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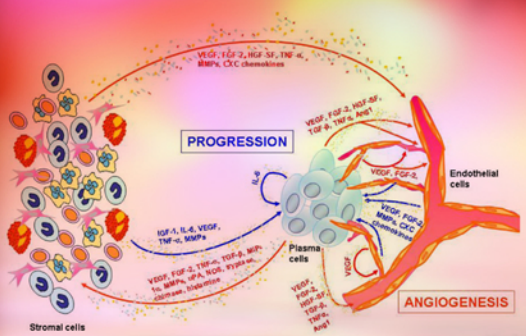

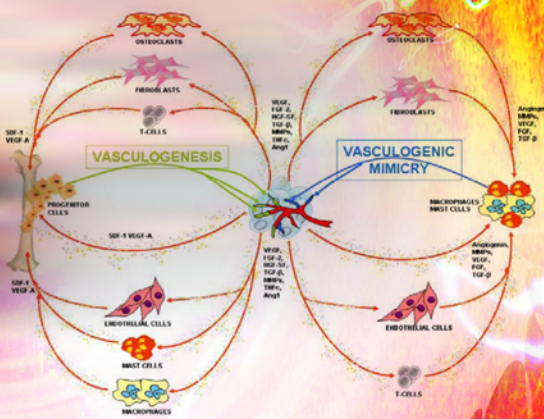

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CELL RESPIRATION AND CELL SURVIVAL: PROCESSES, TYPES AND EFFECTS

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Contents

Preface

In this book, the current understanding of the mechanisms of each beta cell toxins are reviewed, reported toxin resistant insulinoma or immortalized beta cells are summarized, and the different nature of those toxin resistant cells are analyzed. With advancements in cancer stem cell research, the possible involvement of stem cells enrichment after various toxin challenges is also discussed. Moreover, there is a renewed interest in the study of the function of fatty acid synthase (FASN) and fatty-acid synthesis in cancer pathogenesis. This book outlines the role of FASN in cancer development and the preclinical development of FASN inhibitors and their antitumor effects. In addition, Parkinson disease (PD) is a neurodegenerative disorder characterized by a progressive loss of the nigrostriatal dopaminergic neurons. The authors discuss the roles of oxidative modification of the proteins of mitochondrial respiration in the pathogenesis of PD. Furthermore, it has been currently understood that the key role in the physiological and regenerative restoration of adult tissues belongs to adult stems cells. Adult stem cell survival after the action ionizing radiation, hyperthermia and in the conditions of the "ischemia/reperfusion" reaction development are examined.

Chapter I - Basing on the author’s own and literature data, the authors analyze phenomenological problems of the death of several adult stem cells types, including hemopoietic and mesenchymal stem cells of bone marrow, stem cells of intestinal epithelium and other epithelia. The authors discuss potential mechanisms of cell death in vivo and in vitro after the action of ionizing radiation, hyperthermia and in the conditions of the “ischemia/reperfusion” reaction development.

Although the phenomenology of adult stem cells death after the action of ionizing radiation has become classical, the molecular mechanisms of the death of these stem cells types are still being actively studied. It has been shown that after the action of ionizing radiation both the repair of induced damage and programmed cell death are realized in stem cells, the latter process going via several specific molecular pathways. The effects of many modifiers of radiation damage in adult stem cells are related to their participation in a complex combination of the processes of genetic damage repair and cell thanatogenesis.

When adult stem cells undergo hyperthermia, other “targets” are damaged than those in the case of ionizing radiation. However, a similar formal approach developed earlier for the ionizing radiation action can be applied to describe the dependence between the cell survival and the damaging agent dosage. At the same time, the action of hyperthermia is characterized by a number of phenomena with no analogues existing in the case of ionizing radiation, for example, a known phenomenon of thermotolerance resulted from the increased production of the heat shock proteins.

The authors have shown recently that for the two types of adult stem cells (hematopoietic stem cells and intestinal epithelium stem cells) the “ischemia/reperfusion” reaction can be developed in vivo. The damaging action of this reaction onto stem cells can be diminished by an injection of a source of NO-radicals into the animal’s body during reperfusion, since NO-radicals are capable of decreasing the negative effect of radicals produced after oxygen access into the ischemic tissues. The authors believe that these data may be used in the development of new approaches for the protection of cell systems of organism renewal after the damaging action of various agents.

Recently, studies on the cancer stem cells progenitors produced by the action of some carcinogens onto animals have started. The simultaneous studies on the survival of adult stem cells and cancer stem cells progenitors after the damaging action of ionizing radiation and hyperthermia may give rise to the improvement in the existing methods of malignant tumors treatment.

Chapter II - Diabetes is a metabolic syndrome associated with the onset of numerous complications. Among these complications, diabetic cardiomyopathy represents one of the most common causes of death in diabetic patients. In recent years, experimental evidence has indicated a major role of reactive oxygen species (ROS) and lipid peroxidation products in the ethio-pathogenesis of diabetes and its complications. Disagreement, however, exists about the sites and mechanisms involved in ROS formation, the role of

hyperglycemia in exacerbating their formation, and the effectiveness of antioxidant levels in counteracting the formation of ROS and peroxidation products.

Studies from the author's laboratory have provided compelling evidence that type-I diabetes is associated with the endogenous production of 4-hydroxynonenal (HNE). Generated via lipid peroxidation, this highly reactive aldehyde rapidly reacts and forms stable adducts with specific proteins within the cardiac mitochondria of diabetic rats. One of the modified proteins has been successfully identified as the FAD containing subunit of succinate dehydrogenase, and the defect has been correlated to a comparable decline in succinate-supported oxygen consumption in the intact mitochondria and in complex II activity in the purified complex. The process leading to HNE production appears to be independent of glycemia level, but is strictly associated to the decline in circulating insulin level. Streptozotocin-injected animals presenting levels of circulating insulin higher than 65 pmol/L show low levels of HNE-induced modification of mitochondrial proteins and near normal oxygen consumption in intact mitochondria despite presenting hyperglycemia levels comparable to those of frankly diabetic animals (i.e. >400 mg glucose/dl). In contrast, frankly diabetic animals present endogenous circulating insulin levels below 25 pmol/L, high levels of HNE-induced modifications, and defects in mitochondrial respiration. Supplementation of the latter group of animals with exogenous insulin for a minimum of 2 weeks results in a marked decrease in HNE-mediated adducts and the restoration of succinate dehydrogenase activity to levels comparable to those observed in non-diabetic animals. The identification of two distinct pools of hyperglycemic animals that present or lack HNE-induced modifications and related mitochondrial dysfunction has provided a new rationale to explain some of the functional inconsistencies observed in mitochondria purified from various tissues of diabetic animals.

Utilizing a proteomics approach, the authors have identified about 12 mitochondrial proteins that form stable adducts with HNE. Determining to what extent these proteins are functionally impaired by the formation of adducts with HNE will undoubtedly help to shed light on the role of mitochondrial impairment in the onset of diabetic cardiomyopathy.

Chapter III - The growth, survival and proliferation of cancer cells are guaranteed by a crosstalk between cancer cells themselves and surrounding host cells and extracellular matrix. An intense area of research has contributed

to a better understanding of the pathophysiological modification of tumor progression, e.g., the role of microenvironment.

Multiple Myeloma (MM) is a malignancy of immunoglobulin-synthesizing plasma cells with symptoms mainly related to imbalance of bone homeostasis, kidney damage, anemia, impaired humoral immunity, and sometimes nervous system dysfunctions.

Plasma cells home and expand in the bone marrow where cause an unbalanced bone remodelling with increased bone resorption and low bone formation that represent the typical feature in the majority of patients. MM plasma cells are thought to be responsible for the osteolytic bone lesions, which occur by increased osteoclast formation/activity and inhibition of osteoblast formation/differentiation. In physiological conditions, this process is critically regulated by the transcription factor Runx2 and by the Wnt signalling pathway. Moreover, MM plasma cells accelerate the differentiation of resident macrophages to osteoclasts. Finally, plasma cells themselves can transdifferentiate to functional osteoclasts.

Another relevant aspect of the interactions of MM plasma cells with stromal cells in the bone marrow microenvironment is neovascularization, a constant hallmark of disease progression. MM plasma cells induce angiogenesis both directly, via their own factors (vascular endothelial growth factor [VEGF], fibroblast growth factor-2 [FGF-2], hepatocyte growth factor [HGF] and metalloproteinases), and indirectly via recruitment and activation of stromal inflammatory cells to secrete their own angiogenic factors. Macrophages and mast cells play an important role in this sense. They are recruited and activated by tumor plasma cells through the secretion of FGF-2, interleukin-8 (IL-8), and chemokines, such as ITAC, Mig, IP-10. When macrophages and mast cells are activated they secrete potent angiogenic factors (FGF-2, VEGF, granulocyte-colony stimulating factor [G-CSF], granulocyte macrophage-colony stimulating factor [GM-CSF]), which contribute to the tumor neovascularization. Recent evidence demonstrates the vasculogenic ability of active MM macrophages exposed to VEGF and FGF-2, the major angiogenic cytokines secreted by plasma cells, and present in the bone marrow microenvironment at four-to five-fold higher levels than in peripheral blood. Under these stimuli, bone marrow macrophages acquire endothelial cell (EC) markers and transform into cells functionally and phenotypically similar to paired bone marrow ECs (MM patient-derived endothelial cells, MMECs). So they generate capillary-like networks mimicking those of MMECs. Thus, MM macrophages contribute to build the

neovessel wall via a “vasculogenic mimicry”, hence helping MM progression by this way.

Chapter IV - Energetic metabolism is crucial for maintaining the biological function of all cells. Energy sources and redox state depend on metabolism of the oxidative substrates. It is interesting to consider that different cells types have different metabolic patterns. A lot of processes require energy from mitochondrial respiration and cytosol metabolic pathways. Gametes are very special cells and their metabolism and viability allow the fertilization in all species. Mammalian spermatozoa must undergo preparation processes known as capacitation and acrosome reaction that involve biochemical modifications to penetrate the oocyte. Neither molecules nor mechanisms involved in the metabolic pathways leading to sperm capacitation and exocytosis are clearly understood. Capacitation is physiologically dependent on oxidative metabolism where a lot of cytosolic and mitochondrial enzymes are involved. Regarding sperm energy sources, lactate dehydrogenase (LDH) shuttle requires an enzyme which is present in both cytosol and the mitochondria matrix and an aminotransferase that can supply substrates for LDH. Alanine aminotransferase and aspartate aminotransferase are present in the majority of mammalian tissues and they have a important role in aminoacid metabolism including spermatozoa. The shuttle of creatine/phosphocreatine is considered to be responsible for the transfer energy from mitochondria. The level of creatine kinase B indicates a normal spermiogenesis and maturation. Creatine kinase activity is related to redox state and cellular energy sources. NADPH level has an important role in sperm function and its interaction with oocyte. Reduced NAD and NADP levels are associated with isocitrate and malate dehydrogenase activities, enzymes that belong to cytosol pathways and Krebs cycle. These reduced coenzymes are involved in the control of tyrosine phosphorylation. Under aerobic conditions, reduction equivalents can be oxidized in the mitochondrion; NADH is unable to cross mitochondrial membrane so shuttle system must be implicated to transfer the reduction equivalents. Shuttles that are involved in this process depend on the cell types. Mitochondria have a key role in the control of reactive oxygen species (ROS) level and energy production in capacitation induction. Sperm processes require equilibrium between oxidative damage susceptibility and ROS level. Sperm respiration is a crucial process in the production of energy and redox state for intracellular signals and the viability of cells. The author’s purpose is to show the importance of oxidative metabolism on cellular functions. In this case the

model is the spermatozoa a haploid cell with the important function of giving a new life in different species.

Chapter V - Plectin is a cross-linking protein that organizes the cytoskeleton into a stable meshwork. Intermediate filaments (one kind of cytoskeletal component) are important in building the cellular architecture. Hepatocytes have very simple intermediate filaments composed of cytokeratin 8 (type II) and cytokeratin 18 (type I). The authors previously reported that cytokeratin 18 is modulated in human hepatocellular carcinoma. Because hepatocellular carcinoma cells are morphologically different from those of normal liver, the authors speculated that aberrant expression of plectin and the disorganization of intermediate filaments might play some role in the pleomorphism of hepatocellular carcinoma cells. By immunohistochemistry and immunoblotting, the authors confirmed that human hepatocellular carcinoma tissues are deficient in plectin. In *in vitro* experiments, the authors noted that the organization of cytokeratin 18 was altered after plectin knockdown by small interfering RNA (siRNA). In addition, cytokeratin 18 was modulated after plectin degradation in response to staurosporine-induced apoptosis. The authors hypothesize that plectin deficiency plays an important role in the transformation of cells in human hepatocellular carcinoma.

Chapter VI - Alloxan and streptozotocin are two classic diabetogenic agents which are employed for the induction of insulin-dependent diabetes mellitus (IDDM) or insulin-independent diabetes mellitus (NIDDM) when different dosages were used. In addition, due to the autoimmunity in type 1 diabetes, a major loss of pancreatic beta-cell mass occurred after local production of various cytotoxic cytokines, mainly interleukin-1 β, interferon-γ, and tumor necrosis factor-α. Therefore, understanding the mechanisms of actions of those toxins offers good lessons for the development of a good beta-cell protection strategy. Furthermore, studies of beta-cells which survive from those toxic challenges are also informative in terms of understanding intrinsic mechanisms of cell defense. Interestingly, studies of those toxin resistant cells were also drew the attentions from cancer researchers, especially in the field of chemotherapy. In this article, the authors would like to review the current understanding of the mechanisms of each beta cell toxins, to summarize reported toxin resistant insulinoma or immortalized beta cells, and to compare the different nature of those toxin resistant cells. Finally, with the advance of the knowledge for cancer stem cells, the possible involvement of stem cells enrichment after various toxin challenges was also discussed.

Chapter VII - There is a renewed interest in the study of the function of fatty acid synthase (FASN) and fatty-acid synthesis in cancer pathogenesis. With the recent discovery that human cancer cells express high levels of fatty acid synthase and undergo significant endogenous fatty-acid synthesis, the author's understanding of the role of fatty acids in cancer biology is expanding. In spite of an anabolic energy-storage pathway, lipogenesis is now associated with clinically aggressive tumor behaviour and tumor-cell growth and survival and has become a druggable target in many human carcinomas. This review outlines the role of FASN in cancer development and stands out the preclinical development of FASN inhibitors and their antitumor effects.

Chapter VIII - Parkinson disease (PD) is a neurodegenerative disorder characterized by a progressive loss of the nigrostriatal dopaminergic neurons. Several clinical and experimental studies have suggested the roles of oxidative stress and impaired mitochondrial respiration in the pathogenesis of PD. It is believed that oxidative modification of the proteins of mitochondrial respiratory chain alters their normal function leading to the disruption of electron transport and consequently the impairment of oxidative phosphorylation, culminating in the state of energy crisis in neurons. Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mouse is the most extensively used experimental model of PD. MPTP-induced neurotoxicity is accompanied by the blockade of electron flow from NADH dehydrogenase to coenzyme Q at or near the same site as do the rotenone (another parkinsonian toxin). Coenzyme Q is an electron acceptor bridging mitochondrial complexes I and II/III and also serves as a potent antioxidant that has been shown to partially recover the function of dopaminergic neurons. Moreover, the infusion of the ketone bodies in mice confers protection against MPTP-induced dopaminergic neurodegeneration and motor deficits by improving mitochondrial respiration and ATP production. It is highly imperative that novel drug regimens targeting the restoration of mitochondrial respiration, improvement of dopaminergic neurotransmission and alleviation of oxidative stress would offer beneficial effects for the management of PD.

Chapter IX - On the ecophysiological level, the magnitude of aerobic dark respiration (R_D) of a plant organ is considered a measure of the intensity of metabolism and physiological functions of the organ. In this minireview, properties of R_D are reviewed in different types of organs of carnivorous plants from a functional point of view. Although net photosynthetic rate (P_N) in leaves or traps of terrestrial carnivorous plants is usually several times lower than that in leaves of vascular non-carnivorous plants, R_D in carnivorous

plant leaves is relatively high and reaches on average 48 % of P_{Nmax} representing the high metabolic (energetic) cost of carnivory. The metabolic cost of carnivory is markedly high in traps of aquatic carnivorous *Utricularia* species; the traps are structurally quite separate from photosynthetic leaves. In six species, trap R_D was 75-200 % greater than that in leaves but foliar P_N exceeded that in traps 7-10 times. This reflects high metabolic activity of *Utricularia* traps associated with pumping ions and water through the trap walls. However, it has not yet been explained how the internal trap structures (glands taking part in prey digestion, nutrient absorption, and pumping water) provide the ATP for their demanding functions when complete anoxia occurs in the trap fluid.

R_D values of roots (1.6-5.6 nmol g^{-1}_{FW} s^{-1}) as well as water exudation rates of five carnivorous plant species were comparable with those reported in non-carnivorous plants or even higher. A high proportion of cyanide-resistant respiration (65-89 %) was found in the roots. R_D values of turions (winter buds) of some aquatic carnivorous plant species of the genera *Aldrovanda* and *Utricularia* were about 1.5-4 times lower (on FW basis) than those reported in growing shoots/leaves of these or other aquatic plant species. Contrary to true dormant turions, R_D of non-dormant winter shoot apices of *Aldrovanda* and *Utricularia* was comparable to that in aquatic plant shoots/leaves.

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Chapter I

Adult Stem Cells Survival after the Action of Ionizing Radiation, Hyperthermia and in the Conditions of the "Ischemia/Reperfusion" Reaction Development

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Abstract

Basing on our own and literature data, we analyze phenomenological problems of the death of several adult stem cells types, including hemopoietic and mesenchymal stem cells of bone marrow, stem cells of intestinal epithelium and other epithelia. We discuss potential mechanisms of cell death in vivo and in vitro after the action of ionizing radiation, hyperthermia and in the conditions of the "ischemia/ reperfusion" reaction development.

Although the phenomenology of adult stem cells death after the action of ionizing radiation has become classical, the molecular mechanisms of the death of these stem cells types are still being actively studied. It has been shown that after the action of ionizing radiation both the repair of induced damage and programmed cell death are realized in stem cells, the latter process going via several specific molecular pathways. The effects of many modifiers of radiation damage in adult stem cells are related to their participation in a complex combination of the processes of genetic damage repair and cell thanatogenesis.

When adult stem cells undergo hyperthermia, other "targets" are damaged than those in the case of ionizing radiation. However, a similar formal approach developed earlier for the ionizing radiation action can be applied to describe the dependence between the cell survival and the damaging agent dosage. At the same time, the action of hyperthermia is characterized by a number of phenomena with no analogues existing in the case of ionizing radiation, for example, a known phenomenon of thermotolerance resulted from the increased production of the heat shock proteins.

We have shown recently that for the two types of adult stem cells (hematopoietic stem cells and intestinal epithelium stem cells) the "ischemia/reperfusion" reaction can be developed in vivo. The damaging action of this reaction onto stem cells can be diminished by an injection of a source of NO-radicals into the animal's body during reperfusion, since NO-radicals are capable of decreasing the negative effect of radicals produced after oxygen access into the ischemic tissues. We believe that these data may be used in the development of new approaches for the protection of cell systems of organism renewal after the damaging action of various agents.

Recently, studies on the cancer stem cells progenitors produced by the action of some carcinogens onto animals have started. The simultaneous studies on the survival of adult stem cells and cancer stem cells progenitors after the damaging action of ionizing radiation and hyperthermia may give rise to the improvement in the existing methods of malignant tumors treatment.

An adult organism consists of a big number of different cell types (approximately 250-300) which constitute various cell populations of all the tissues and organs [1-2]. These cells can be damaged upon the action of different physical, chemical and biological agents. From the viewpoint of radiobiology, considering ionizing radiation as a possible cause of lethal damage of any cells in body, all the cell populations of an adult organism can be divided into two big groups based on their organization and reaction to

irradiation. The first group involves cells fast or slowly regenerating during postnatal life due to cell division, while the second group represents systems not regenerating during this period of life [3-4]. Fast regenerating systems include bone marrow, intestinal epithelium, spermatogonial epithelium etc.; vascular endothelium and fibroblasts regenerate slowly. The cells which almost never regenerate under normal conditions are populations of parenchymal cells of liver, kidneys and lungs, though their regeneration can be stimulated by special conditions [3, 5-6]. It has been currently understood that the key role in the physiological and regenerative restoration of adult tissues belongs to adult stem cells [2, 5-8]. This fact applies to both fast and slowly regenerating tissues and organs, and even such tissues as nerve and muscle, which currently have no methods of successful stimulation of cell proliferation and until recently have been considered as systems unable to regenerate [9-11]. In the last few years, new data appeared on the presence of stem cells and, hence, on the possibility of cell reproduction in such cell populations, to replace dying cells and provide repair after damage [9-11].

The systems of fast cell renewal have been most extensively studied; they are usually designed by a so-called hierarchical type - cell hierarchy from stem cells to functional elements. A. Michalowsky [3] suggested to call their reaction onto irradiation “H-systems (or hierarchical cell population) reaction”. Non-renewal (or, in some cases, slowly renewal) cell systems suggested to be called “F-systems” (flexible cell lineage) are less well understood. The latter consist mainly of a homogeneous population of functionally competent cells being in the G_o –phase of cell cycle. Although the cells of H- and F-systems are pretty much similar in their radiosensitivity (tested as the loss of ability to “infinitely” reproduce), they are significantly different in the dynamics and level of adult stem cells death after irradiation, and also in the picture of radiation damage development.

The systems of fast cell renewal usually involve three or more compartments: 1) Adult stem cells compartment. Those are divided into primitive, pluripotent (capable of generating not one, but several cell lineages simultaneously), and committed stem cells (which are the initial component of a certain cell lineage); 2) Proliferative-increasing cell pool. These cells reproduce and differentiate into the elements of the following compartment (often a maturing cells pool is considered as a subpool within this pool); 3) Pool of functional cells. Adult stem cells form the basis for such cell renewal systems and usually are the only source of their repopulation after massive loss caused by irradiation, action of chemicals or, less common, action of

other damaging agents. Adult stem cells are characterized by such two fundamentally important properties as capability of long-lasting self-renewal (it is sometimes referred to as an infinite proliferative potential) and the ability to produce cells going into differentiation, in doing so they can initiate various cell lineage. For example, in the hematopoietic system a pluripotent stem cell is a common progenitor cell for all hematopoietic cell lineages [12]; in the intestinal epithelium it is the only common progenitor cell of four cell types: columnar epithelium or enterocytes in the small intestine and colonocytes in the large intestine, mucosecreting or goblet cells, enteroendocrine cells and Paneth cells [13]. In the last years, two more properties of adult stem cells have been discovered – their plasticity (a possibility of reprogramming the following differentiation of adult stem cells) [14] and a possibility of producing various types of pluripotent stem cells from embryonic stem cells in vitro [15].

There exist different methods for revealing adult stem cells, first of those methods were developed by radiobiologists in the 60-70s of the last century [16-20]. Following irradiation, an acute deficit of survived stem cells is created and formation of the cell colonies is observed in the regions of their progeny proliferation. During 20 sequential divisions, one survived stem cell produces more than a million of cells-progeny. These new cells form a peculiar “colony” that may be morphologically revealed and recorded. When applying this method to studying survival of hematopoietic stem cells (HSC), the analysis of colonies formation in the spleen appeared to be possible not only for own cells survived the radiation (endogenous colony test), but also for bone marrow cells transplanted into a lethally irradiated organism (exogenous colony test), as shown in pioneer works by Till and McCulloch [16-18]. In the same decade, Withers and Elkind developed the methods of intestinal "microcolonies" and "macrocolonies", identical in their approach to endogenous colony test for HSC, for studying post-radiation survival of stem cells in murine intestinal epithelium [19-20]. Later on, methods applying primary and stable cell cultures were developed for human and animal hematopoietic stem cells (and some other types too) [21-22]. A population of non-hematopoietic stromal stem cells found in bone marrow [23-24] and later named mesenchymal stem cells (MSC) also rapidly became an object of radiobiological studies [24, 27-28]. Another population of adult stem cells - spermatogonial stem cells – had drawn attention of Withers et al [29] and other radiobiologists [30] who used the previously described method of radiation devastation of this cell renewal system in this case as well.

Analogous works on peculiar equivalents of "exocolonies" of non-irradiated and irradiated thyroid epithelium stem cells and other tissue-derived progenitor cells upon transplantation into different places in the body have been substantially less developed [31-34].

Before discussing the general laws of the death of different stem cells upon the action of ionizing radiation, here we briefly refer to the methods of quantitative description of the dependencies between the radiation dose (D) and cell survival (S). A relatively small number of defined schemes are currently used in the modern radiobiology for the description of dose dependencies of various cells' survival in vivo and in vitro. One of the most widely used schemes is a single-hit multitarget model [35-37]. Its widespread popularity is due to the fact that it allows one to extract the parameters characterizing cell radiosensitivity from the graph of cell survival vs. radiation dose. The formal description of this model is given by the equation:

$$S = 1- [(1 – \exp (-D/D_0)]^n \text{ or, identically, } S = 1- [(1 - e^{(-D/D_0)}]^n \qquad (1),$$

where S is the fraction of survived cells (survival) after irradiation in the dose D, D_0 and n are constants (parameters) called "mean cell lethal dose" and "extrapolation number", correspondingly. The dose-effect curves plotted using semi-logarithmic scale (with the linear X-scale of the dose and the logarithmic Y-scale of the survival) have sigmoidal shape with the initial smoothly bent region followed by an almost linear (i.e. exponential in linear coordinates) dependence. It is easy to verify that, at sufficiently high D values the equation (1) transforms as follows:

$$\ln S \cong \ln n - D/D_0 \qquad (2),$$

or, using decimal logarithms,

$$\lg S \cong \lg n - D/D_0 \times \lg e \qquad (3).$$

These equations (2 and 3) describe the straight line which the dose-effect plot approaches at higher irradiation doses. They describe only a part of the "dose- cell survival" curve to be experimentally estimated using the methods of stem cells survival similar to endogenous colonies method for HSC. The measurement of this curve slope allows estimation of the mean cell lethal dose D_0, which is numerically equal to the dose decreasing cell survival by a factor

of *e*, for example, from 1 to 1/e (i.e., from 1 до 0,37, or from 100% to 37%; therefore, sometimes the parameter D_{37} is used instead of D_0). In the formal approach of the "target theory" [38] the mean cell lethal dose is an absorbed dose which, for the evenly distributed events of target inactivation, is sufficient for precisely one hitting of the sensitive cell "target" with 100% cells damage. However, due to random distribution of events of target inactivation, according to the Poisson statistics such events will happen only in 63% of cells, while 37% of cells will remain intact. The point of the intersection of the linear part of the curve with Y-axis (i.e. at the zero dose of radiation) gives a value of the logarithm of "extrapolation number" (in formal terms of the "target theory" it corresponds to the number of "targets" in a cell). Thus, having plotted the linear part of the curve based on experimental data, a researcher gets an opportunity to simultaneously estimate two parameters characterizing cells radiosensitivity. Often one more parameter is used - "width" of the initial shoulder region (or quasithreshold dose) D_q, which is numerically equal to the dose of radiation, cut on the X-axis by the linear part of the dose-effect curve at the level of 100% survival. This parameter is related to the other two ones via the following expression:

$$D_q = D_0 \text{ x } \ln n \qquad (4).$$

It is conventional that D_q may be used to quantitatively characterize cells' ability to repair radiation damage, and that increase in this parameter means enhancement in the irradiated cells ability to repair after radiation damage. It is especially related to the so-called "sublethal radiation damage" which does not directly result in the irradiated cell's death, but makes it sensitized to the further action of ionizing radiation [35-36].

In a number of cases, instead of an S-shaped survival-dose curve one may observe exponential curve, i.e. the dependence of the form

$$S = \exp(-D/D_0) \qquad (5).$$

Such dependence is a special case of the described model at n=1, and D_q in this case is accordingly equal to zero. Such a scheme is referred to as a single-hit single-target model. In this case only one parameter - D_0 - is needed to characterize cells radiosensitivity. Same cells may produce different dose-effect curves (sigmoidal or exponential) depending on the different linear energy transfer (LET) of irradiation. The first type of curves is usually observed upon the action of low-LET radiation, while the second type is

observed for high-LET radiation. An important point is that the initial slope of S-shaped dose-effect curves is equal to zero, i.e. the tangent to the curve at the zero dose of radiation is parallel to the X-axis. At the same time, in the radiobiological practice S-shaped curves with non-zero (usually negative) initial slope are frequently observed, with the rest of the curve described by the equation (1). In this case experimental points are best approximated by the so-called modified single-hit multitarget model with the equation of a form

$$S = \exp(-D/{}_1D_0)\{1 - \exp(-D/{}_2D_0)]^n\} \quad (6).$$

It is commonly assumed for such a case that there are two types of cell inactivation in a homogeneous population that can be regarded both to the cells characteristics and radiation characteristics, or to both factors simultaneously. The mean cell lethal dose D_0 for the exponential region in this case can be derived using the following simple relationship:

$$1/D_0 = 1/{}_1D_0 + 1/{}_2D_0 \quad (7).$$

Another approach to the description of the dose-effect dependence which has gained acceptance in the last decades is based on a so-called linear-quadratic model [36-38]. In this case the dependence of the cell survival on the radiation dose is given by the following relationship:

$$S = \exp[-(aD + bD^2)] \quad (8),$$

where parameters a and b characterize contributions of the linear and quadratic components into the gradient of the fraction of survived cells (note that many authors use greek characters α and β instead of coefficients a and b; note also the dimensionality of these values – it is Gy^{-1} for the linear coefficient and Gy^{-2} for the quadratic one). The initial slope of such a curve is determined by the a (or α) value, while its following shape at high radiation doses is given by the b (or β) value. It is noteworthy that in this case in the range of high doses the curve "dose-effect" does not tend to the exponential function but is similar to a parabola, i.e. its curvature grows with the radiation dose. When the range of the radiation dose is limited and experimental points are scattered, separate parts of such a curve can obviously be approximated by any dependence, including the exponential one, so the choice of a model depends on the researcher's preferences.

In the first works [16-17], dedicated to the survival of murine hematopoietic stem cells after the action of low-LET radiation (X-rays of 280 kV), it was found that the dose-effect curves for HSC in the exogenous colony test (colony on spleen of lethally irradiated mice 10 days after donor bone marrow transplantation, irradiation of bone marrow cells in vitro and in vivo) are very similar and have the following parameters characterizing their radiosensitivity: 1. D_0 is 1,05 Gy, and n is equal to 2,5 (when irradiating bone marrow in vitro); 2**.** D_0 is 0,95 Gy, and n is equal to 1,5 (when irradiating bone marrow in vivo). Similar value of D_0 , close to 1 Gy, was obtained in the experiments using the method of spleen endocolonies [36, 39]. Thus, murine hematopoietic stem cells did not differ in their radiosensitivity from the majority of other mammalian cells, which were grown in the culture and whose post-radiation survival was evaluated by change in their ability to form clones [35]. This conclusion about similarity of the radiation reaction of this type of stem cells and mammalian cells cultured in vitro were confirmed by other radiobiological data. The same conclusions were made when the methods of culturing progenitor cells for different hematopoietic lines were developed, and the curves of the radiation survival were obtained [36, 40-42]. The progenitor cells under study included early and late progenitor cells of erythroid series (BFU-e and CFU-e), progenitor cells of granulocytes and macrophages (GM-CFU, G-CFU и M-CFU) and others. As a rule, progenitors differentiated into a certain cell line were slightly more resistant to ionizing radiation than pluripotent stem cells, but this difference was not significant. At the same time, more committed stem cells, but still remaining a source of several hematopoietic lines, are characterized by a higher radiosensitivity as compared to polypotent HSC [43]. Under the action of high-LET radiation (mainly neutrons, protons, heavy charged particles etc.) D_0 for HSC and their committed cell progeny drops by several times (proportionally to the magnitude of the relative biological effectiveness of such radiation – RBE), while n – extrapolation number – decreases to 1.0, i.e. the dose-effect curves convert into exponential functions [36, 44-45]. After the action of high-LET radiation, hematopoietic stem cells, as well as other types of mammalian cells, are almost incapable of repairing radiation damage, as opposed to the action of low-LET radiation [35-36, 42, 46-48]. Similar to mammalian cells in vitro, this type of stem cells was able to repair both sublethal cell damage and potentially lethal damage [35, 46-48]. It was shown later that repair of the damage in HSC genetic structures caused by low-LET radiation along with their proliferation at sufficiently long irradiation are the reasons for the dose-

rate effect [36, 48]. HSC and their partially differentiated cell progeny have served as first types of adult stem cells to investigate the effect of radiomodification of various biological, physical and chemical agents, which can either attenuate or enhance their damage from irradiation in vivo and in vitro [21, 36, 49-61]. These studies substantially expanded the knowledge about the nature of fetal and adult stem cells and also about the mechanisms promoting their survival or death in the different whole-organism conditions during fetal and postnatal life, as well as during growth in the conditions of primary and stable cell cultures. Some of these data will be considered in more detail later, in the discussion on the mechanisms of stem cells death in body and their importance for sustaining cell homeostasis in the cell renewal systems.

After the methods of cell culture and survival estimation were developed for pluripotent HSC and HSC-generated progenitor cells for different large animals and human hematopoietic lines, extensive research was conducted to characterize their radiosensitivity [36, 62-69]. It turned out that, with few exceptions, the quantitative parameters of cell radiosensitivity for these cells were of the same order of magnitude as those for laboratory mice. In other words, for such cells the value of D_0 under the action of low-LET radiation is about 1 Gy, and the values of extrapolation number n are in the range of 2-5 [32, 36]. When applying the "linear-quadratic model" to describe the dose-effect curves for survival of human hematopoietic stem cells, the main quantitative parameter of radiosensitivity – "α/β ratio" - was found to be 3-5 Gy, i.e. of the same order as that for mice [36-37, 70]. Therefore, human HSC cultures as well as laboratory animal stem cells have become a popular object not only for solely radiobiological studies, but also for evaluation of chemotherapy agents especially those involved in the cancer therapy [71-74]. Besides, they are used for evaluation of different modifiers which can be utilized to stimulate the reparative processes in the case of disorders of hematopoiesis in humans [75-76]. Along with the modern molecular biology tests, methods based on the consideration of the death level of stem cells with different degree of committing are still applied very frequently in such studies [65, 77].

Another system of adult stem cells, first investigated by radiobiologists, are intestinal stem cells (ISC) [6, 19-20, 32, 36, 78-80]. As it was for HSC, the first elaborated approaches to estimate ISC survival were developed for mice and by their concept they were analogous to the method of "spleen endogenous colonies". The mice underwent total irradiation in the doses

leading to a substantial loss of ISC in the lower region of intestinal crypts. Before the "intestinal" form of radiation death began, 3 days after irradiation, fragments of mice small intestine were fixed for the following histological treatment and obtaining transverse cross-sections of small intestine. The cross-sections were examined for presence of regenerating crypts and those crypts where regeneration had not begun. The estimation was based on a reasonable assumption that regenerating crypts must have one or more ISC remaining after irradiation, while non-regenerating crypts must have no viable ISC, and that the probability to find survived ISC in the small intestine crypts was described by Poisson distribution. Thus, using rather simple mathematics, a mean number of survived ISC per one crypt and also for the entire transverse cross-section in a histological slide could be calculated [19-20, 36, 78]. This allowed one to plot the curve of ISC survival vs. irradiation dose in a certain dose range and to estimate a value of "mean cell lethal dose" D_0 for them. D_0 for murine ISC appeared to be approximately the same as that for HSC and was equal or a little higher than 1 Gy under the action of low-LET radiation 19-20]. However, full curves "dose-effect" for these two types of stem cells are significantly different, since ISC have considerably greater "shoulder dose" D_q . For stem cells of the small intestine epithelium D_q is 4-5 Gy according to different estimations, and the value of "extrapolation number" n is of several tens [78-83]. This fact reflected higher ability of ISC to repair sublethal cell radiation damage (Elkind-type repair) during the early postradiation period, as was soon confirmed by the experiments on the effects of fractionated irradiation for ISC [84-85], and also in the low-dose experiments [36], when reparative processes in the damaged stem cells could be realized fast. In a number of such radiobiological experiments the "linear-quadratic model" was used to describe the "dose-effect" dependence. According to this model, the "α/β ratio" is 13.3 Gy at a high radiation power dose (1.2 Gy/min) and is equal to 96 Gy at a low radiation power dose (0.08 Gy/min) [86]. The conclusion about higher ISC ability to repair radiation damage of low-LET radiation is consistent with the data on a higher biological activity of high-LET neutrons of various energies and other high-LET radiation for these cells as compared to HSC [36, 87-90]. In [91], a relatively infrequently used "two-component" model was applied (see Eq. 6 and 7) to describe dose dependence of ISC survival after γ –irradiation of mice. According to this model, the D_0 value for the first component is 1.5 Gy, for the second component it is 4.5 Gy, with the value of "extrapolation number" n equal to 20. These data also showed better expressed reparative processes for

ISC after the action of low-LET radiation, additionally confirmed by a study of fractionation effect and by comparison of effects of Co^{60} γ-radiation and fast neutrons.

The research on the ISC radiobiology allowed creation of a new experimental model for some problems which were mentioned already in the works on HSC, but were difficult to analyze using one object only. First of all, it concerns the concept of "stem cells niche" first formulated for HSC [92] which suggested that the adult cell renewal systems contained certain structures capable, by producing cytokines and growth factors, and also by providing necessary cell interactions, of generating conditions for self-sustainable population of stem cells during life span. A concept of "stem cells microenvironment" is widely used along with this term nowadays which extends to all known stem cells types [93], although a detailed research on such structures is only beginning and probably will not be finished soon. This concept is utilized also for analysis of different problems of radiation damage and the following HSC and ISC population regeneration [93-95]. It has been found that survival of ISC in the lower part of mice intestinal crypts after massive irradiation increases significantly when this zone is supplied with FGF-2 produced by pericryptal fibroblasts. The latter have a mesenchymal nature and play a role of "niche" elements for ISC [94]. Thus, the "niche" influence is realized not only in the conditions of sustained cell homeostasis in adult cell renewal systems, but also in the conditions of its disruption resulting from radiation inactivation of stem cells.

Using experimental ISC models of transgenic and knockout mice, researchers successfully investigated the effects of a number of genes onto the apoptosis development and onto radiation damage of stem cells [96-98]. A number of genes (***p53***, ***p21, ATM, Ku80, PARP-1, Msh2 etc.***) directly relevant to DNA repair were shown to play an essential role in the regulation of the programs of cell survival. For example, products of p53 gene served as inducers of apoptotic form of cell destruction in intestinal crypts or led to the growth arrest at cell cycle checkpoints [96, 99]. It was observed for control non-irradiated mice that 1-2 ISC underwent spontaneous apoptosis in the small intestine crypts during 1 day, the fact which was probably related to homeostatic regulation for sustaining the number of stem cells in a crypt, with the participation of p53 genes and genes of the bcl-2 family [96, 99]. Deletion of p53 gene had a differing effect onto ISC survival in mice after low-LET irradiation: their survival in small intestine did not change, though regeneration of damaged crypts slowed down, while in large intestine ISC

survival was lower for p53(-/-) mice than that for the wild type mice [98]. It was believed that in this case attenuation or absence of stem cells arrest in G_2-phase of cell cycle did not give them enough time for the reparative processes to go and resulted in the following decrease in cell survival. Under the action of high-LET radiation p53-independent apoptosis was induced in ISC [100]. No difference in the extent of apoptosis within the whole pool of intestinal crypt cells was found between adult wild-type (WT) and p21(-/-) mice, but p21(-/-) mice showed 3 times higher crypt survival than WT mice 3.5 days after irradiation in the dose of 13 Gy [101]. The increase in the survival of ISC and their proliferating and differentiating cell progeny in irradiated animals was associated presumably with increased numbers of Msi-1- and survivin-expressing cells in regenerative crypts. Besides, it was shown that p53 gene products participated in the development of early apoptosis induced by very low radiation doses in ISC located in the base of small intestine crypts [102]. Late manifestations of radiation apoptosis for ISC are p53-independent [99, 102]. Although no change in the probability of spontaneous or radiation-induced apoptosis was observed for mice with the deletion of bcl-2 gene family, but *bad* gene products were expressed in the crypt cells and villi after the action of radiation [102]. An unexpected phenomenon of significant increase in radioresistance of ISC was revealed for the cells located in the zone of patches of Peyer upon the action of both low-LET and high-LET radiation [103-104]. It was suggested that the increase in the ISC radioresistance in this case was due to “physiological shut down” of the p53 gene activity and/or increased expression of the bcl-2 and bcl-x genes [105].

A unique property has been discovered for ISC of small intestine – preservation of stability of two template DNA strands after cell division in the remaining stem cell, while the second cell going into differentiation receives two newly synthesized DNA strands [106-107]. Such segregation of template and newly synthesized DNA strands in stem cells of small intestine provides a peculiar “immortality” of DNA in this type of adult stem cells and stability toward development of cancer that makes them strikingly different from stem cells of large intestine epithelium. At the same time, it is worth mentioning that radiobiological aspects of many signaling pathways regulating cell homeostasis, including cell proliferation, cell death, possible carcinogenesis (such as Wnt/β-catenin, BMP/SMAD4, Notch, Hedgehog, PTEN/aKt, TGF-β etc.) have been studied absolutely insufficiently. The basic file of new radiobiological data concerns effects of different modifiers of stem cells’ radiosensitivity, with initiation of a variety of these signaling pathways. For

example, epithelial growth factors (TGF-β, KGF) and cytokines related to hematopoiesis and immune functions (IL-1, SCF и IL-11, IL-12), usually in the form of human recombinant polypeptides, appeared to be powerful exogenous modifiers of ISC survival/death [108]. Interleukin 1α (IL-1α) was one of the first cytokines which was found to have radiomodifying properties toward ISC [109]. Administration of IL-1α to animals 4-8 hrs before irradiation resulted in the aggravation of crypts damage. Only if administered 1-7 days before irradiation, it led to a decrease in the ISC death, though repopulation of the damaged crypts was not faster [110]. It has been known that IL-1α radioprotective action onto HSC also appears in case of preliminary injection performed several hours or days before irradiation [111]. Interleukin-12 (IL-12), secreted under the action of bacterial products by monocytes/macrophages and lymphocytes plays an important role in the generation of T1-helper cells. With other growth factors, it synergetically stimulates proliferation of early hematopoietic progenitor cells thus providing radioprotective action [112]. At the same time, this cytokine is capable of suppressing tumor growth and metastases production, probably by inhibiting cancer stem cells, and also of decreasing the proliferative activity of ISC [112-113]. This fact is demonstrated by a significant drop in the number of regenerating crypts and a twofold decrease in the lifespan of mice having received this cytokine in the dose of 15 Gy either 18 hrs before irradiation, or 1 hr after irradiation. Antibodies to interferone-gamma (aIFN-γ) cancelled this sensitizing effect [113]. Since a controversial action of IL-12 onto the radiation effect for different tissues was found, the prospective of its application as well as application of similar cytokines as radioprotectors seems to be somewhat illusive. Radioprotective action of FGF-2 was noticed earlier via the test of ISC survival [94]. An extensive study on the mechanisms of this effect has shown that they are not associated with direct action of FGF-2 onto the cells of intestinal epithelium, since its receptors are present only in the endothelium of microvessels surrounding crypts [114]. In the endotheial cells FGF-2 inhibited activity of acid sphingomyelinase producing a well-known thanatogenic mediator – ceramide. FGF-2 receptors are not expressed in the intestine, while acid sphingomyelinase is presented in the vessels endothelium in the amount which is 20 times higher than that in other tissues. Taking the above facts into account, it was suggested that the primary cause of intestinal mucosa exhausting and, correspondingly, ISC death, was early apoptotic destruction of the microvessels endothelium. Morphologically, FGF-2 radioprotective action was expressed in the limiting of crypts shrinking, but

not in the acceleration of their regeneration after irradiation [114]. At the same time, it should be mentioned that the extent of the radioprotective effect for FGF-1 and FGF-2 depends greatly on the experimental mice strain [115]. Transforming growth factors (TGFβ1, TGFβ2, TGFβ3) are known as inhibitors of epithelial cells proliferation, arresting them in the G_1-phase of the cell cycle, thus they are capable of affecting ISC radiosensitivity [116]. For example, exogenous TGFβ3, injected into mice 24, 8, 4 hrs or immediately before irradiation, essentially increased the number of regenerating crypts in the small intestine (3-12 times increase, depending on the radiation dose). In the large intestine, the effect was less noticeable, and the number of regenerating crypts increased no more than 2.5 times [116]. A similar radioprotective action was found for the keratinocytes growth factor (KGF) which is a member of the fibroblast growth factor family (FGF-7). KGF stimulates reparative processes, as was shown in the models of skin wounds and experimentally induced colitis [117-118]. KGF radioprotective effect onto intestinal epithelium is observed only if it is administered before irradiation; the number of regenerating crypts increases by a factor of 3.5 for the 14 Gy radiation dose. Since its receptor is expressed in any epithelial cells, KGF presumably directly affects ISC survival, possibly via the induction of seleno-independent glutathione-peroxidase activity [118]. However, it should be pointed out that intestinal crypts of glutathione-peroxidase deficient mice ($Gpx1^{-/-}$) were more resistant to radiation, than those of the wild-type mice [119]. KGF radioprotective action may also be related to the increase of ISC population and/or their accumulation in the radioresistant S-phase of the cell cycle [102, 120]. A combination of KGF and stem cell factor (SCF) did not additionally boost ISC radioresistance [121], though individually applied SCF improved survival of stem cells of this type in irradiated mice [122]. This fact may indirectly show that the radioprotective effect of these agents is realized via the same mechanisms.

Another class of extensively studied anti-radiation agents for adult stem cells is lipopolysaccharides (LPS). LPS, components of a cell membrane of gram-negative bacteria, are one of the most well-known and powerful modifiers of various mammalian cells (including HSC and ISC) survival upon irradiation in vivo [52, 123]. Note that the important details of the radioprotective action of LPS onto ISC have been found only in the last decade [124]. The regulatory circuit appears as follows: introduction of LPS increases expression of the factor of tumor necrosis alpha (TNF-α) in the small intestine (exact cell origin is not identified) by almost 4 times; TNF-α binds to

TNFR1 in pericryptal fibroblasts and/or villus enterocytes and promotes synthesis of cyclooxygenase Cox-2 and prostaglandin PGE_2 in these cells. RNA-binding protein Apobec-1 is found in the same chain [125]. It should be noted that PGE_2 is not synthesized under the action of LPS in the cells forming a crypt and carrying TNFR1. Such an effect is observed also for murine enterocytes expressing mutant Apobec-1. The increase in ISC survival due to prostaglandin may be relevant to the inhibition of the mechanisms of apoptotic destruction and to the arrest of irradiated stem cells in the G_2-phase that gives them time for the reparative processes [126]. It is not improbable that LPS ability to inhibit apoptosis via suppression of p53 activity plays a certain role in its antithanatogenic action [127]. In general, it is conventional to consider the radioprotective effect of LPS onto stem cells and their microenvironment as a result of the LPS-induced prostaglandins production [124, 128].

Among the low-molecular stimulators of ISC post-radiation survival, dimethylsulfoxide ($OH^{\bullet}$ -radicals scavenger) and retinoic acid (a well-known inducer of cell differentiation) were found [129]. cAMP-phosphodiesterase inhibitors (diethylamino-1-reserpine, 1-methyl-3-isobuthyl-xanthine, theophylline and caffeine) administered to mice shortly before irradiation boosted the number of regenerating crypts by 6-7 times in respect to the control group [130]. The effect in this case did not visibly differ from that for the standard radioprotector WR-2721, which acts at the physico-chemical level via decreasing the degree of DNA radiation damage in various types of adult stem cells [131-132]. Two more groups of substances, opposite in their general effect, are of special interest for radiomodification of stem cells. These two groups include anti-inflammatory agents (by the example of indometacin) promoting death of irradiated ISC, and carcinogens (by the example of azomethane and 1,2-dimethylhydrazine) promoting survival of irradiated stem cells [126,133-135]. For example, indometacin, non-selective inhibitor of cyclooxygenases 1 and 2 (Cox-1, Cox-2), decreases the concentration of prostaglandin PGE_2 both in intact and in irradiated animals, though the latter ones show a rise in the Cox-1 level after irradiation. Administration of this drug to the animals 1 hr after irradiation and then every 8 hrs for 3 days resulted in the dramatic reduction in the number of regenerating crypts [133]. Selective Cox-2 inhibitors did not affect the crypts' survival, as well as the deficit of these genes ($Cox\text{-}2^{(-/-)}$) in irradiated mice. However, neutralizing antibodies to PGE_2 and $Cox\text{-}1^{(-/-)}$-genotype decreased the number of regenerating crypts [136]. As the analysis of these data has shown, prostaglandins switch into the mechanisms of ISC death only in the damaged

epithelium, in the conditions of stress reaction onto cytotoxic action [133]. In contrast, carcinogens - azomethane and 1,2-dimethylhydrazine – administered to mice 1 day before irradiation were able to essentially enhance ISC survival as compared to the control group, where the animals did not receive carcinogens [134-135]. This effect may be related to the damage brought by the carcinogens to the mechanisms of apoptotic removal of stem cells containing radiation-damaged DNA, for example, via inhibiting the activity of p53 gene. At the same time, it was shown using the ISC model that combined application of a carcinogen and indometacin in irradiated mice attenuates radioprotective action in comparison with the effect of the carcinogen only [134], i.e. prostaglandins participate in the realization of carcinogens radioprotective action, either. We have shown recently that 1,2-dimethylhydrazine is capable of exhibiting the same radioprotective action onto murine HSC [135], irradiated in vivo, though additional indometacin administration did not suppress this effect. It is possible that a specially synthesized p53 gene inhibitor - pifithrin-α - has a radioprotective action onto adult stem cells similar to the carcinogens effect [137]. On the other hand, in the mechanisms of pifithrin-α and its analogues action, their ability to inhibit NO-synthase activity may play a certain role, which is characteristic for many radioprotectors with physico-chemical mechanism of action [55].

Rafiomodifying action of the majority of agents investigated mainly using HSC and ISC remains absolutely insufficiently studied for other types of adult stem cells. Among those, we earlier mentioned mesenchymal stem cells (MSC) [6, 23-28], stem cells of spermatogonial epithelium and other epithelia [29-31, 34], and also so-called cancer stem cells [138-140]. At the same time, even those few radiobiological studies conducted with the use of the above types of stem cells allowed finding a number of interesting phenomena. For example, it was revealed that MSC of bone marrow were characterized by a higher radioresistance than HSC of bone marrow in vitro and in vivo [24, 27-28, 36, 141-143]. The magnitude of the mean cell lethal dose D_0 for low-LET irradiation of human and laboratory animals' MSC is 1.4-2.0 Gy and can be even higher (up to 2.5 Gy) after prolonged irradiation [144]. MSC population obtained by cell culture of rats' bone marrow is inhomogeneous and contains fibroblastoid precursor cells forming "compact" and "diffuse" clones which significantly differ in their radiosensitivity [143]. If the linear-quadratic model for the description of the survival-dose dependencies is used for these two MSC subpopulations in vitro, the "α/β ratio" is 12.7 ± 5.5 Gy for precursor cells forming "diffuse" colonies, and is 4.5 ± 3.0 Gy for precursor cells

forming “compact” colonies. These data can be explained on the hypothesis that precursor cells forming “compact” colonies belong to a category of more “primitive” and less committed MSC, while more radioresistant precursor cells forming “diffuse” colonies belong to more committed cell progeny, with partially exhausted proliferative potential. This assumption is consistent with the data obtained in our laboratory on the MSC cultures of patients with Hodgkin’s lymphoma. In these patients, large portions of bone marrow underwent intense radiotherapy resulting in a high proliferative load onto different populations of bone marrow cells. We have shown that the MSC cultures grown from non-irradiated regions of the patients’ bone marrow contain a greater fraction of “diffuse” colonies [145]. We have also found in the experiments on animals that the “oxygen effect” is expressed much weaker for irradiation of this MSC subpopulation in vivo, i.e. these cells are under hypoxia conditions in an organism [146]. There is no question that MSC and ISC remain a good object for studying cell nature of radiation aging [147-148].

As we have recently discovered in the experiments on 5-azacytidine-induced human and rats’ MSC differentiation toward cardiomyocytes, their radioresistance increased [149]. This fact is in a good agreement with the general understanding that this is a characteristic phenomenon in the process of pluripotent adult stem cells differentiation into progenitor cells of certain cell lineage. It would be interesting to observe how MSC radioresistance changes when they differentiate into other numerous cell lineages (for example, progenitor cells for osteoblasts, chondrocytes, adipocytes etc.), but such data have been non-existent so far. Another prospective direction in the radiobiological studies with the use of MSC may be investigation of radiosensitivity of gene-modified MSC cultures, as it is this cell type that is believed to be the most promising agent for gene delivery into different tissues [150-151]. Radiobiological data could be useful in the development of optimal schemes for cell and gene therapy applying MSC and their partially differentiated cell progeny, while the data quantitatively characterizing these cells may be utilized for a peculiar “quality control” of cell cultures industrially manufactured for therapeutic purposes.

A very interesting phenomenon was detected in the radiobiological studies using spermatogonial stem cells - cells existence in the conditions of physiological hypoxia in an intact organism, the fact making this type of adult stem cells relatively radioresistant (the value of D_0 for low-LET radiation is about 1.6-2.4 Gy [29, 36, 152]). Spermatogonial stem cells showed fast

increase in the sensitivity to repeat irradiation due to reoxygenation in the first hours after the first action of ionizing radiation, and also efficient radiosensitization in the presence of electron affinic substances [152-154]. This pool of adult stem cells is characterized by exponential decrease in its size with age, in contrast with stem cells in other systems of cell renewal [155]. A local hyperthermic action (in the range of 41-43^0 C, 30 min long) onto mice testicles before irradiation significantly increased radiation damage of spermatogonial stem cells [156]. A research has been started on the "niche" for this interesting system of adult stem cells [157], which provides both self-maintenance of the stem cell pool and their following differentiation up to the spermatozoa production. Although the key role of the Sertoli cells in this system functioning is of no doubt, studies on the specific programs of interaction of different components of this cell renewal system and their reaction onto damaging agents are in their beginning stage only.

Very little radiobiological information exists on the characteristics of cancer stem cells. These cells are traditionally considered as relatively resistant to ionizing radiation and many chemotherapy drugs [158-160], but the nature of this resistance remains almost unknown. This might be related to the fact that they exist in the conditions of hypoxia in an organism, or they can have p53 function shut down or genes of bcl family activated, they can possess a powerful system of reparative enzymes etc. Study of these mechanisms for developing radio- and combined therapy for patients with resistant forms of tumors has become an urgent task. It is not impossible that the model based on the observation of radiobiological characteristics of adult stem cells for animals treated with carcinogens will become one of the most convenient models, as mentioned earlier [134-135]. Both expanding the set of adult stem cells types (first, by using MSC and their partially differentiated progeny) and monitoring the effects not only immediately after the carcinogen administration but also in a more distant time appear to be promising.

Stem cells within the "critical" systems of cell renewal which preserved their viability after total irradiation are currently considered as certain "determinants" of an organism's survival in the acute phase after a lethal dose irradiation leading to "bone marrow" and "intestinal" forms of radiation death [36, 161]. This is due to the fact that they are the only sources of proliferative recovery of the corresponding systems of cell renewal devastated by radiation. In this case the adult stem cells which survived radiation escaped the death which manifests itself in the form of radiation-induced apoptosis or mitotic catastrophe. Radiobiological analysis performed using "intestinal" form of

mice death has shown that at such radiation doses an average of one or less stem cells survives in the crypts of small intestine. These surviving stem cells may recovery the initial cell population of a crypt relatively fast via proliferation [16]. Those intestinal crypts where no survived stem cells after irradiation still may restore their structure due to the effect of "fission" of near by repopulated crypts [162]. For the hematopoietic system the recovery of hematopoiesis is possible due to the phenomenon of HSC "migration" [163-164]. Death and damage of stem cells also play a crucial role in the development of late non-tumorous radiation damage of different tissues [4]. However, other effects, such as still poorly investigated effects of radiation-induced senescence of stem cells, as well as accumulation of somatic mutations and clonal stabilization, may contribute into the pathogenesis of late radiation damages [147-148]. As for the development of radiation-induced tumors, epigenic disorders and the possibility of the "plasticity"effect (dedifferentiation) may greatly contribute along with somatic mutations in stem cells and disorders in their "microenvironment" [165-166], but the real progress in this direction is expected only in the future research.

Going to the description of the effect of another physical factor - hyperthermia – capable of lethally damaging adult stem cells in vitro and in vivo, we emphasize that thermal biology of stem cells has been poorly developed so far. The research in this direction was stimulated by first attempts of utilizing methods of general and local hyperthermia to boost the efficiency of radiotherapy for resistant tumors in the second half of XX century [167-168]. As has been established in the experiments on the biological basis for such hyperthermia application, heating of normal and tumor cells of humans and laboratory animals in vitro and in vivo to $40\text{-}41^0$ C and more increases their sensitivity to ionizing radiation, besides, heating has its own damaging action onto cells [169-170]. The damaging and radiosensitizing action of hyperthermia at the cell level depends on the temperature and duration of heating, and also on such factors as cells' nature, pH of the medium etc. Hyperthermia is capable of aggravating damaging action of not only ionizing radiation, but also of many chemotherapy drugs used in the cancer treatment [171]. One rather unusual phenomenon is characteristic for the biological action of hyperthermia as compared to the majority of other damaging agents – development of a higher resistance to the repeat hyperthermia observed in a short (1-2 days) time after a mild heating. This phenomenon was called "induced thermotolerance" [169-172]. The basis for the thermotolerance phenomenon is induction of heat shock proteins and a

raise in the activity of DNA polymerase-beta (a key enzyme; suppression of its activity under hyperthermia leads to cell death) [173]. Heat shock proteins are known to perform important functions in the body on sustaining homeostasis and providing reparative processes. They are synthesized not only in response to hyperthermia, but also under the action of many stress agents [174-176]. Therefore, it seemed interesting to investigate damaging and radiosensitizing action of hyperthermia and its ability to induce thermotolerance in adult stem cells. It was shown in the experiments conducted on murine HSC heated in vitro that hyperthermic "dose-effect" curves plotted in a manner similar to that for radiation (X-axis is the duration of heating for each of 4 temperatures in the range of 41-44^0 C, Y-axis is the logarithm of survived fraction of cells) had a typical sigmoidal shape with a small "shoulder dose" and the subsequent exponential region [177]. This finding means that the "single hit multi-target" model suggested in radiobiology can be used for the description of the dependence of adult stem cells survival on the "heat dose". The latter is estimated by the duration of heating at a given temperature. The D_0 for murine HSC heated in vitro and then transplanted to lethally irradiated mice for the survival estimation using spleen "exocolonies" was found to be 29.3, 22.6, 8.1 and 2.8 min for the temperatures of 41^0, 42^0, 43^0 and 44^0 C, respectively. An additional study on the distribution of spleen colonies by their morphological forms showed that it did not differ statistically significantly for stem cells survived hyperthermia and for control intact HSC. In other words, the choice of differentiation for stem cells after hyperthermia in the studied range of temperatures and time of heating was not disrupted. The analysis of the character of D_0 change with temperature showed that this dependence was consistent with the second order kinetics of chemical reactions describing reaction rate vs. temperature [169-170, 178]. The main parameter of such dependence – "activation energy" - was estimated to be about 120-150 kcal/mol for the process of heat inactivation of murine HSC. This value is close to similar parameters for other mammalian cells (but not stem cells) in vitro and in vivo [178-179], i.e. similar mechanisms can be assumed for the damaging action of hyperthermia onto adult cells with different functions. In the same work the radiosensitizing action of hyperthermia onto murine HSC was described. The cell suspension was heated to 43^0 C for 30 min that decreased stem cells survival up to 10% of the initial value. The subsequent irradiation of the preliminary heated cell suspension resulted in the reduction of HSC radiosensitivity according to the test of clonogenic survival with the D_0 being decreased from 0.97 Gy to 0.61 Gy, or about 1.5 times.

Approximately the same degree of HSC radiosensitization was observed if the cells were heated 1 hr before irradiation. However, when the HSC were heated 1 hr after irradiation, the observed reduction in the cell survival was only a simple summative (additive) effect of radiation and heating. These data can be explained as a result of fast reparative processes in the irradiated stem cells which can be blocked only by preliminary heating. The latter conclusion is confirmed by the data on the hyperthermia blocking the activity of DNA repair enzymes, in particular, DNA-polymerase-beta [173, 178, 180]. Similar results on the thermal inactivation and radiosensitization were obtained by our group and other authors for other types of adult stem cells of humans and laboratory animals – progenitor cells of granulocytes-macrophages [181-182], spermatogonial stem cells [156, 183], mesenchymal stem cells [175, 182, 184]. The general conclusion based on these data is that the prepared suspensions of adult stem cells are characterized by almost the same sensitivity toward hyperthermia, while transplanted tumor or leukemic cells often demonstrate higher sensitivity to the heating [168, 179, 182, 185-186]. For the murine leukemic myeloid cell line L1210, it was possible to bind their elevated heat sensitivity to a disorder in the balance of protein expression for the Bcl-2 family, with inclination toward the family members possessing proapoptotic action [186]. At the same time, there are indications that in a tumor tissue some cells can be different in their heat sensitivity which was demonstrated in the study of this sensitivity for several different cell clones obtained from human colon adenocarcinoma [187]. It remains unclear how precisely the data of testing adult stem cells thermosensitivity in vitro correspond to their thermosensitivity in vivo. In our works performed on MSC of rat bone marrow, we obtained "dose-effect" curves for the 43^0 C heating of a prepared suspension of bone marrow cells and for a local heating of a lower extremity (by a controlled microwave radiation). We found that the MSC thermosensitivity in vitro and in vivo did not noticeably differ [182, 184]. In these experiments we also discovered a possibility to induce thermotolerance of MSC in vivo via local action of microwave radiation. This allowed us to create a method of temporary "labeling" of stem cells in an organism to observe certain physiological processes, for example, stem cells migration into other parts of the body upon their damage or when using different actuators of cell migration. These data appear to be of top interest in connection with the development of cell therapy often supplemented with stimulation of autologous bone marrow stem cells migration into various damaged organs and tissues [188].

Taking into account an important role of p53 gene in apoptosis, attempts were made to ascertain this system participation in the hyperthermic death and radiosensitization of normal and tumor cells in vivo. It was shown that hyperthermic sensitization for different types of cells which underwent action of radiation with different LET values manifested itself in the form of activation of apoptotic processes and possibly necrosis regulated by expression of p53, Bcl-2 and Bax [189-193]. The damaging action of hyperthermia alone was found to have no close relation to the activity of p53 gene and was observed for the mutant cells or in the absence of this gene [194-195]. This may be regarded to the fact that a medium-efficiency heat dose may disrupt the process of apoptosis, and also in this case necrosis processes may be realized [196]. Thus, the analysis of the realization of the hyperthermia mechanisms at the cell level and its application in the new methods of anticancer and cell therapy remains an urgent task of the modern biomedical research.

One more interesting problem in the field of agents acting on adult stem cells in vivo is an "ischemia/reperfusion" reaction recently discovered by our group for stem cells of two “critical” cell renewal systems of a whole ogranism’s [197]. This reaction is known to result in cell apoptosis or necrosis in different tissues of a whole organism after acute hypoxia caused usually by temporary disruption in the delivery of oxygen and other necessary components for energetic and plastic demands of tissues. Until recently, it has been investigated only for highly differentiated, usually non-proliferating parenchymal cells of vitally important organs (heart, kidneys, nervous system etc.) [198-200]. A possible development of this reaction in the stem cells within the tissues designed by the principle of cell renewal almost did not attract attention of researchers. However, stem cells in such tissues (primarily, in bone marrow and small intestine epithelium) play a crucial role both in sustaining their physiological regeneration and in the response to the action of damaging agents, especially ionizing radiation and cytostatics [36]. Besides, it has become clear in the last years that the regeneration processes in an organism with damaged vitally important organs (including the case of "ischemia/reperfusion") can be markedly enhanced by delivery of MSC into the “target” tissues. This can be realized through transplantation of autologous bone marrow MSC or by activation of own MSC migration [201-203]. Therefore, we projected a search for indications of the reality of the "ischemia/reperfusion" reaction in stem cells of two “critical” systems of cell renewal (bone marrow and intestinal epithelium). The cells survival was

studied by radiobiological methods after total irradiation using a known radioprotector serotonin to generate a short-lasting acute hypoxia in the tissues during irradiation [204-205]. Sodium nitroprusside (SNP) was used as an agent for testing the "ischemia/reperfusion" reaction in stem cells. SNP is a donor of NO radicals and is known to significantly enhance the cells' probability to survive when administered to animals in the period of "reperfusion (reoxygenation)". NO radicals compete with active forms of oxygen (AFO) and thus diminish their damaging action [198, 206]. In the experiments on the mice from the control group which underwent only total irradiation in the dose of 6 Gy, the number of endogenous spleen colonies was 1.9±0.2 colonies/spleen on the 8th day after irradiation. The injection of hypoxic radioprotector serotonin into mice 10 min before irradiation increased this level to 6.1±0.5 colonies/spleen, i.e. by about 3 times. This was consistent with the results of similar experiments on the evaluation of "bone marrow" survival of mice or on the HSC survival [36, 204, 207]. However, when SNP was administered to serotonin-protected mice immediately after irradiation, it resulted in the further essential growth of the number of endogenous spleen colonies to 13.2±0.7 colonies/spleen on the 8^{th} day after irradiation. At the same time, administration of SNP alone to mice did not produce radioprotective effect, and the number of registered endogenous spleen colonies was only 2.2±0.3 colonies/spleen which did not differ statistically significantly from the control group. The discovered effect of the strong radiomodifying action of the post-radiation SNP administration to mice after prior protection with serotonin before irradiation was quite unexpected and was obtained for the first time for adult stem cells. This effect can be explained as an additional suppression of the "ischemia/reperfusion" reaction in HSC irradiated in the conditions of rather substantial but short-term hypoxia. It may be suggested that the real anti-radiation protection due to acute hypoxia in vivo is actually higher than the observed one, but the majority of survived stem cells (about 50%) undergo apoptosis or necrosis at the stage of reoxygenation because of the "ischemia/reperfusion" reaction. The validity of this hypothesis was tested in the experiments where the post-radiation survival of HSC with different degree of committing (CFU-S-8 and CFU-S-12) was determined by the method of exogenous spleen colonies. In this case the same groups of mice as those in the experiment with exogenous spleen colonies were used as donors of bone marrow. However, the total mice irradiation was conducted in the dose of 2 Gy, and the bone marrow of these animals was obtained 2 hrs after irradiation, when the completion of the

"ischemia/reperfusion" reaction in HSC was expected. The number of colonies (formed by the remaining live HSC) was measured grown in the spleen 8 and 12 days after transplantation of a given amount of donor bone marrow cells into lethally irradiated recipients. The level of the counted spleen colonies was normalized to the number of transplanted cells of bone marrow. The stem cells survival was calculated in comparison with the survival of the same cells from non-irradiated animals. The "ischemia/reperfusion" reaction was also established in this case, being more expressed for CFU-S-8 than for CFU-S-12. This may be related to the fact that more “primitive” CFU-S-12 are likely to exist in an organism in the conditions of relative physiological hypoxia and proliferative resting thus being less vulnerable to the "ischemia/reperfusion" reaction. These considerations are consistent with the previously reported data on the radiobiology of these two HSC subpopulations [208-209]. A similar picture of the enhancement of the serotonin radioprotective effect by the post-radiation SNP administration was also detected in the test of the intestinal stem cells survival using the method of intestinal “microcolonies”. Note that the radioprotective action of serotonin for the stem cells of small intestine epithelium was less marked, and the increase in the survival due to additional SNP after initial serotonin protection was lower, correspondingly. It is possibly related to a lower degree of hypoxia created by serotonin in the intestine, and also to a shorter duration of hypoxia than that in the hematopoietic tissues [210]. The same enhanced radioprotective effect at additional SNP administration was detected in the test on the mice survival for the “bone marrow” and “intestinal” form of death, which reflects the importance of the "ischemia/reperfusion" phenomenon in the organism reaction to the acute radiation damage.

Thus, using radiobiological methods for estimation of adult stem cells survival, for the first time it has become possible to reveal the "ischemia/reperfusion" reaction for such cells in vivo. The primary tasks for the further research in this direction involve elucidating the dynamics of this reaction in the whole organism and study of different modifiers for this reaction. In the latter task, the presence of many inducers of apoptosis and necrosis should be considered, which were found in the experiments on the cell death in tissue culture or in the short-lasting cultures prepared from specially separated parenchymal cells of vitally important organs [211]. From this viewpoint, a “preconditioning” technique is of importance when using adult stem cells (primarily, MSC) for their systemic transplantation into a damaged organism. The “preconditioning” is essential so that transplanted

cells would be able to avoid attack of active forms of oxygen after they reach the damaged parts of tissues in the process of “homing” [212-214]. Our scheme of experiments on the studying the "ischemia/reperfusion" reaction may become useful for studying various ways of “preconditioning” and other methods for creation of favorable conditions to sustain stem cells survival after their transplantation into an organism. On the other hand, it seems important to attempt applying the "ischemia/reperfusion" reaction to destroy cancer cells or at least increase their sensitivity to radiation of chemotherapy drugs. This experiment can be performed using the previously considered model of adult stem cells treatment with carcinogens in vivo.

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Chapter II

Identification of Specific Mitochondrial Proteins Forming Stable Adducts with 4-Hydroxynonenal within Cardiac Tissue of Type-I Diabetic Animals: Implications for Bioenergetics Dysfunction and Onset of Diabetic Cardiomyopathy

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Abstract

Diabetes is a metabolic syndrome associated with the onset of numerous complications. Among these complications, diabetic cardiomyopathy represents one of the most common causes of death in diabetic patients. In recent years, experimental evidence has indicated a major role of reactive oxygen species (ROS) and lipid peroxidation products in the ethio-pathogenesis of diabetes and its complications. Disagreement, however, exists about the sites and mechanisms involved in ROS formation, the role of hyperglycemia in exacerbating their formation, and the effectiveness of antioxidant levels in counteracting the formation of ROS and peroxidation products.

Studies from our laboratory have provided compelling evidence that type-I diabetes is associated with the endogenous production of 4-hydroxynonenal (HNE). Generated via lipid peroxidation, this highly reactive aldehyde rapidly reacts and forms stable adducts with specific proteins within the cardiac mitochondria of diabetic rats. One of the modified proteins has been successfully identified as the FAD containing subunit of succinate dehydrogenase, and the defect has been correlated to a comparable decline in succinate-supported oxygen consumption in the intact mitochondria and in complex II activity in the purified complex. The process leading to HNE production appears to be independent of glycemia level, but is strictly associated to the decline in circulating insulin level. Streptozotocin-injected animals presenting levels of circulating insulin higher than 65 pmol/L show low levels of HNE-induced modification of mitochondrial proteins and near normal oxygen consumption in intact mitochondria despite presenting hyperglycemia levels comparable to those of frankly diabetic animals (i.e. >400 mg glucose/dl). In contrast, frankly diabetic animals present endogenous circulating insulin levels below 25 pmol/L, high levels of HNE-induced modifications, and defects in mitochondrial respiration. Supplementation of the latter group of animals with exogenous insulin for a minimum of 2 weeks results in a marked decrease in HNE-mediated adducts and the restoration of succinate dehydrogenase activity to levels comparable to those observed in non-diabetic animals. The identification of two distinct pools of hyperglycemic animals that present or lack HNE-induced modifications and related mitochondrial dysfunction has provided a new rationale to explain some of the functional inconsistencies observed in mitochondria purified from various tissues of diabetic animals.

Utilizing a proteomics approach, we have identified about 12 mitochondrial proteins that form stable adducts with HNE. Determining to what extent these proteins are functionally impaired by the formation

of adducts with HNE will undoubtedly help to shed light on the role of mitochondrial impairment in the onset of diabetic cardiomyopathy.

Keywords: type-I diabetes, streptozotocin, insulin, diabetic cardiomyopathy, mitochondria, HNE, succinate dehydrogenase, proteomics

Introduction

Since its identification in 1980 [1], more than 2000 references in the literature have highlighted the involvement of 4-hydroxy-2-nonenal (HNE[1]) in a variety of physio-pathological conditions. Generated as a result of free radical formation within the cell, HNE is one of the most reactive aldehydes originated via lipid peroxidation of unsaturated fatty acids such as linoleic or arachidonic acid [2]. The presence of an aldehydic group, a hydroxyl group and an unsaturated double bond within the first 4 carbons of the molecule provide an area of electron density that favors the rapid interaction of the aldehyde with various biological structures, phospholipids and proteins being chief among them, near the site of formation but also at a distance from it. Following its generation and interaction with nearby structures, HNE undergoes a series of intermediate reactions that culminate in the formation of a stable fluorophore by cross-linking to specific amino acids [3,4], or to carbonyl functions present in acyl residues of peroxidized phospholipids in cellular membranes [5]. In both cases, the stable adducts formed can resist degradation and actually tend to accumulate within the cell. Hence, highly specific antibodies have been developed by various groups to identify proteins cross-linked by HNE [3,4], whereas a histo-chemical approach based upon the reactivity of lipid peroxidation products with the Schiff's reagent has been utilized to detect peroxidized phospholipids within biological membranes [5]. Both these techniques have the intrinsic advantage of detecting modifications in proteins and membrane phospholipids under conditions in which no massive HNE production is required, often at an early stage upon its generation.

Numerous studies have capitalized upon these techniques and HNE properties to effectively detect and identify proteins or phospholipids modified by the aldehyde, and assess whether HNE is generated during the aging process or under various pathological conditions. Owing to the hypothesis that aging and cancer are the result of an increased formation of reactive oxygen

species (ROS), free radicals, and peroxidation products [6,7] that overpower the natural antioxidant defenses of the host, these two areas of research were the first to take advantage of HNE detecting antibodies or reaction to Schiff's reagent. Based upon the positive results obtained in these areas, research has subsequently expanded to other conditions including Parkinson's and Alzheimer's diseases, diabetes, and ischemia-reperfusion just to name a few.

The first part of the present review will summarize the key point of HNE formation and its role in the most common diseases. The second part of the review will focus on diabetic cardiomyopathy and the potential role that HNE plays in the onset of this complication. Particular attention will be paid to recently identified cardiac mitochondrial proteins that form stable adducts with the aldehyde, and the implications that possible defects in their functions may have for the cardiac bioenergetics and the overall organ performance under diabetic conditions.

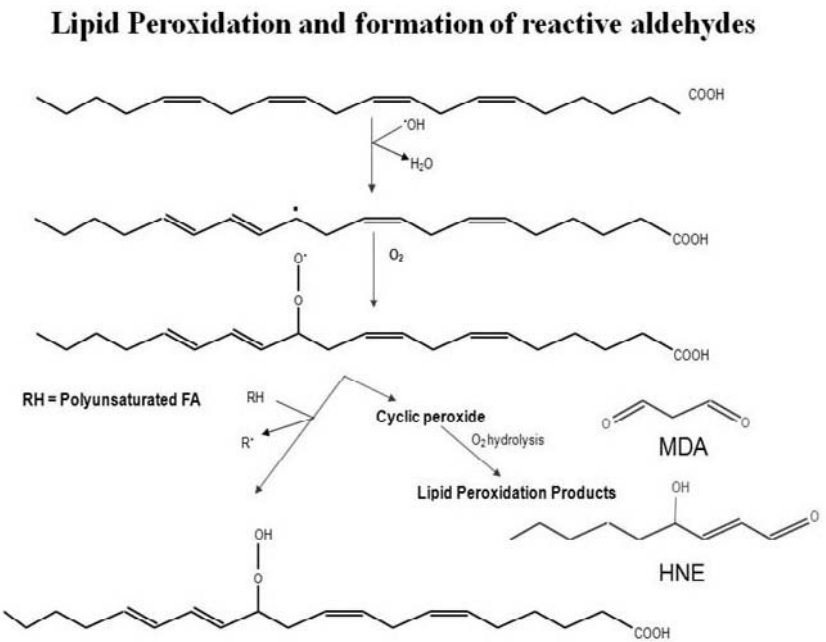

Figure 1. Formation of HNE via lipid peroxidation of unsaturated fatty acids. The figure reports a schematic of the generation of HNE from unsaturated fatty acids (e.g. arachidonic or linoleic acid) upon their interaction with reactive oxygen species.

HNE Formation

Trans-4-hydroxy-2-nonenal (HNE) is a small aldehyde with a molecular weight of 156 Daltons. Formed by a nine carbon backbone, its exact formula is $C_9H_{16}O_2$ [8]. HNE is one of several degradation products resulting from the lipid peroxidation of n-6 polyunsaturated fatty acids, namely linoleic and arachidonic acids, via interaction with superoxide anion radicals (O_2-), hydrogen peroxide (H_2O_2), or hydroxyl radicals (OH•) (Figure 1). Hydroperoxidation of linoleic acid was shown by Schneider et al. to yield, among other products, 9-(S)-hydroperoxy-octadecadienoic acid, which can further decompose into 9-oxo-nonaic acid and nonenal [9]. The nonenal can then be peroxidized into hydroperoxy-nonenal and then reduced to form HNE [10, 11].

HNE is a highly reactive molecule. Its exceptional reactivity can be attributed to three functional groups present within the first four carbons of the molecule. These groups can act in conjunction to enhance the reaction rate of HNE with other biological entities. The C=C double bond between carbons 2 and 3 and the C=O carbonyl group at carbon 1 put a partial positive charge on carbon 3, making it especially prone to nucleophilic attack [12]. In addition, the generated HNE is racemic, yielding (R)- and (S)- enantiomers, with the chiral center being at carbon 4. This results in enantio-selective interaction between HNE and specific enzymes [13]. HNE participates readily in a large array of reactions. Many of these reactions specifically involve at least one of the three reactive sites of HNE. The α,β-unsaturated carbonyl group and the C=C double bond can react with nucleophilic molecules via 1,2- and 1,4 Michael addition (Figure 2). In a 1,2-Michael addition, an amino compound reacts with the carbonyl group of HNE, shedding water and yielding a Schiff base. The Schiff base reaction is a prime contributor to the HNE cross-linking of proteins into fluorophores [14]. In a 1,4-Michael addition, carbon 3 of the C=C double bond in the HNE molecule reacts with a nucleophilic group (e.g. thiol, amine, etc.) ultimately yielding a lactol product (Figure 2) [2,15]. These Michael additions are competitive with one another when primary amines are involved. Nadkarni and Sayre have shown that, in the case of lysine products, the 1,4 Michael addition is the predominant product in aqueous solution, whereas the 1,2 Schiff base product is the primary outcome in the organic phase of a two-phase aqueous-organic solution [15]. In addition to these Michael additions, the C=C double bond can be reduced by an

alkenal/alkenone oxido-reductase using NAD(P)H as a cofactor to form 4-hydroxynonanal, or an epoxide in the presence of a hydroperoxide to insert an oxirane (ethylene oxide) ring into the HNE molecule [14].

The carbonyl group has its own host of common reactions in addition to Schiff-base-forming Michael additions. Reaction of an alcohol with the carbonyl group of HNE results in a stable acetal product. Oxidation of the carbonyl group by aldehyde dehydrogenase and cofactor NAD^+ yields 4-hydroxy-nonenoic acid, and reduction of the carbonyl by alcohol dehydrogenase and NADH results in 1,4-dihydroxy-nonenol. The hydroxyl group on HNE can undergo intramolecular secondary reactions with its own aldehyde group after a Michael addition resulting in lactol hemi-acetal formation, or can be oxidized into a ketone [8, 14].

As a result of any of these reactions, once formed, HNE has a vast array of potential reaction partners within the cell. These partners vary based upon the cell type, the conditions responsible for HNE formation, and the level of antioxidants present within the cell or the extracellular environment. With a half-life of ~3 minutes, the HNE molecule has ample time to traverse the cell in which it has been generated to interact with proteins, lipids, and nucleic acids at sites that can be distant from the site of HNE formation. The reaction of HNE with proteins typically involves specific amino acids such as lysine, histidine, and cysteine, with which HNE forms Michael adducts. If occurring within the active site of a given enzyme, these adducts - which are irreversible for the most part - can hinder or completely inhibit the enzyme activity. It is not necessary, however, that the HNE adduct is formed within the catalytic site of an enzyme to inactivate the protein. In the case of an enzyme formed by different subunits, it is sufficient that HNE interacts with a side chain in one subunit to disrupt the overall enzyme function. This applies, for example, to the observed modification of the mitochondrial FAD containing subunit of SDH [16]. Modified and/or non-functional proteins are normally degraded through the proteosome apparatus. However, the proteosome itself is a target of HNE within the cell [17]. The HNE-induced modification of this degradation structure results in its functional inhibition, and in the consequent intracellular accumulation of stable adducts between HNE and other proteins, ultimately giving rise to the formation of lipofuscins [18]. In the case of lipids, Guichardant et al. [19] have shown that HNE can form Schiff-bases with primary amino groups present in the lipids. This interaction can have far reaching consequences when these lipids are part of phospholipid components within biological membranes (cell membrane or subcellular compartments).

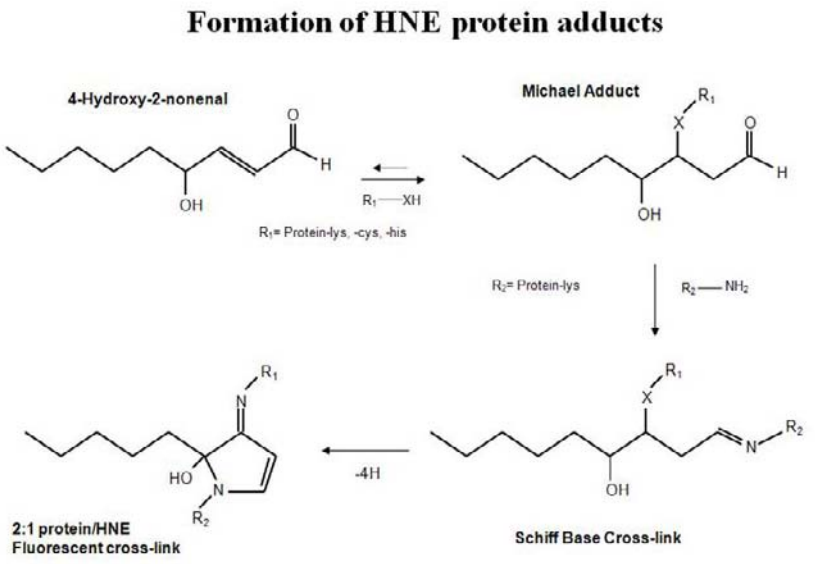

Figure 2. Formation of HNE protein adducts. The figure reports a schematic of the formation of Michael adduct, Schiff Base cross-link, and 2:1 protein/HNE fluorescent cross-link following the interaction of HNE with lysine, cysteine and histidine in target proteins

Table 1. Pathological conditions in which HNE production has been detected.

General Diseases	Refs.
Chronic ozone exposure	122
Diabetes mellitus	34, 123
Genetic hemochromatosis	124
Perinatal asphyxia	125
Post-surgery lymphedema	126
Systemic inflammatory response syndrome	127
Cardiovascular Diseases	
Acute myocardial infarction	128

Table 1. (Continued)

Dilated cardiomyopathy	129, 130
Atherosclerosis	131, 132
Circulatory shock	133
Deep venous thrombosis / Pulmonary embolism	134
Stroke	135
Liver Diseases	
Chronic iron overload	136
Chronic alcoholic disease	136, 137, 138
Chronic hepatitis B	139
Chronic hepatitis C	139
Cirrhosis	136
Non-alcoholic fatty liver disease/steatohepatitis	140, 141
Primary biliary cirrhosis	136
Wilson's disease	136
Neurological Diseases	
Alzheimer's disease	142, 143
Amytrophic lateral sclerosis	144
Brain ischemia / reperfusion injury	145
Cognitive impairment	146
Creutzfelt-Jakob disease	147
Global cerebral ischemia	145, 148
Huntington's disease	149, 150
Multiple sclerosis	151, 152

Parkinson's disease	153
Spinal chord injury	154
Kidney Diseases	
Chronic kidney failure	155, 156
Congenital nephritic syndrome	157
Intestinal Diseases	
Crohn's disease	158
Autoimmune Diseases	
AIDS	159
Lupus erythematosus	160
Multiple sclerosis	151, 152
Rheumatoid arthritis	161, 162
Lung Diseases	
Acute respiratory distress syndrome	163
Chronic obstructive pulmonary disease	164
Deep venous thrombosis / pulmonary embolism	134
Eye Diseases	
Macular degeneration of the retina	165

As for its interaction with nucleic acid, HNE is known to possess mutagenic potential. There are two known mechanisms for the interaction between HNE and nucleic acids. One involves the formation of a standard HNE adduct with guanosine within DNA. The second mechanism entails the post-oxidation epoxide of HNE forming an ethanol-adduct also with guanosine [20-22]. Lastly, experimental evidence has also shown that HNE is capable of inhibiting DNA repair mechanisms [23], *de facto* preventing the removal of the modified guanosine.

Despite the potential damaging effects of HNE, the cell is not without defense to metabolize the aldehyde and mitigate its effects. Under basal conditions, the concentration of HNE seems to fall in the range of 0.05 to 0.15μM [24], but it has been demonstrated by Esterbaur and collaborators that even at a concentration of 100μM HNE can be metabolized within minutes by hepatocyte suspensions [25]. In the body, the two main organs deputed to HNE metabolism are the kidney and the liver. Two groups of HNE metabolites can be found in the urine. The first group of metabolites is represented by mercapturic acid conjugates. In this metabolic pathway, HNE first conjugates with glutathione (GSH) via glutathione-S-transferases (GSTs). The generated conjugate can then be processed in one of three ways [26]. First, the aldehyde of HNE-GSH can be reduced by a member of the aldo-keto reductase superfamily to 1,4-dihydroxynonene (DHN). Second, the aldehydic group can be oxidized by aldehyde dehydrogenase to form a hydroxynonenoic acid (HNA)-lactone-GSH conjugate. In the third catabolic pathway, this HNA-lactone-GSH conjugate can be hydrolyzed into an HNA-GSH product. In the case of all three of these processes, the HNE metabolites ultimately form mercapturic conjugates of the three GSH products. The second type of urinary metabolite is generated by cytrochrome P450 4A, which ω-hydroxylates HNA or HNA-lactone. This lactone can then undergo GSH conjugation to be further metabolized into mercapturic acid. All of these urinary entities are the primary modality of HNE disposal in the body. HNE is also deposited in the bile in the form of GSH and/or mercapturic acid conjugates of HNE, DHN, and HNA. The amount of these biliary metabolites ultimately excreted in the feces is limited by the biliary enterohepatic cycle, which returns them to the liver from the small intestine [26].

HNE and Diseases

An increase in steady-state level of HNE has been observed in numerous human diseases [reviewed in 27]. A list of the most common conditions organized by organs can be found in Table 1. As observed, the list includes neurodegenerative disorders including Parkinson's and Alzheimer's diseases, or amyotrophic lateral sclerosis, cardiovascular diseases such as atherosclerosis, myocardial infarction, or dilated cardiomyopathy, liver pathologies related to alcoholism (cirrhosis, ASH) or independent of alcohol consumption (NAFLD, or NASH), and autoimmune diseases (e.g. lupus

erythematosus). The interaction of HNE with various cellular targets (MAPKs, signaling molecules, enzymes, etc.) results in a broad array of functional modifications for the cell. The interaction of the aldehyde with these various cellular targets will ultimately lead to the activation of programmed cell death or aberrant functions. The most common cellular dysfunctions and related targets affected by HNE are reported in Figure 3.

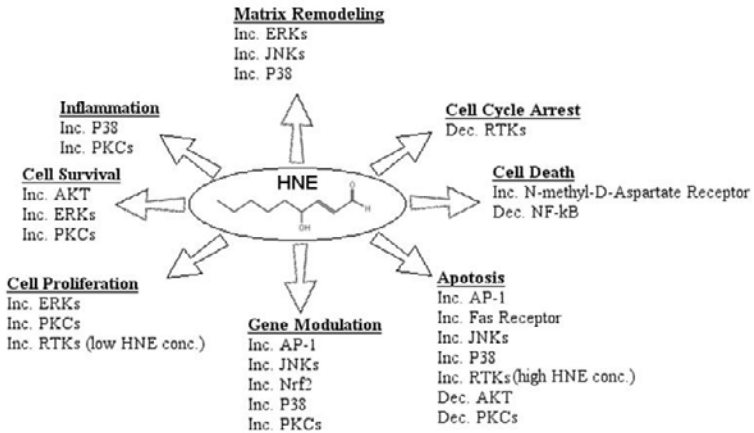

Figure 3. Cellular targets of HNE. A schematic of the various cellular functions and targets affected by HNE is reported.

A description of the potential role of HNE in the development of the various pathologies reported in Table 1, and the modality by which HNE alters the various cellular targets reported in Figure 3 is outside the scope of the present review and therefore we refer the interested reader to the listed references for more detailed information. For the purposes of this review, we will primarily focus on the role HNE plays in diabetes, both type-1 and type-II, and the development of diabetic cardiomyopathy, to conclude with some recent evidence generated in our laboratory.

HNE and Diabetes

Several lines of evidence propose a key role for HNE in the pathogenesis of diabetes and several diabetes-related complications. As for the pathogenesis

of diabetes, increased levels of HNE, among other lipid peroxidation products, have been detected in the beta-cells of GK rats, a model of non-obese rat model [28]. An increased level of HNE has also been observed in rat beta-cell islets incubated with a cocktail of cytokines including IL-1β, TNFα, and interferon-gamma [29]. In this model, cytokines elicit an increased production of HNE and other alkanals, which results in the destruction of beta cells and an accelerated onset of type-I diabetes through a mechanism that specifically involves malondialdehyde (MDA), HNE, and hexanal [29]. Reduced beta-cell mass and presence of oxidative-stress related DNA damage have also been observed in humans [30], further corroborating the results obtained in animal models. In an *in vivo* model of beta-cell damage by oxidative stress, an increased expression of p21 and a decreased expression and production of insulin have been observed [31]. Because p21 acts as an inhibitor of cyclin dependent kinase, an increase in its expression together with a decrease in insulin production suggest the suppression of beta cell proliferation and insulin synthesis, two conditions that contribute to the onset of diabetes. Unfortunately, this study did not determine whether the decreased expression of insulin was related to HNE production, thus preventing us from attributing the decline in hormone synthesis to a direct or indirect effect of the aldehyde. A subsequent study, however, filled this vacuum by providing direct evidence that HNE can indeed inhibit glucose-induced insulin secretion in pancreatic beta islets [32] through a mechanism that is not fully elucidated. Experimental evidence, however, indicates that HNE can alter and inactivate insulin by modifying histidine residues within the hormone peptide chain [33].

Beta cells are not the only site in which production of HNE or modification of proteins by HNE-related adducts has been observed under diabetic conditions. For one, elevated levels of HNE modified albumin have been detected in the serum of patients with type-2 diabetes [34]. Modification of proteins by HNE has been observed in diabetic nephropathy, highlighting a positive correlation between the amount of oxidative stress bioproducts detectable in the urine and the severity of the pathological damage [35]. Immuno-histochemical determinations have confirmed the presence of an increased amount of protein carbonyls modified by oxidative stress in areas of renal glomerular lesions in diabetic patients [36]. Increases in HNE production and in the amount of proteins forming stable cross-link adducts with HNE have also been reported in a pilot study in children affected by cystic-fibrosis-related diabetes [37], and in patients affected by diabetic retinopathy [38].

The exact mechanism by which oxidative stress is elicited under diabetic conditions has not been precisely identified. One of the proposed modalities is through the elevated and sustained glycemia resulting from lack of insulin (type-I diabetes) or from insulin resistance (type-II diabetes). In either condition, the elevated glycemia results in an accelerated and accentuated production of advanced glycation end-products (AGEs) [39]. These products promote an oxidative stress condition in susceptible cells via glycation of specific cellular proteins or by interacting with specific receptors (RAGEs, or receptor for advanced glycation end-products) on the cell surface [40]. The activation of RAGEs then triggers a specific cascade of events within the cell that ultimately results in an enhanced oxidative stress condition and in the generation of ROS and lipid peroxidation products including HNE [40].

Diabetic Cardiomyopathy

Diabetic cardiomyopathy represents one of the most common complications of diabetes, and the primary cause of death for diabetic patients [41-43]. It has been estimated that, irrespective of the type of diabetes, 65% to 70% of all diabetics die of heart-related disease [44]. The incidence is so high that the American Heart Association, the European Association for the Study of Diabetes (EASD), and the Juvenile Diabetes Research Foundation (JDRF) have all identified diabetes as a serious and potentially fatal cardiovascular disease [45 and also http://www.jdrf.org/research/feature/res082201.php].

The incidence and severity of diabetic cardiomyopathy are elevated in both male and female diabetic patients, with the latter population actually exhibiting a greater incidence than their male counterparts [41]. In the UK, the standardized mortality ratios for ischemic heart disease in diabetic patients versus the non-diabetic population are 9-fold higher in males and 42-fold higher in females who are <40 years of age [46]. For patients in the range between 40 to 84 years of age, the mortality ratios attenuate to 4 fold higher in males and 7 fold higher in females for diabetic versus non-diabetic patients [46]. Even when myocardial infarction does not result in immediate death, diabetic patients are three times more likely to progress to congestive heart failure than non-diabetic patients [47]. These results are consistent with those previously provided by the Framingham study, which indicated heart failure incidences in diabetic men and women to be 2- and 5-fold greater, respectively, than in their non-diabetic counterparts [48].

The main contributing factor to the development of diabetic cardiomyopathy has not been clearly identified as of yet. On one hand, major emphasis has been and still is placed on hyperglycemia, considered to be the most likely culprit in the development of cardiovascular complications in type-I diabetic patients. Results from two large studies, the Diabetes Control and Complications Trial (DCCT) and the subsequent Epidemiology of Diabetes Intervention and Complications (EDIC) have consistently indicated that diabetic patients undergoing intensive insulin therapy have fewer cardiovascular insults and a reduced thickening of the intima-media of aorta and main arteries, a criterion largely used as a measure of atherosclerosis, as compared to patients undergoing conventional insulin therapy [49]. The beneficial effects of the intensive insulin therapy were not restricted to the period of the DCCT study but extended to more than 10 years after its completion [50]. These results suggest, although indirectly, that hyperglycemia can indeed have a significant role in the development of cardiovascular insults. This is corroborated by the post-mortem evidence that diabetic hyperglycemia accelerates the presence of fatty streaks in young patients with type-I diabetes even in the absence of dyslipidemia [51-53].

On the other hand, a large percentage of all cases of diabetic cardiomyopathy have been attributed to atherosclerosis, which is frequently present in the same cohort of patients. Unfortunately, it is not always feasible to discriminate between the direct effect of type-1 (or type-II) diabetes and the contributing role of underlying atherosclerosis on the pathogenesis of diabetic cardiomyopathy [reviewed in 54, 55]. This becomes far more complicated in cases in which dyslipidemia is severely accentuated with elevated levels of triglycerides and/or cholesterol. In this respect, the animal models more routinely utilized have not been extremely useful. While still widely used to investigate the potential role of dyslipidemia in the pathogenesis of diabetic cardiomyopathy, many of these murine models present significant drawbacks. One drawback is that the mouse models do not fully mimic the human disease in terms of levels and nature of the altered lipid moieties. Moreover, there is a broad variability of triglycerides level in the blood under basal physiological conditions among murine strains, which complicates the interpretation of the obtained results [54]. A second drawback is that many of these murine models do not show a high degree of atherosclerosis unless a severe hyperlipidemia is also induced either through diet or through genetic manipulations [55]. Lastly, mice do not develop atherosclerotic lesions at the same coronary artery site affected in humans. Humans, in fact, are primarily affected at the first branch

of all the major coronary arteries, a site that is usually protected from disease in mice [56]. More recently, genetic models have been utilized, which more closely resemble the dyslipidemia observed in humans. These models essentially present an Apo-E null mice background in which modest levels of hyperlipidemia (triglycerides and/or cholesterol) are induced through diet manipulations [54, 55]. Provided that the hyperlipidemia remains modest and comparable to that observed in humans, the information obtained in these models has provided some useful insight about the role of dyslipidemia on the onset of cardiomyopathy under diabetic conditions.

Based upon the obtained results, several factors appear to be implicated in the pathogenesis of diabetic cardiomyopathy, including metabolic [57], biochemical [58-60], and ultra-structural changes within the cardiac myocyte [61, 62]. Histo-morphological studies have indicated the presence of defects in cellular organelles including myofibrils, mitochondria, sarcoplasmic reticulum and sarcolemma [63]. Calcium handling and cycling are also severely depressed in the diabetic heart, and result in the derangement of cardiac contraction and relaxation. This derangement is already detectable 3-4 days following the induction of diabetes, at least in animal models [63], and progresses rapidly towards heart dysfunction and heart failure [64, 65]. At least in its initial phase, the heart dysfunction has the stigmata of a left ventricular diastolic dysfunction (LVDD) [66]. As the morphological changes within the myocytes, the extracellular space, and the microvasculature progress, systolic contractility also deteriorates, ultimately resulting in the onset of heart failure [66].

Diabetic Cardiomyopathy: Is There a Role for the Mitochondrion?

Mitochondria are the primary source of energy in the myocardium Under physiological conditions, 60% of all heart energy is provided by fatty acid utilization, with the remaining 40% of the energetic requirement being supplemented by glucose utilization through aerobic glycolysis. In uncontrolled diabetes, glucose utilization decreases to almost zero, whereas free fatty acid utilization through the mitochondrial beta-oxidation cycle increases to provide nearly 100% of the heart ATP requirement [65]. The reduced glucose utilization occurring under diabetic conditions can be ascribed to: 1) a defective glucose transport into the myocytes through the

insulin-dependent glucose transporter Glut4 [67], 2) a reduced expression of the hexokinase II that converts glucose to glucose 6-phosphate for its utilization in the glycolytic pathway [65], and 3) an altered operation of the pyruvate dehydrogenase (PDH) complex, which controls the entry of glucose into the citric acid cycle within the mitochondrion [68]. In the case of PDH complex, an increase in PDH kinase, possibly coupled to a decrease in PDH phosphatase, has been detected in diabetic hearts [66], ultimately leading to a decrease of PDH in its active non-phosphorylated form.

Because of its role in cardiac bioenergetics, mitochondrial function has undergone intense scrutiny. Disruption of the respiratory function of the organelle and the ensuing decrease in ATP production have been regarded as key factors in the development of complications in the diabetic heart as well as other tissues. Consensus about the involvement of mitochondria in diabetic complications, however, is not univocal. Experimental observation about altered mitochondrial functions in diabetic tissues, however, has provided discrepant and often contradictory results. For example, in the case of liver mitochondria isolated from alloxan-treated diabetic rats a deficient ATP synthesis rate and a low ADP/O ratio have been observed [69], a result that corroborates the occurrence of a functional deficit in mitochondria respiration under diabetic conditions. Other reports, however, indicate no significant differences in state 3 and 4 respiration despite a decrease in ADP/O ratio in liver mitochondria from rats rendered diabetic by streptozotocin-injection [70]. A decrease in protein synthesis in conjunction with a decline in state 3 respiration has been observed in mitochondria from skeletal muscles of diabetic rats [71]. Similarly, the assessment of respiratory function in mitochondria isolated from hearts of streptozotocin-injected diabetic rats indicates a decrease in state 3 but not state 4 respiration [72]. Based upon these reports, it is glaringly evident that uncertainty still plagues the field, making it difficult to understand whether mitochondrial respiration is defective under diabetic conditions, and consequently relate any cardiac complication of diabetes to such a defect.

The supporters of a role of mitochondria in the development of diabetic complications in general, and diabetic cardiomyopathy in particular, have advocated the occurrence of defects at both the systemic level and in the mitochondria. Changes in cardiac microvasculature [73], disturbances in metabolism and the release of (neuro)-hormones [60,74], and hyperglycemia and related protein glycation [75-78] have all been indicated as the most probable causes of systemic defects under diabetic conditions. Defects in

mitochondria have been predominantly attributed to an increase in free radical formation, possibly associated with variations in the level of antioxidant or scavenger enzymes. As of yet, there is not definitive proof as to whether either of these factors is the primary cause of mitochondrial dysfunction. In the case of free radicals involvement, for example, the scenario is complicated by the increased production of these moieties within cardiac cells during episodes of micro-ischemia, which amplify the extent of mitochondrial damage in diabetic hearts, making it difficult to discriminate between cause and effect of defective mitochondrial functions and ischemic damage. This raises a second point of contention: are the cardiac myocytes from diabetic animals or patients more susceptible to ischemia/reperfusion injury as a result of a mitochondrial dysfunction? If so, which is the real cause of the cardiac complication, the metabolic or functional changes within the cardiac myocytes (and the mitochondria), or the increased susceptibility to episodes of myocardial ischemia/reperfusion?

From a clinical stand-point, general consensus exists about an increased sensitivity of diabetic hearts to ischemic insult [41,42]. Experimental animal models, however, have provided contradictory results, as both an increased [79-86] and a decreased sensitivity [87-95] of diabetic hearts to ischemia/reperfusion have been reported. To add a further level of complication and uncertainty, different laboratories have reported contrasting results in terms of cardiac function perturbation in diabetic hearts undergoing episodes of ischemia/reperfusion under seemingly similar experimental conditions [96-102]. The problem is clearly multifaceted. Experimental animal models differ in the dose of streptozotocin or alloxan injected to induce diabetes, the duration and severity of diabetic condition, the time of ischemia, the presence of fatty acids or insulin in the extracellular milieu, the level of cellular acidosis, and the duration of zero- or low-flow rate of perfusion during ischemia and the subsequent reperfusion time [86,95].

Tani and Neely [87] and several other groups as well [88-93] have reported a reduced sensitivity to ischemia. All of these studies used streptozotocin in a range of doses varying from 40 to 90 mg/kg bw to induce type-I diabetes for a period ranging from 2 days [87] to 12 months [93]. No clear cause-effect relationship, however, could be established between the onset of diabetes and the reduced sensitivity to ischemia. In fact, based upon the individual experimental conditions, the reduced sensitivity was tentatively explained by the maintenance of cellular ATP via an enhanced glycolitic flux under anaerobic condition [68], a reduced intracellular acidosis via a

decreased Na^+ and Ca^{2+} overloads through the reduced activity of Na^+/H^+ and Na^+/Ca^{2+} exchangers [88,89], and an increased level of free radical scavengers offsetting free radical production and related damage [103].

Just as many studies, however, have reported an increased sensitivity to ischemia [79-85]. Common denominators in these reports are an increased severity and/or a longer duration of the diabetic conditions, often as a result of a higher dose of streptozotocin (or alloxan) utilized to induce the pathology, a low-flow rate ischemia model as opposed to zero-flow rate, which hampers anaerobic glycolysis, and the persistence of a reduced cellular acidosis. Furthermore, several of these studies have utilized long-chain acyl-carnitine and acyl-CoA fatty acids and insulin supplementation in the perfusion medium. The addition of fatty acids especially to the system represents a confounding component as it often results in an increased susceptibility to arrhythmias [58]. In contrast, insulin supplementation would induce in a rapid change in the energetic substrates of choice within cardiac cells during ischemia and subsequent reperfusion, i.e. at a time when the heart is more susceptible to depletion in ATP and ATP-regenerating moieties (e.g. phosphocreatine), and cellular damage is more likely to occur, thus making more difficult the interpretation of the obtained results.

In recent years, the occurrence of oxidative stress under diabetic conditions has been indicated as a possible unifying link between diabetic complications and mitochondrial dysfunction [104,105]. Experimental evidence indicates an increased production of reactive oxygen species and lipid peroxidation products within diabetic mitochondria [106-110], including HNE and MDA [111]. In contrast, over-expression of superoxide dismutase or catalase, or utilization of high doses of antioxidants, which act as ROS scavengers, have all resulted in the decreased formation of lipid peroxidation bioproducts and an amelioration of the diabetic pathology [105,106,111,112]. Whether the increased oxidative stress is the result of hyperglycemia and enzyme glycation as mentioned earlier, or is a consequence of abnormal oxygen utilization by the mitochondrial electron chain due to the absent or reduced flow of proper energetic substrates is still largely debated.

Under normal conditions, less than 2% of all the oxygen utilized by the mitochondria is converted into reactive oxygen species (H_2O_2, OH^- and O_2^-) by the interaction of oxygen with electrons not utilized by the respiratory chain. Scavenging mechanisms, however, keep the formation of ROS under control so that no major lipid peroxidation occurs. The percentage of oxygen re-routed towards ROS formation can be expected to increase when

respiration is altered by defects in complex function, and electrons interact with oxygen rather than redox components of the respiratory chain. While the precise site of formation of ROS within the mitochondria has not been clearly identified, compelling evidence suggests their generation at the level of complex I [113] or complex III [114]. The generated reactive oxygen species have extremely short half-lives spanning from 1 ns (in the case of $^{\cdot}OH$) to 1 μs (for $^{\cdot}O_2^-$), to 1 ms (for H_2O_2), which restricts their area of interaction to their site of formation. HNE and MDA, instead, have a longer half-life, which allows the interaction of both these products with proteins and phospholipids located away from the site of formation, thus enlarging the area of damage.

The identification of proteins modified by HNE is one of the main interests of our laboratory. In particular, our group has been interested in determining whether HNE is endogenously produced within the mitochondrion during diabetic conditions, and identifying which proteins within the mitochondrion and the cardiac myocyte form specific and stable adducts with HNE, thereby yielding a loss of function.

By treating each animal as an individual sample, we have observed the presence of two distinct sub-populations following the experimental induction of diabetes by streptozotocin injection [115]. One population, which accounts for the majority of injected animals (~80%), presents the classical stigmata of diabetes including a marked decrease in body and heart weight (>35%), and serum insulin level (≥75%) as compared to age-matched non-diabetic animals. This population also presents a high level of urinary ketones [16,115]. The second population, which accounts for the remaining 20% of all the streptozotocin-injected rats, shows a level of hyperglycemia comparable to that presented by the first population but little loss in body and heart weight (≤10%), absence of urinary ketones, and levels of circulating insulin equivalent to ~80% of those measured in non-diabetic animals [115]. It is important to stress that the level of glycemia is not a criterion to distinguish the two sub-populations, as glucose level in the blood is ~450 mg/dl in both groups at 4 weeks after streptozotocin injection [115]. It is also worth noting that a 20% decrease in circulating insulin level appears to be already sufficient to affect glycemia. All the mentioned differences between the 2 populations of animals were fully evident within a week from the induction of diabetes and persisted unaltered for the duration of our studies (12 weeks) [115]. For practical purposes, we refer to the first population as *diabetic* and to the second as *hyperglycemic non-ketotic* or *HNK* [115]. Interestingly, only the *diabetic* population presented a ~35% to 40% decrease in state 3 oxygen

consumption in cardiac mitochondria with either glutamate/malate or succinate as energetic substrates [16,115], as well as a 35% to 50% increase in state 4 oxygen consumption with either substrate. The decrease in state 3 and the increase in state 4 oxygen consumption resulted in poor RCR (respiratory control ratio) in the mitochondria from diabetic animals. In contrast, HNK animals did not present any detectable decline in state 3 respiration with either substrate, their function being essentially undistinguishable from that of age-matched non-streptozotocin injected (control) animals [115]. The only functional defect observed in the HNK animals was elevated oxygen consumption in state 4 respiration and only with glutamate/malate and not with succinate as energetic substrate [115]. This increase was approximately 20% at 2 weeks and reached ~30% at 4 weeks after injection of streptozotocin, not changing significantly thereafter. Glycemia was already ~250mg/dL at 2 weeks after streptozotocin injection in both diabetic and HNK animals. Therefore, it does not appear that the increase in state 4 oxygen consumption observed in HNK animals is directly proportional to the glucose level in the blood. It cannot be excluded, however, that the defect is related to the elevated blood glucose level observed in the animals and the consequent glycation of key mitochondrial proteins.

Enzymatic *in vitro* assessment of complex I and complex II activity confirmed the defect in state 3 respiration observed in intact cardiac mitochondria from diabetic animals energized with glutamate/malate and succinate, respectively [16]. When similar assessments were carried out on complex I or complex II activity of cardiac mitochondria from HNK animals, no defect was observed irrespective of the time elapsed from the injection of streptozotocin (Table 2).

Table 2. Enzymatic activities of complex I and complex II (succinate dehydrogenase) in sub-mitochondrial fractions isolated from control, diabetic, and HNK animals.

	Complex-I	**SDH**
2 weeks		
Control	234.9±3.2	48.9±1.4
Diabetic	164.2±2.8$^{\#}$	32.2±2.0$^{\#}$

HNK	nd	49.9±1.9
4 weeks		
Control	246.0±6.0	50.3±3.8
Diabetic	189.4±3.4[#]	33.6±3.4[#]
HNK	nd	49.6±2.3
6 weeks		
Control	226.1±5.8	51.4±2.6
Diabetic	147.0±3.6[#]	31.1±3.5[#]
HNK	223.3±8.9	50.2±2.6
8 weeks		
Control	235.6±5.0	50.2±1.9
Diabetic	166.9±3.3[#]	32.3±3.0[#]
HNK	233.2±5.8	48.9±2.3
12 weeks		
Control	238.7±4.7	49.8±2.4
Diabetic	173.5±2.3[#]	32.7±2.8[#]
HNK	238.9±5.0	51.5±3.6

Values are nmol NADH oxidized/mg protein/min for complex -I activity, and nmol DCPIP reduced/mg protein/min for succinate dehydrogenase (SDH) activity. Data are means±S.E. of n=5 for all experimental groups. [#]Statistically significant vs. corresponding values of mitochondria from non-diabetic or HNK animals.

Supplementation of diabetic animals for 2 weeks with exogenous insulin at a daily dose sufficient to renormalize glycemia returned both oxygen consumption and enzyme activities to normal levels in mitochondria from diabetic animals [16]. Administration of insulin for less than 2 weeks was

insufficient to renormalize these parameters, although it restored euglycemia (~100 mg/dL). The beneficial role of insulin on mitochondria functions is supported by the results obtained in HNK animals. These animals, which present circulating levels of insulin significantly higher than the diabetic animals but fairly comparable to those of non-injected (non-diabetic) animals, do not exhibit major functional defects in terms of succinate-supported state 3 respiration or complex II activity [16].

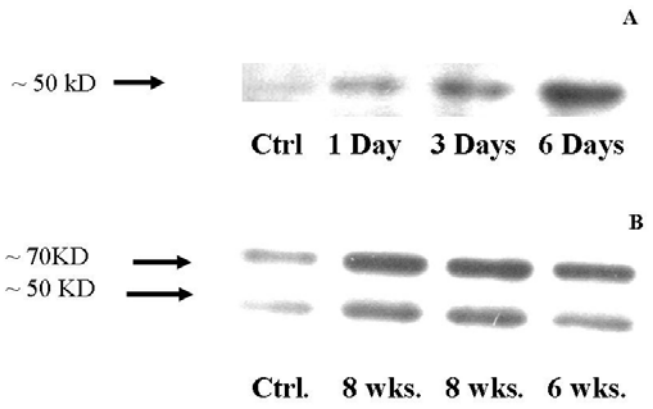

Figure 4. Western blot analysis of mitochondrial HNE adducts. The protein pattern of cardiac mitochondria isolated from 3 days (Figure 3A) and 8 weeks (Figure 3B) diabetic animals and age-matched non-diabetic (Control) animals were separated by SDS-PAGE and western blot analyzed using primary antibodies recognizing 2:1 amino acid:HNE cross-link. A typical experiment out of 4 for each experimental groups is reported.

Western blot analysis of cardiac mitochondria from diabetic animals with antibodies that recognize non-specific modifications of protein carbonyls by ROS indicates the occurrence of wide-spread alteration of mitochondrial protein by these reactive molecules [16]. The use of antibodies that specifically recognize the cross-linked adduct between HNE and side chain amino acids [116] confirmed that the onset of diabetes is accompanied by the endogenous mitochondrial production of HNE [16]. The formation of stable adducts between HNE and selective mitochondrial proteins was already evident within 2-3- days from the induction of diabetes and increased over time, although not in a clear time-dependent manner [16, and also Figure 4A]. The number of proteins modified by HNE appeared to be relatively small,

accounting for about eight bands on SDS-PAGE. The actual number of modified proteins, however, can be expected to be slightly higher than 8 due to the fact that several mitochondrial proteins have a relatively close molecular weight that cannot be accurately resolved by SDS-PAGE, making their recognition and identification rather difficult by Western Blot analysis.

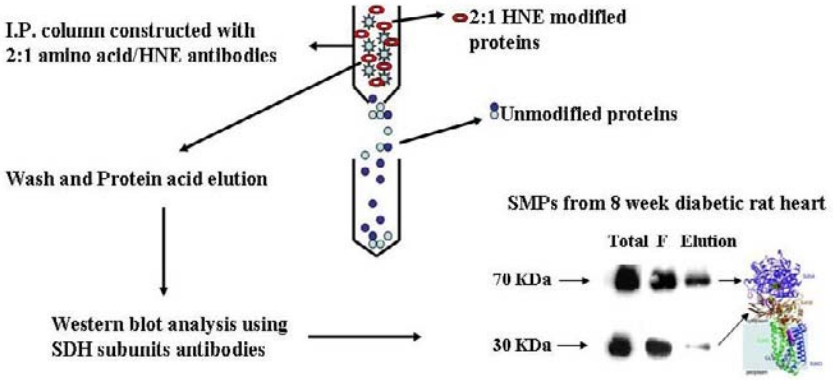

Figure 5. Immuno-precipitation procedure and Western Blot analysis of the 70 kDa subunit of SDH modified by HNE. Antibody-affinity columns were constructed by immobilizing antibodies recognizing 2:1 amino acid:HNE adducts to protein A gel included in the Seize X Protein A immunoprecipitation kit (Pierce) using disuccinimidyl suberate. Detergent-treated mitochondrial membrane fractions from the different experimental groups indicated in the figure were then applied onto the column overnight at 4-C. The column was washed with a buffer containing (in mM): 140 NaCl, 8 Na2HPO4, 2 KH2PO4, and 10 KCl, pH 7.4, to remove unbound antigen. The antigen (HNE-modified proteins) bound to the column was dissociated from the supporting antibody using an elution buffer containing a primary amine (pH 2.8) supplied with the kit. The samples eluted from the column were collected and rapidly neutralized by adding 10 µl of 1 M Tris (pH 9.5) per 200 µl of elution buffer.

One of the proteins modified by HNE that specifically attracted our attention had an apparent molecular weight of ~70 kDa (Figure 4B). Succinate dehydrogenase (complex II) is formed by 4 subunits [117], one of which (the FAD containing subunit) is in a molecular range consistent with the modified 70 kDa band. Furthermore, the activity of this complex was decreased by ~35% under diabetic conditions, both in intact mitochondria and at the

enzyme level. Using a combination of Western blot analysis and immuno-precipitation technique (Figure 5), we identified the FAD containing subunit of SDH as one of the proteins modified by HNE in the 70 kDa molecular range [16]. When a similar analysis was conducted on mitochondria from HNK animals or from 8 weeks diabetic animals treated for 2 weeks with insulin, the levels of HNE-induced modifications were significantly lower than in diabetic animals not treated with insulin, and fairly comparable to those detected in age-matched control animals (Figure 6).

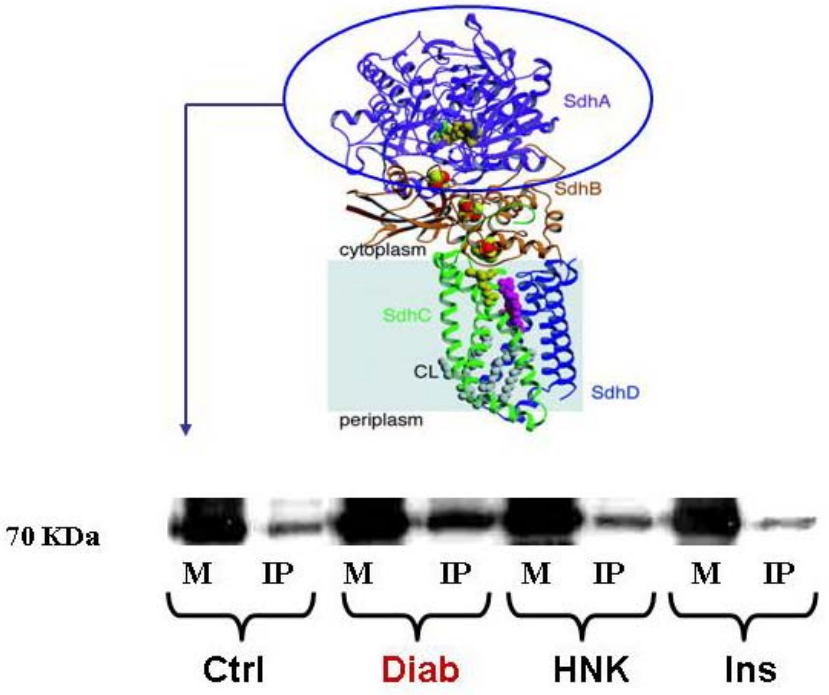

Figure 6. Comparison of the amount of the 70 kDa subunit of SDH forming an adduct with HNE in age-matched non-diabetic (control) animals, diabetic animals, diabetic animals treated with insulin, and HNK animals. Total mitochondria membrane fraction from 8 weeks diabetic (D8w), age matched HNK and control animals, and 8 weeks diabetic animals treated for 2 weeks with exogenous insulin (D8w+2) were purified by differential centrifugation and applied onto an affinity immuno-purification column constructed with antibodies recognizing the 2:1 amino acid:HNE cross-link. Aliquots of the fraction prior to (Total = T) to and post immuno-purification (IP) column were western blot analyzed using monoclonal antibodies recognizing the 70 kDa subunit of SDH. A typical experiment out of 5 for each experimental condition is reported.

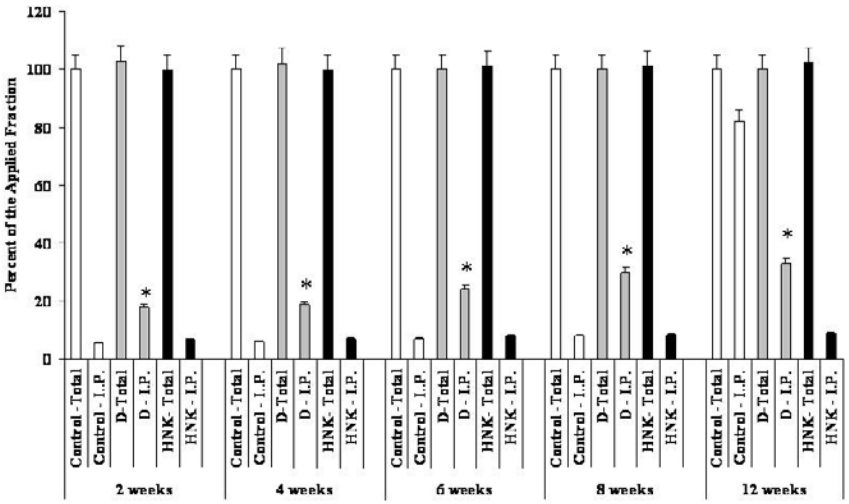

Figure 7. Amount of the 70 kDa subunit of SDH forming an adduct with HNE in age-matched non-diabetic (control) animals, diabetic animals, and HNK animals at periods of time varying from 2 to 12 weeks. Total mitochondria membrane fraction from 2, 4, 6, 8, and 12 weeks diabetic (D), age matched HNK and control animals were purified by differential centrifugation and applied onto an affinity immuno-purification column constructed with antibodies recognizing the 2:1 amino acid:HNE cross-link. Aliquots of the fraction prior to (Total = T) and post immuno-purification (IP) column were western blot analyzed using monoclonal antibodies recognizing the 70 kDa SDH subunit. Densitometry analysis of the IP bands was carried out as described under Materials and Methods. Data are means$\pm$S.E. of 6 different preparations for each experimental group. *Statistically significant vs. corresponding values from ND and HNK samples.

The immuno-precipitation technique we utilized requires a wash-out from the IP column with a very acidic amine buffer (pH 2.8), a procedure that results in the denaturation of the eluted protein independent of HNE modification. This technical limitation has prevented us from confirming that the modification of the protein by HNE results in its functional inactivation. Therefore, we resorted to a series of correlative studies including *in vitro* pre-incubation of non-diabetic mitochondria with varying concentrations of HNE prior to the functional assessment of complex II activity. Although indirect, this approach confirmed the impairment of complex II activity by HNE [16]. We then extended the IP approach and Western Blot identification to animals diabetic for varying periods of time. As Figure 7 indicates, the fraction of

SDH subunit forming an adduct with HNE appears to be a relatively early event, fully detectable within 2 weeks from the induction of diabetes. The percentage of modified protein increases progressively from ~20% to ~35% of the total expression during the first 6 weeks from diabetes induction, remaining relatively stable around this percentage thereafter, irrespective of the duration of diabetes. Similarly, enzyme activity remained consistently depressed by ~30%-35% (Table 2), supporting a close percent correspondence between the amount of SDH subunit modified by HNE and the functional decline in enzyme activity. Throughout the 12 weeks of our study, the percentage of HNE-induced modifications remained relatively low and stable in mitochondria from HNK animals (Figure 7).

These results generate several considerations. First, they would argue against hyperglycemia being the direct cause or being sufficient *per se* to induce the decrease in state 3 respiratory function observed in cardiac mitochondria from type I diabetic rats, and provide compelling evidence that a marked decrease in insulin level has to be attained in order for these modifications to take place. Second, it would appear that insulin plays an essential role in preventing (HNK animals) as well as in reverting (diabetic animals supplemented with insulin) the formation of ROS and the consequent generation of lipid peroxidation products. Third, caution has to be exerted in evaluating changes in mitochondrial function in so-called '*diabetic*' animals without assessing multiple parameters including insulin level. All these aspects need to be taken into proper consideration to determine whether diabetes impairs mitochondrial function, which can then alter the sensitivity of diabetic hearts to ischemia- reperfusion injury. At the same time, these results raise some interesting questions. One of them revolves around the role of insulin and the mechanism(s) this hormone may activate to prevent or reverse oxidative stress damage. The absolute requirement of two weeks of insulin treatment for these defects to be corrected would be consistent with insulin having a major effect at the genomic level in promoting the neo-synthesis of proteins to be incorporated within the mitochondrial membrane as a replacement of the copies modified by HNE, and/or used to counteract free radicals and HNE formation (e.g. GSH transferases). A non-mutually exclusive alternative is that insulin supplementation by restoring euglycemia in diabetic animals prevents glycation of cellular proteins involved in the catabolism and removal of the HNE/protein adduct. The possibility that insulin *per se* enhances the degradation of HNE/protein adducts via the cellular and/or mitochondrial protein degradation pathways is not supported

by experimental results [110]. It is possible, however, that by favoring proper substrate utilization within the mitochondria, insulin prevents the formation of reactive oxygen species and consequently limits lipid peroxidation and HNE generation. Alternatively, insulin could maintain a steady turnover of cellular-encoded SDH (and possibly other proteins) within the mitochondrial membrane. It has to be kept in mind that mitochondrial complexes do not usually operate at a maximal rate and that there is a redundancy of enzyme copies within the organelle. Therefore, the level of protection provided by insulin does not have to be total as mitochondria can tolerate defects in function that affect ≤10% of a specific protein. All of these possibilities open interesting new aspects of research and investigation that have been only marginally tapped.

Another consideration generated by our study is whether the formation of stable adducts between HNE and specific mitochondrial proteins in diabetic tissues is restricted to cardiac mitochondria, or is instead a more general phenomenon. Examination of other major tissues from diabetic animals indicates that HNE adducts within a molecular range similar to those detected in the heart can be observed in liver mitochondria (Figure 8). In the case of the kidney, however, a different pattern of modified proteins is observed (Figure 8). Furthermore, both glutamate/malate and succinate supported state 3 oxygen consumption in kidney mitochondria from diabetic animals are not decreased – as in the case of the heart – but are actually increased by 25% to 35% (Table 3). This result is rather peculiar for several reasons. First, the kidney is one of the main organs undergoing diabetic complications (diabetic nephropathy). Second, experimental evidence supports the formation of ROS and lipid peroxidation products within the nephron [36]. Third, there is a large body of clinical and experimental evidence suggesting an essential role of diabetic nephropathy in the development of diabetic cardiomyopathy [118,119]. The increased oxygen consumption and enzyme activity observed in kidney mitochondria is consistent with a previously published observation [120], but the underlying reason is undefined. This increase in oxygen consumption is not observed in liver mitochondria, which present instead a decline in function (Table 3) as well as a modification of the 70 kDa subunit of SDH by HNE (Figure 8). Hence, it can reasonably be excluded that the observed increase in oxygen consumption and complex activity are related to the gluconeogenic properties of the organ. As the expression of both 30 and 70 kDa SDH subunits in kidney mitochondria are not increased (Figure 9), the

enhanced SDH activity rate appears to be due to a post-translational mechanism not yet identified.

Table 3. Oxygen Consumption in Heart, Kidney, and Liver Mitochondria from Type-I Diabetic Rats

		GLUTAMATE		SUCCINATE	
		State 3	State 4	State 3	State 4
Heart	(ND)	228.6±14.3#	43.8±7.1#	142.2±10.9#	32.4±3.4
	(D)	169.4±12.5	62.8±9.4	111.9±6.4	43.8±10.1
Kidney	(ND)	125.6±19.0#	35.4±3.5	185.9±8.0#	54.5±10.7
	(D)	182.8±18.2	43.3±4.1	236.5±19.7	61.2±7.8
Liver	(ND)	174.2+4.5#	36.8+3.9	195.8+4.7#	48.8±6.5#
	(D)	137.4+9.7	47.1+10.1	172.8±8.1	69.8±9.8

Values are nanoatoms of oxygen/mg protein. Mitochondria were energized by either glutamate (15 mM), or succinate (15 mM) plus rotenone (2 μM). State 3 was initiated by addition of 0.5 mM ADP [**16**]. Data are means±S.E. of n=8 different preparations for cardiac and kidney mitochondria, and n= 4 different preparations for liver mitochondria from both non-diabetic (ND) and diabetic (D) rats. Rats were diabetic for 4 weeks at the time mitochondria were isolated. Age-matched non-diabetic rats were used as controls.

#Statistically significant ($p<0.05$) vs. corresponding values of mitochondria from diabetic animals.

Proteomics Approach

While succeeding in identifying the 70 kDa subunit of SDH as one of the cardiac mitochondrial proteins modified by HNE, our previous study had not unveiled the identity of the other cardiac mitochondrial proteins specifically modified by HNE. Using a proteomic approach in combination with antibodies recognizing adducts between specific proteins and HNE, we have

now succeeded in identifying the entire set of modified mitochondrial proteins (Table 4). While the investigation has been restricted to the mitochondria, it is nevertheless comforting to note that this approach has confirmed the presence of the 70 kDa subunit of SDH in the list of modified proteins. It is also extremely interesting to note that the PDH phosphatase is one of the listed proteins modified by HNE. As mentioned earlier, one of the defects in glucose metabolism under diabetic conditions is the altered operation of the PDH complex [68]. This enzymatic complex controls the entry of glucose into the citric acid cycle within the mitochondria under physiological conditions, and is also responsible for generating sufficient level of CoA for metabolic purposes. The active form of PDH is the dephosphorylated state, whereas phosphorylation of the enzyme by PDH kinase inactivates it. The increased activity of the PDH kinase under diabetic conditions in conjunction with the possible decrease in activity of the phosphatase (modified by HNE) would then result in an increased phosphorylation of the enzyme, with consequent inactivation. Additionally, PDH activity appears to be modulated by PKC-mediated phosphorylation [121], and it is presently unknown whether the enzyme retains this form of regulation under diabetic conditions.

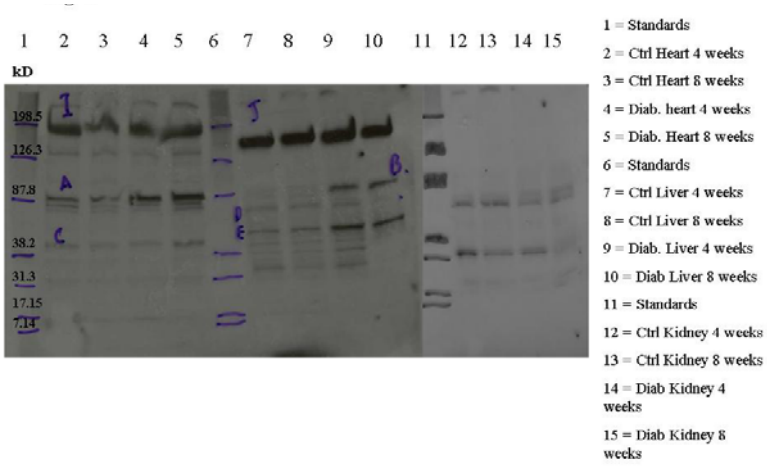

Figure 8. Comparison of HNE/protein adducts among cardiac mitochondria, liver mitochondria, and kidney mitochondria from 4 weeks (Figure 8A) and 8 weeks (Figure 8B) diabetic animals versus age-matched non-diabetic (control) animals. The mitochondrial protein pattern of each experimental sample was separated by SDS-PAGE and western blot analyzed using primary antibodies recognizing 2:1 amino acid:

HNE cross-link. A typical experiment out of 5 for each experimental group at each time point is reported.

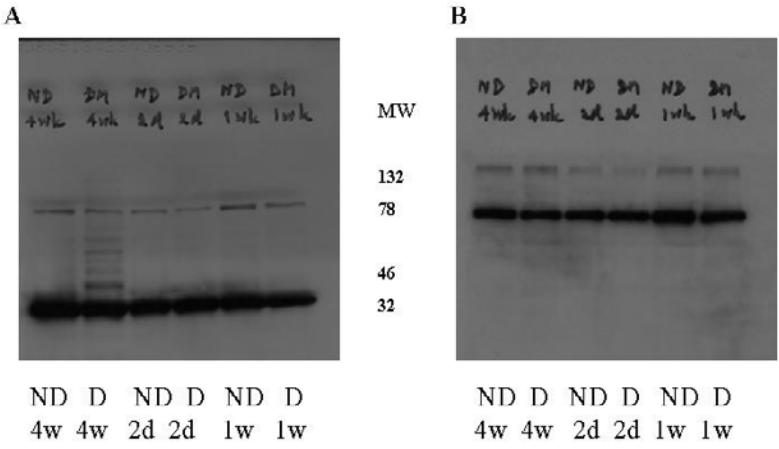

Figure 9. Western Blot analysis of 30 kDa and 70 kDa subunits of SDH in kidney mitochondria from diabetic animals. A typical experiment out of 4 is reported in panel A. Densitometry analysis of 30kDa and 70 kDa subunits expression, normalized per amount of protein applied per lane (20 μg), is reported in panel B. Data in Figure 10B are means±S.E. of 4 different preparations for both non-diabetic (control) and 8 weeks diabetic animals.

Table 4. List of Mitochondrial Proteins Modified by HNE in Hearts of Diabetic Rats.

Dihydrolipamide S-succinyltransferase
Succinyl-CoA Syntetase, beta chain
Aspartate Transaminase (precursor)
Trifunctional Protein beta subunit (hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase)
Trifunctional Protein, alpha subunit (hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase)
Dihydrolipamide S-acetyltransferase, precursor

Propionyl-Coenzyme A carboxylase, alpha unit
TNF receptor-associated protein 1
Hydroxysteroid dehydrogenase-like 2 (HSDL2)
Optic atrophy 1 like protein
Pyruvate dehydrogenase phosphatase
Transferrin precursor
succinate dehydrogenase, chain A[#]

Protein modified by HNE previously identified in our laboratory [16].

In collaboration with the Case Proteomics Center, our laboratory is now involved in determining whether the modification of the listed proteins, in particular PDH phosphatase, correlates with a decline in their function, and in assessing the implications this defect may have for the overall organelle bioenergetics and possibly the onset of diabetic cardiomyopathy.

Conclusions

As evidenced by the information reported here, the field of diabetic cardiomyopathy is still plagued by inconsistencies and uncertainties about the possible involvement of oxidative stress and free radicals in the development of this complication. It is also unclear to what extent oxidative stress and the consequent modification of mitochondrial proteins by HNE and/or other lipid peroxidation products predispose the diabetic heart to ischemia-reperfusion injury. The identification of two distinct subpopulations of streptozotocin-injected animals with very different responsiveness to oxidative stress and mitochondrial dysfunction can help to clarify some of the inconsistencies reported in the field. At the same time, the identification of the mitochondrial proteins modified by HNE can help to design better experimental conditions to test more specific hypotheses about the role of (some of) these modified proteins in the pathogenesis of diabetic cardiomyopathy and its progression towards heart failure.

[1]Abbreviations

HNE	4 hydroxy-2,3, *cis-trans*-nonenal
ROS	reactive oxygen species
SDH	succinate dehydrogenase (E.C. 1.3.5.1)
GSH	reduced glutathione
GST	glutathione S-transferase
DHN	1,4-dihydroxynonene
HNA	hydroxynonenoic acid
MDA	malonyldialdehyde
AGEs	advanced glycation end-products
RAGEs	receptor for advanced glycation end-products
SDS-PAGE	sodium dodecyl-sulfate-polyacrilamide gel electrophoresis
PDH	pyruvate dehydrogenase.

Acknowledgments

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Chapter III

The Bone Marrow Microenvironment in Multiple Myeloma: Cellular and Molecular Basis of Disease Progression

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Abstract

The growth, survival and proliferation of cancer cells are guaranteed by a crosstalk between cancer cells themselves and surrounding host cells and extracellular matrix. An intense area of research has contributed to a better understanding of the pathophysiological modification of tumor progression, e.g., the role of microenvironment.

Multiple Myeloma (MM) is a malignancy of immunoglobulin-synthesizing plasma cells with symptoms mainly related to imbalance of

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bone homeostasis, kidney damage, anemia, impaired humoral immunity, and sometimes nervous system dysfunctions.

Plasma cells home and expand in the bone marrow where cause an unbalanced bone remodelling with increased bone resorption and low bone formation that represent the typical feature in the majority of patients. MM plasma cells are thought to be responsible for the osteolytic bone lesions, which occur by increased osteoclast formation/activity and inhibition of osteoblast formation/differentiation. In physiological conditions, this process is critically regulated by the transcription factor Runx2 and by the Wnt signalling pathway. Moreover, MM plasma cells accelerate the differentiation of resident macrophages to osteoclasts. Finally, plasma cells themselves can transdifferentiate to functional osteoclasts.

Another relevant aspect of the interactions of MM plasma cells with stromal cells in the bone marrow microenvironment is neovascularization, a constant hallmark of disease progression. MM plasma cells induce angiogenesis both directly, via their own factors (vascular endothelial growth factor [VEGF], fibroblast growth factor-2 [FGF-2], hepatocyte growth factor [HGF] and metalloproteinases), and indirectly via recruitment and activation of stromal inflammatory cells to secrete their own angiogenic factors. Macrophages and mast cells play an important role in this sense. They are recruited and activated by tumor plasma cells through the secretion of FGF-2, interleukin-8 (IL-8), and chemokines, such as ITAC, Mig, IP-10. When macrophages and mast cells are activated they secrete potent angiogenic factors (FGF-2, VEGF, granulocyte-colony stimulating factor [G-CSF], granulocyte macrophage-colony stimulating factor [GM-CSF]), which contribute to the tumor neovascularization. Recent evidence demonstrates the vasculogenic ability of active MM macrophages exposed to VEGF and FGF-2, the major angiogenic cytokines secreted by plasma cells, and present in the bone marrow microenvironment at four-to five-fold higher levels than in peripheral blood. Under these stimuli, bone marrow macrophages acquire endothelial cell (EC) markers and transform into cells functionally and phenotypically similar to paired bone marrow ECs (MM patient-derived endothelial cells, MMECs). So they generate capillary-like networks mimicking those of MMECs. Thus, MM macrophages contribute to build the neovessel wall via a "vasculogenic mimicry", hence helping MM progression by this way.

Keywords: angiogenic switch; CXC-chemokines; fibroblast growth factor-2; mast cells; myeloma; plasma cells; stromal cells; tumor progression; vasculogenesis; vascular endothelial growth factor.

Introduction

In the past decades, the major focus of cancer research has been the malignant cell itself. This has led to the identification of oncogenes and tumor suppressor genes and associated signalling pathways by which they modulate growth, survival and proliferation of tumor cells [1]. More recently, newly developed technologies have enabled us to investigate cancer cells at the genomic level. Such gene profiling studies are providing insight into the pathogenesis of the most common human cancers, and help to predict both prognosis and treatment response [2].

Evidence is also accumulating that cancer cells interact with surrounding host cells and extracellular matrix (ECM) [3], this crosstalk affecting the most important aspects of the malignant phenotype, both at primary and secondary tumor sites [4]. Therefore, the role of host cells or the niche microenvironment and ECM is becoming an intense area of research, finalized at a better understanding of the pathophysiological modifications of the complete tumor entity, i.e., malignant cells and microenvironment.

Multiple Myeloma (MM) is a malignancy of immunoglobulin (Ig)-synthesizing plasma cells, that home to and expand in the bone marrow [5]. Although presenting with the same histological features, MM is characterised by a high genomic heterogeneity, as shown by conventional karyotyping, fluorescence *in situ* hybridization (FISH), and by gene expression profiling [6-9]. From the gene expression signature studies, distinct molecular entities have been recently identified, thus contributing to a prognostically relevant molecular classification of MM, and to the identification of molecular features of tumor cells in high-risk disease.

Genetic and genomic studies of MM plasma cells have therefore contributed to the identification of genetic abnormalities involved in pathogenesis [10] and to the new molecular classifications of the disease. However, there is a growing awareness that the interaction between MM plasma cells, stromal cells, hematopoietic cells, and ECM is as important as the genetic changes in the disease progression. Pathophysiological interactions of myeloma cells in the bone marrow microenvironment are highlighted by the progression-associated bone disease and neovascularization, and are witnessed by autocrine/paracrine circuits that activate multiple signalling pathways and affect the most important aspects of malignant phenotype, *i.e.*, apoptosis/survival, proliferation, invasion, and angiogenesis [11].

Unbalanced bone remodelling with increased bone resorption and low bone formation is the typical feature of the majority of patients with bone lesions [12]. MM plasma cells are thought to be responsible for the osteolytic bone lesions, which occur by increased osteoclast formation/activity and inhibition of osteoblast formation/differentiation [12].

A clinically relevant aspect of the interactions of MM plasma cells in the bone marrow microenvironment is neovascularization, a constant hallmark of disease progression [13]. This process is only partially supported by factors such as Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor-2 (FGF-2) and metalloproteinases, which are directly secreted by the tumor cells. In fact, the presence in the bone marrow microenvironment of cytokines, in particular interleukin-6 (IL-6), as a consequence of plasma cell-stromal cell interactions, induces the production and secretion of angiogenic factors by other cells present in the bone microenvironment, thus contributing to the angiogenic switch during the progression of the disease.

Multiple Myeloma

MM is a debilitating malignancy that is part of a spectrum of diseases ranging from monoclonal gammopathy of unknown significance (MGUS) to plasma cell leukemia. First described in 1848, multiple myeloma is a disease characterized by a proliferation of malignant antibody-forming cells (ie, plasma cells) and a subsequent overabundance of monoclonal (M) paraprotein. They cause an unbalanced bone remodelling with increased bone resorption and low bone formation that represent the typical feature in the majority of patients [5].

This imbalance of bone homeostasis with osteolytic lesions cause pain, the main symptom of MM [5]. Occasionally, plasma cells infiltrate multiple organs and produce a variety of other symptoms [14]. The plasma cell clone produces a monoclonal (M) protein that can lead to renal failure caused by light chains (Bence Jones protein) or hyperviscosity from excessive amounts of M protein in the blood [14]. Other symptoms are related to anemia, impaired humoral immunity, and sometimes nervous system dysfunctions [5].

The diagnosis of MM is based on the criteria recommended by the International Myeloma Working Group (IMWG): a) clonal bone marrow plasma cells $\geq$ 10%; b) presence of serum and/or urinary monoclonal protein

(except in patients with true non-secretory MM); c) evidence of end-organ damage that can be attributed to the underlying plasma cell proliferative disorder (hypercalcemia, renal insufficiency, anemia, bone lesions -CRAB) [15]. Other criteria occur to differentiate Symptomatic MM from MGUS (monoclonal gammopathy of undetermined significance), and SMM (smoldering MM or asymptomatic MM) [15].

Regarding staging the current standard is the International Staging System that consists of three stages through the determination of β2-microglobulin and serum albumin [16].

Genomic Sudies in MM

Conventional cytogenetic studies show an abnormal karyotype in only one-third of patients with MM [17]. However, the presence of hypodiploidy [18] or the deletion of chromosome 13 predicts a significantly reduced survival [6]. FISH reveals abnormalities in more than 90% [17] of patients with MM: t(4;14), t(14;16), 17p- that are associated with poor prognosis [19].

MM is characterized by a profound genomic instability involving both numeric and structural chromosomal rearrangements [6]. Nearly half of MM tumors are non-hyperdiploid, and frequently show chromosome 13 deletion, 1q gain and constitutively activated *CCND1* (11q13), *CCND3* (6q), *MAF* (16q24), *MAFB* (20q), or *FGFR3/MMSET* (4p16.3) as a result of chromosomal translocations involving the immunoglobulin heavy chain locus (14q32) [20-23]. The remaining tumors are hyperdiploid, showing multiple trisomies of non-random odd chromosomes and a low prevalence of *IGH* translocations and chromosome 13 deletion [6]. Almost all MM patients are affected by deregulation of one of the *Cyclin D* genes, suggesting their potential important role in the pathogenesis of the disease. The patients can be molecularly stratified into five groups (TC classification) on the basis of known *IGH* translocations and *Cyclin D* deregulation [7;24].

The occurrence of specific transcriptional patterns associated with the major genetic lesions in MM has been extensively demonstrated [7;9;24-28]. In addition, efforts have been made to correlate the expression and clinical data, but it is still difficult to evaluate the clinical relevance of the different molecular alterations, and data seem to suggest that other molecular events apart from those already known may also be relevant [9;29]. More recently, comprehensive cytogenetic and array-based genomic profiling analysis have

been performed, which revealed that virtually the entire plasma cell genotype in MM is affected by local or wide numeric alterations, and identified allelic imbalances and loss-of-heterozygosity (LOH) associated with the clinical and/or biological features of MM [30-33]. Furthermore, the combination of genome-wide DNA analysis with transcriptomic profiles has been performed in order to identify more reliably potential candidate genes [34;35].

Nevertheless, several aspects of MM pathogenesis still remain to be elucidated, and the available molecular data are not sufficient to explain the biological and clinical heterogeneity of the disease.

The recent discovery of microRNA (miRNA) genes, encoding for a class of small non-coding RNAs involved in the regulation of cell cycle, survival and differentiation programmes has added a further level of complexity to normal and cancer cell biology. Through complementary base pairing to specific protein-coding transcripts, miRNAs direct mRNA silencing by message degradation and translational repression [36]. Impaired miRNA expression has already been demonstrated in a number of solid tumors and, more recently, in some hematological disorders [37-39]. To date, only little evidence of miRNA expression/deregulation in MM has been reported: recently, it has been demonstrated that miR-21 can be induced by STAT3 and mediate IL-6-dependent human myeloma cell lines (HMCLs) survival [40]. Successively, Pichiorri et al reported a miRNA microarrays and quantitative PCR (Q-RT-PCR) analysis of HMCLs and PCs from patients with MM, MGUS and normal controls, showing a set of differentially expressed miRNAs that can be associated with neoplastic transformation and progression [41]. Recently, an integrative genomic approach that revealed coordinated expression of some intronic miRNAs with their deregulated host genes has been performed [42]. In particular, it has been monitored host transcript expression values generated on Affymetrix oligonucleotide microarrays in a panel of 20 HMCLs and identified miRNA host genes whose expression varied significantly across the dataset. Moreover, the expression levels of the corresponding intronic miRNAs by Q-RT-PCR has been evaluated, and it has been identified a significant correlation between the expression levels of MEST, EVL, and GULP1 genes and those of the corresponding miRNAs miR-335, miR-342-3p, and miR-561, respectively. Notably, miRNAs and their host genes were overexpressed in a fraction of primary tumors with respect to normal plasma cells, and interestingly, the predicted putative miRNA targets and the transcriptional profiles associated with the primary tumors suggested

that MEST/miR-335 and EVL/miR-342-3p may play a role in plasma cell homing and/or interactions with the bone marrow microenvironment.

These first evidences suggest that, as already extensively observed in other tumours, miRNAs could play a critical role also in MM, and their expression profiling could add a further level to our understanding of its pathogenesis. Important evidences have been shown by Roccaro et al, who has identified a MM-specific microRNA signature characterized by down-expression of microRNA-15a/-16 and overexpression of microRNA-222/-221/-382/-181a/-181b. MicroRNA-15a and -16 regulate proliferation and growth of MM cells by inhibiting AKT serine/threonineprotein-kinase (AKT3), ribosomal-protein-S6, MAP-kinases, and NF-κB-activator MAP3KIP3. Moreover, miRNA-15a and -16 exert their anti-MM activity even in the context of the bone marrow microenvironment. They reduce VEGF secretion from MM cells at the protein level, thereby reducing MM cell–induced proangiogenic activity on endothelial cells. So miRNA-15a and -16 are critical regulators of MM pathogenesis both directly by targeting clonal plasma cells, and indirectly by reducing BM neoangiogenesis and the interaction between tumor cells and BM milieu [43].

Bone Marrow Microenvironment and Disease Progression in Multiple Myeloma

The IMWG has defined the criteria for progressive disease [15], and the American Society of Haematology/Food and Drug Administration (ASH-FDA) has defined specific criteria for disease progression to active myeloma in patients with smouldering myeloma [14]. The cellular and molecular basis of disease progression are fovourited by mechanisms involving the bone marrow micoenvironment: a) bone disease mediated by interactions between plasma cells, osteoblasts, osteoclasts and macrophages; b) neovascularization, that represents the main feature of disease progression and that is supported by all the cellular and extracellular elements of the bone marrow microenvironment; c) bone marrow microenvironment and inflammatory cells as real protagonists.

Stromal Cells in Multiple Myeloma Progression

The MM microenvironment is characterized by the presence of plasma cells, ECM proteins, hematopoietic stem cells and bone marrow stromal cells, including fibroblasts, osteoblasts, osteoclasts, chondrocytes, endothelial cells, endothelial progenitor cells, T lymphocytes, neutrophils, macrophages and mast cells [44]. We have already explained some of the interactions between these components, which determine the proliferation, migration and survival of plasma cells, as well as drug resistance and formation of bone disease [45]. So a permissive stromal environment is important in supporting tumor progression in combination with genetic alterations [46].

Endothelial Cells

Tumor endothelial cells differ greatly from those of quiescent healthy vessels [47]. They proliferate rapidly in keeping with the enhanced angiogenesis that accompanies tumor progression [48]. Their intercellular adhesion and to the ECM during sprouting (that implies cell proliferation and migration) is greatly reduced since they have different profile and level of cell adhesion molecules [49]. Their survival is markedly dependent on growth factors secreted by the tumor and its microenvironment, and on their expression of specific receptors for these factors [50]. They are abnormal in shape and highly permeable due to the presence of fenestrae, vesicles, transcellular holes, widened intercellular junctions, and a discontinuous basement membrane [51]. They share the lining of new vessels with tumor cells able to mimic vessels [52]. The fast growth of endothelial cells and tumour cells, coupled with their structural and functional abnormalities make tumor vessels thin, tortuous, and arborized [53]. As a consequence, tumor blood flow is chaotic and variable and leads to hypoxic and acidic environment that stimulate further angiogenesis [54].

MM endothelial cells intensely express markers of vivid angiogenesis such as VEGFR-2 and Tie/Tek. This implies synergistic activity of VEGF and Ang-2, produced by plasma cells, in the induction of sprouts from existing vessels [47]. MM endothelial cells sizably express CD133, a marker of the progenitor endothelial cells involved in pre-natal vasculogenesis [55]. It has been proved that some CD133+ hematopoietic stem and progenitor cells contribute to the formation of the vessel wall of newly forming blood vessels together with FVIII-RA+, VEGFR-2+, and VE-cadherin+ MM endothelial cells [56]. MM plasma cells and inflammatory cells secrete high levels of

VEGF, FGF-2, and insulin-like growth factor (IGF), which recruit bone marrow and circulating hematopoietic stem and progenitor cells into the tumor microenvironment [57], where they differentiate into MM endothelial cells and participate to the formation of the new vessel wall. High expression of β3-integrin, which prevents apoptosis of endothelial cells and favours their adhesion to the ECM, proliferation, migration, and capillarogenesis [58], also implies vivid neovascularization. Overexpression of endoglin, that enhances the expression of the adhesion molecule CD31, which is the ligand of the plasma cell CD38, by endothelial cells suggests enhanced opportunities for plasma cells to interact with the new-formed blood vessels, enter circulation and disseminate [47]. Frequent interactions between plasma cells and new-formed blood vessels are also mediated by the high expression of E-selectin by endothelial cells [59]. Moreover, MM endothelial cells intensely express a water transporter, namely aquaporin 1, which enhances vascular permeability, facilitates plasma extravasation, increases interstitial pressure, induces hypoxia, and upregulates hypoxia inducible factor-1 alpha (HIF-1α) and VEGF [60].

A paracrine loop for tumor angiogenesis and growth has been demonstrated in MM patients, mediated by VEGF-A and FGF-2 [61,62]. Plasma cells secrete VEGF-A and this induces endothelial cell proliferation and chemotaxis through VEGFR-2, prevalently expressed on these cells, which display constitutive autophosphorylation of VEGFR-2 and the associated kinase ERK-2 [61, 63].

Another important role is played by the paracrine loop existing between MM endothelial cells and plasma cells involving CXC-chemokines and their cognate receptors, which mediate plasma cell proliferation and chemotaxis [64]. Bone marrow endothelial cells express and secrete high amounts of the CXC-chemokines CXCL8/IL-8, CXCL11/interferon-inducible T-cell alpha chemoattractant (I-TAC), CXCL12/stromal cell-derived factor (SDF)-1α, and CCL2/monocyte chemotactic protein (MPC)-1 [64]. Several MM cell lines display a complex expression pattern of chemokine receptors (CXCR, CCR) [64], some of which also mediate the interactions between plasma cells and stromal cells in the bone marrow microenvironment [65].

To summarize, MM endothelial cells show constitutively ultrastructural features of enhanced metabolic activation, an high expression of typical endothelial markers (Tie2/Tek, VEGFR-2, FGFR-2, CD105-endoglin, and VE-cadherin), an high secretion of matrix metalloproteinases-2 and -9, and up-regulation of angiogenic genes (VEGF, FGF-2, Gro-α chemokine,

transforming growth factor beta (TGF-β), Tie2/Tek, HIF-1α, ETS-1, and osteopontin) [47]

Macrophages

Macrophages contribute to tumor angiogenesis, and there are several reports describing an association between macrophage infiltration, vascularity and prognosis [46]. Tumor-associated macrophages accumulate in poorly vascularised hypoxic or necrotic areas [66] and respond to experimental hypoxia by increasing the release of VEGF and FGF-2 and a broad range of other factors, such as tumor necrosis factor alpha (TNF-α), urokinase and matrix metalloproteinases [67]. Moreover, activated macrophages synthesize and release inducible nitric oxide synthase, which increases blood flow and promotes angiogenesis [68]. Lastly, macrophages recruit mast cells [69].

We have demonstrated that bone marrow macrophages in patients with active MM contribute to build neovessels through vasculogenic mimicry, in parallel to progression of plasma cell tumors [70]. Macrophages display oblong and spindle shape with thin cytoplasmic expansions, some of which are either arranged to form microvessel-like lumen or anastomosed with each other and with those of nearby macrophages to form tubular-like structures [70]. Macrophages retain their own CD14 and CD68 lineage markers, indicating that they do not transdifferentiate into endothelial cells, but only adapt functionally, phenotypically and morphologically [70].

Under a synergistic stimulation by VEGF-FGF-2, macrophages undergo a phenotypic and functional adaptation [71], starting to behave like MM endothelial cells. VEGF and FGF-2 bind to VEGFR-1 and FGFR-1, -2 and -3 expressed on monocytes/macrophages surface [72]. VEGFR-1 is involved in macrophage chemotaxis [72] and vasculogenesis [73], but not in the definitive vessel assembly, which is closely dependent on VEGFR-2 [74]. On the other hand, FGF-2/FGFRs system is involved in vasculogenesis [75].

In active MM, plasma cells secrete VEGF and FGF-2 [76;77] and induce macrophage to secrete their own VEGF and FGF-2 [57].

Mast Cells

Mast cells density is strictly correlated with the extent of pathological angiogenesis, occurring in chronic inflammation and tumors [78,79]. Mast cells accumulation has been associated with enhanced growth and invasion of several solid and haematological malignancies [67] and they also act as a host response to neoplasia and display tumoricidal activity in experimental settings

[80]. Mast cells are recruited via several mediators produced by tumor cells, such as c-kit receptor or Stem Cell Factor (SCF) [79, 81], FGF-2, VEGF and platelet derived growth factor (PDGF). Mast cells contain several angiogenic factors including tryptase, chymase, heparin and histamine [82, 83], TGF-β, TNF-α [84], IL-8 [85], FGF-2 [86] and VEGF [87]. Heparin may induce endothelial cell proliferation and migration [80]; histamine has angiogenic effect through both H1 and H2 receptors [82] and also contribute to the hyperpermeability of new formed microvessels during tumor angiogenesis, increasing leakage of plasma proteins and hence deposition of fibrin [80]. Degradation products of fibrin, in turn, are angiogenic in vivo [88]. Moreover, in vitro experiments demonstrated that histamine induces VEGF production in the granulation tissue [89]. Tryptase is the predominant protease in mast cells and it is a potent mitogen for fibroblasts, smooth muscle cells, and epithelial cells [90, 91] and could play an important role in neovascularization favouring the formation of capillary structures via a direct action on endothelial cells [92] or by activating latent metalloproteinases and plasminogen activator [93]. It has been demonstrated that the new-vessels wall appear lined also by typical tryptase-positive mast cells, which are connected by a junctional system with the endothelial cells. Because mast cells keep their lineage marker, they can be regarded as cells that do not transdifferentiate into endothelial cells. This behaviour of mast cells can thus be regarded as an example of vasculogenesis mimicry [94].

It has been also demonstrated that bone marrow angiogenesis, evaluated as microvessel area, and mast cells counts are highly correlated in patients with MM [67]. Both parameters increase simultaneously in active MM [93]. Moreover it has been demonstrated a significant correlation between vessel count and the number of both mast cells and VEGF-expressing cells revealing that mast cells express VEGF mRNA [95].

Multiple Myeloma and Bone Disease

MM plasma cells home and expand in the bone marrow where cause an unbalanced bone remodelling with increased bone resorption and low bone formation that represent the typical feature in the majority of patients [5]. This imbalance of bone homeostasis with osteolytic lesions cause pain, the main symptom of MM [5].

The biological mechanisms by which myeloma plasma cells are able to inhibit formation and differentiation of osteoblasts are not completely clarified. In physiological conditions, this process is critically regulated by the transcription factor Runx2 and by the Wnt signalling pathway [96, 97]. In MM, tumor cell-dependent alterations of these pathways in the bone marrow microenvironment have been postulated. In parallel, myeloma plasma cells accelerate the differentiation of resident macrophages in osteoclasts and plasma cells themselves can transdifferentiate to functional osteoclasts [98].

A significant increase in both the recruitment of new osteoclasts and single osteoclast activity occurs in the close vicinity of MM plasma cells, suggesting that bone disease results from local production of Osteoclast Activating Factors (OAF) secreted by either MM plasma cells or stromal cells [99]. Examples of molecules that are implicated in bone resorption are: IL-6, IL-1α or –1β, IL-11, TNF-α, TNF-β, M-CSF [99]. In particular, the Receptor Activator of Nuclear Factor Ligand (RANKL), the decoy receptor osteoprotegerin (OPG), and the chemokine Macrophage Inflammatory Protein-1α (MIP-1α) [100] are involved in bone resorption. RANK ligand is expressed by stromal cells and binds to its receptor (RANKR), which is present on osteoclasts, triggering differentiation and activation signals in osteoclasts precursors, and thus promoting bone resorption. OPG is a naturally occurring factor that antagonises the effects of RANKL and so preserves bone integrity. Adesion molecules, such as β1-integrins, mediate the binding of MM plasma cells to stromal cells and VCAM1 induces overexpression of RANKL in both cell types and suppresses OPG production by stromal cells. In addition, MM plasma cells internalise and degrade OPG within their lysosomal compartment. MIP-1α secreted by MM plasma cells induces expression of RANKL in stromal cells and probably acts directly on osteoclast precursors to induce recruitment, late-stage differentiation and finally their activation [100]. Furthermore, tumor cells interfer with the regulation of the bone resorption mediated by the transcription factor Runx2 and by the Wnt signalling pathway [96, 97]. Osteoblast differentiation from bone marrow osteoprogenitor cells is critically regulated by the transcription factor Runx2 [12], whereas the Wnt signaling pathway acts through its canonical pathway mediated by beta-catenin nuclear translocation. Myeloma cells exert a strong inhibition of Runx2 through the cell-to-cell contact [98], and the secretion of IL-7 and DKK1, a Wnt inhibitor released by malignant plasma cells, has been correlated to the occurrence of bone lesions in MM [101].

Moreover, the excess of RANK-L by stromal cells and malignant plasma cells primarily accelerates the differentiation of resident macrophages in osteoclasts. In parallel plasma cells themselves can transdifferentiate to functional osteoclasts [13]. Myeloma cells may directly resorb the bone tissue in MM bone disease in relation to their transdifferentiation to functional osteoclasts [13]. $\alpha_v\beta_3$ is greatly up-regulated by myeloma cells from patients with severe MM bone disease and drives their extravasation, adhesion through a RGD (arginine-glycine-aspartic acid) domain and invasion of the bone substrate. Deranged intracellular signaling by $\alpha_v\beta_3$ through RGD may prompt the expression of genes committed to regulate the production of osteoclastogenic factors such as TRAcP, vacuolar ATPase, carbonic anhydrase and cathepsin K that are normally produced by osteoclasts in their resorptive functions of both organic and inorganic components of the bone tissue. Molecular events driving these functions involve ITAM-bearing proteins, Syk, c-Src, and c-Fms transcription factors, leading to NFATc1 as final osteoclast transdifferentiating factor in myeloma cells.

Angiogenesis in Multiple Myeloma

Angiogenesis is the sprouting of new blood vessels from a pre-existing vasculature and it is a tightly regulated process [102].

During embryogenesis two major processes of blood vessel formation are implicated in the development of the vascular system: vasculogenesis and angiogenesis [53;103]. Vasculogenesis starts from mesodermal-derived cells, the hemangioblasts, which differentiate both into angioblasts-endothelial cells and into hematopoietic stem cells [103]. Vasculogenesis prevails in the embryo but it may have physiological roles in health and disease in adults [104]. Both mechanisms, angiogenesis and vasculogenesis, occur in ischemic and tumor tissues in response to growth factors, such as VEGF and bFGF, produced by tumor and stromal cells [105]. In some aggressive tumors the vessel wall is lined with only cancer cells as a mosaic of cancer cells and endothelial cells. This phenomenon is called *"vasculogenesis mimicry"* [106].

Angiogenesis is uncontrolled and unlimited in time, and essential for tumor growth, invasion and metastasis during the transition from the avascular to the vascular phase [11]. The angiogenic switch is preceded by the expression of oncogenes (c-myc, c-fos, c-jun, ets-1) coding for angiogenic factors, and activated as a consequence of immunoglobulin translocations and

genetic instability of plasma cells [107]. So tumor plasma cells acquire an angiogenic phenotype due to clonal expansion and epigenetic modifications (hypoxia, shear stress) [58]. There is a shift from CD45-positive to CD45-negative plasma cells that produce VEGF [108]. VEGF stimulates proliferation and chemotaxis in both endothelial cells and stromal cells [58]. These cells are rapidly phosphorylated by the interaction with VEGF, and signal via extracellular signal-related kinase-2 [109]. VEGF acts as an autocrine inducer of growth and chemotaxis via VEGER-1 [110]. It increases IL-6 (a major growth and survival factor for MM plasma cells) production by bone marrow stromal cells via VEGFR-2 and thus forming a paracrine loop for tumor growth [111] and angiogenesis. Moreover, adhesion of plasma cells to bone marrow stromal cells increases VEGF secretion by both cell types [112], and so enhances angiogenesis. VEGF production by plasma cells is also regulated by TNF-α of bone marrow stromal cells [113]. TNF- α mediates upregulation of adhesion molecules of plasma cells and bone marrow stromal cells, and thus enhances heterotypic adhesions and activates IL-6 secretion by bone marrow stromal cells [114]. TNF-α secreted by plasma cells induces upregulation of adhesion molecules on both MM plasma cells and bone marrow stromal cells [115], thereby increasing the binding of MM plasma cells to bone marrow stromal cells with associated cell adhesion mediated-drug resistence and induction of IL-6 and VEGF secretion by bone marrow stromal cells [116;117], which mediates MM cell homing and migration, as well as angiogenesis [118]. VEGF signalling also contributes to inhibit antiangiogenic signals such as semaphorin3A (SEMA3A) whose autocrine loops are usually activated to self-limit physiologic angiogenesis [119].

FGF-2 is an other important angiogenic growth factor, and it represent a potent activator of endothelial proliferation and can thus stimulate angiogenesis, promote stromal fibroblast proliferation and extracellular matrix formation leading to excessive bone marrow fibrosis and can directly affect neoplastic cells by acting on their high affinity FGFRs [120]. FGF-2 increases IL-6 secretion; conversely IL-6 enhances FGF-2 expression and secretion by MM plasma cells [120], thus forming a paracrine IL-6/FGF-2 cross-talk between MM plasma cells and bone marrow stromal cells that triggers neovascularisation as well as MM cell growth and survival [121].

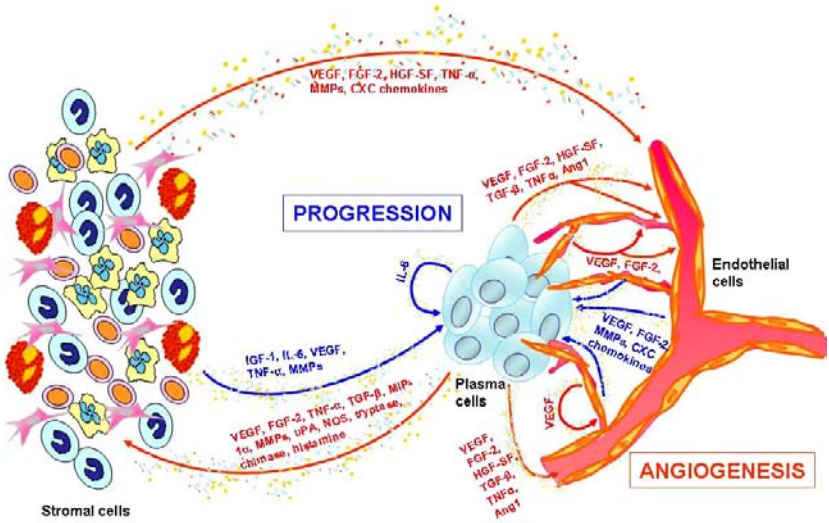

Figure 1 Angiogenesis in MM: autocrine and paracrine loops in the myeloma bone marrow.

Vasculogenesis in MM

Vasculogenesis, i.e. the in situ differentiation of the primitive endothelial progenitors known as angioblasts from groups of mesodermal cells into endothelial cells that aggregate into a primitive capillary plexus, is responsible for the primary development of the vascular system during embryogenesis [122].

This process is particularly important for vascular development , e.g., the formation of the yolk sac vasculature, of the heart, and of the dorsal aortae [123]. Important evidences suggest how the bone marrow neovascularization is partly formed by postnatal vasculogenesis [124].

Various studies have suggested that endothelial stem cells may persist into adult life, where they contribute to the formation of new blood vessels [125], and that in the post-natal life vasculogenesis may also occur [124]. Isolation of putative endothelial progenitor cells from peripheral blood was initially suggested by Asahara *et al* [126]. Peripheral blood $CD34^+$ cells expressing VEGFR-2 were cultured on fibronectin-coated plates for 4 weeks, and the attached cells sotein couplehowed a typical spindle-shaped morphology, the

uptake of acetylated low-density lipoproteins (Ac-LDL) and the expression of several endothelial cell markers (CD34, CD31, Flk-1, Tie-2 and E-selectins). More recently, it has been confirmed that 0·1-0·5% of circulating $CD34^+$ cells express VEGFR-2/KDR receptors and that pluripotent haematopoietic stem cells are restricted to this fraction [127].

It has been demonstrated that angiogenesis is an important process in MM progression and represents an important prognostic factor [57;77]. Moreover, the in vitro generation of endothelial cells from haematopoietic stem cells mobilized in MM patients and their expansion and differentiation into endothelial cells in the presence of angiogenic cytokines has been obtained [56]. These data also demonstrate that in the bone marrow of MM patients, but not of MGUS patients, some isolated endothelial cells express on their surface the typical endothelial cell markers, such as factor VIII-related antigen (FVIII-RA), vascular endothelial-cadherin (VE-cadherin), VEGFR-2, and TIE/Tek, as well as the CD133 staminal antigen whose expression was found in the microvascular wall together with FVIII-RA or VE-cadherin in some active MM patients.

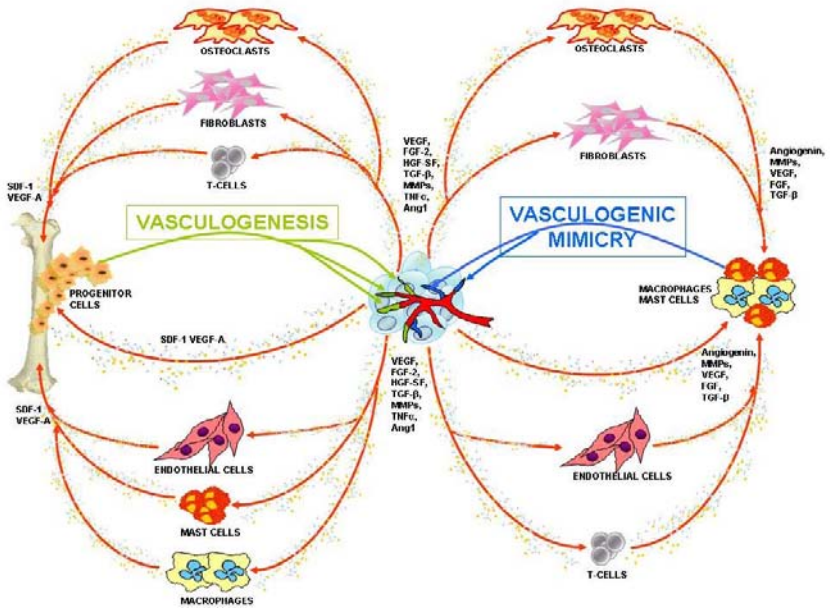

Figure 2 Vasculogenesis in MM: autocrine loops in the myeloma bone marrow.

In healthy subjects, cells of monocyte lineage (other mesodermal-derived cells) can generate endothelial cell progenitors [55]or act as pluripotent stem cells [128]. They can develop an endothelial cell phenotype, especially when stimulated by VEGF and/or bFGF [128;129], and produce a functional capillary-like mesh [130] permeable by blood cells [131], hence recapitulating embryo vasculogenesis [103].

Bone marrow monocytes and macrophages of MM patients can be induced to assume a number of endothelial cell properties and form capillary-like structures *in vitro* through vasculogenesis. Moreover, macrophages contribute to build neovessels in MM through vasculogenic mimicry, and in MGUS they are prone to a vascular switch that marches in step with the progression toward MM [73]. In fact, MM bone marrow macrophages exposed to VEGF and bFGF develop a number of phenotypic properties similar to those of paired bone marrow endothelial cells, and form capillary-like structures morphologically mimicking those produced by MM endothelial cells. At the ultrastructural level, MM macrophages exhibit numerous cytoplasmic extroversions arranged in tube-like structures [73]. All these features are lacking or minimal in macrophages of patients with MGUS or with benign anemia which, however, will become phenotypically and functionally similar to those of MM under angiogenic stimulation [73]. Bone marrow biopsies of MM, but not of MGUS, harbour 'mosaic' vessels since these are formed by MM endothelial cells, endothelial cell-like macrophages and macrophages themselves [73].

All these observations are indicative that: *i)* angiogenic stimuli provided by VEGF, FGF-2 and IGF released in the bone marrow microenvironment of MM patients by myeloma plasma cells are sufficient to recruit haematopoietic stem cells and macrophages to the tumor bed and induce their differentiation into endothelial cells contributing to the tumor vasculature; *ii)* haematopoietic stem cells and macrophages may be a source of endothelial cells in the bone marrow of MM patients during disease progression; *iii)* vasculogenesis, contributing together with angiogenesis in MM, is induced by cytokines secreted by myeloma plasma cells.

Targeting the Bone Marrow Stromal Cells

The actual therapeutic strategies of MM consists of conventional chemotherapy in combination with biologically based therapies in various settings, targeting not only the MM plasma cells but also its microenvironment and new therapeutic targets are currently available [131].

Proteasome inhibitor bortezomib (Velcade, formerly PS-341), a boronic acid dipeptide, is a potent, highly selective, and reversible proteasome inhibitor that targets 26S proteasome complex and inhibits its function [132]. The 26S proteasome is an ATP-dependent multicatalytic protease mediating intracellular protein degradation [133]. Proteasomal degradation of misfolded or damaged proteins proceeds by recognition of polyubiquitinated proteins by the 19S regulatory subunit of the 26S protease and subsequently hydrolysis to small polypeptides [133]. Besides eliminating damaged/misfolded proteins, the proteasome also regulates key cellular processes, including modulation of transcription factors, such as NF-κB, cell cycle progression, inflammation, immune surveillance, growth arrest, and apoptosis [134].

Bortezomib has inhibitory effects on the NF-κB activity in MM cells. NF-κB is a major transcriptional factor which mediates the expression of many proteins including cytokines, chemokines, cell adhesion molecules, as well as those involved in anti-apoptosis and cellular growth control [134]. Its activity is regulated by association with IκB family proteins [135]. Varius stimuli, including cytokines, such as TNFα and IL-1β, trigger phosphorylation of IκB protein by IκB kinase [136]. Phosphorylated IκB is subsequently polyubiquitinated by specific enzymes and degradated by the 26S proteasome [136], which allows p50/p65 NF-κB nuclear translocation and binding to consensus motifits in the promoter region of target genes [134]. Expression of adhesion molecules, such as ICAM-1 and VCAM-1, on both MM cells and bone marrow stromal cells are also regulated by NF-κB [115, 137]. Thus inhibition of NF-κB by bortezomib downregulates these adhesion molecules, thereby enhancing susceptibility of MM cells to therapeutic agents in the context of the bone marrow milieu [134]. Another important aspect is that induction of IL-6 transcription and secretion by bone marrow stromal cells is mediated via NF-κB activation which, in turn, increases secretion of other cytokines, such as VEGF, from MM plasma cells [111]. Furthermore, MM cell adherence to the bone marrow stromal cells triggers IL-6 secretion via NF-κB activation, associated with an increased MM cell growth [134] and

leads to a reduction of VEGF secretion. Bortezomib significantly blocks both constitutive and MM cell adhesion induced by IL-6 secretion from bone marrow stromal cells [134]. Bortezomib is also directly cytotoxic, triggering stress response and apoptotic signalling via multiple pathways [134]. As the result of inhibition of proteasome activity, it causes the accumulation of misfolded polyubiquinated proteins, resulting in endoplasmic reticulum stress which triggers caspase-4 and downstream signalling [138]. Bortezomib also induces ROS which play a critical role in the initiation of the apoptotic cascades by disruption of membrane potential and the release of cytochrome c from mitochondria, followed by caspase-9 activation [139]. Proteasome inhibitors have a potent activity against mitotic endothelial cells, so they target aberrant blood vessel development associated with tumor growth, in fact, bortezomib inhibits the proliferation of MM endothelial cells associated with downregulation of VEGF, IL-6, IGF-I, Ang-1 and Ang-2 [132]. Moreover, bortezomib inhibits DNA repair activity by cleavage of DNA dependent protein kinase catalytic subunit (DNA-PKcs), thereby restoring sensitivity to DNA-damaging chemotherapeutic agents, such as doxorubicin and melphalan [140]. Bortezomib also down-regulates caveolin-1 tyrosine phosphorylation, which is required for VEGF-mediated MM cell migration, and also blocks the caveolin-1 phosphorylation induced by VEGF (transcriptional target of NF-κB) in endothelial cells, thereby inhibiting ERK-dependent cell proliferation. It inhibits the transcription of important adhesion molecules such as ICAM-1, VCAM1 and E-selectin [141].

Thalidomide has a direct tumoricidal activity, an antiangiogenic effect and modulates TNF-α signalling through direct and/or indirect effects on the tumour microenvironment [142], reduces FGF-2 [143], VEGF and IL-6 secretion in bone marrow stromal cells and by MM cells [144]. It also stimulates the activation and expansion of T cells and augments NK-cell – mediated cytotoxicity through its direct effect on T cells with a consequent increase in IL-2 and interferon gamma (IFN-γ) secretion [145], and interferes with NF-κB activity by blocking its ability to bind to DNA or suppresses IκB kinase activity, thus abrogating normal inflammatory cytokine production [146]. Thalidomide also disrupts the host marrow-MM cell interactions by selective modulation of the density of cell surface adhesion molecules [147]. Treatment with thalidomide is associated with sedation, fatigue, constipation, rash, deep-vein thrombosis, and peripheral neuropathy [148]. Lenalidomide, a derivative of thalidomide, is less toxic and more potent than the parent drug [149]. In patients with relapsed or refractory MM, lenalidomide can overcome

resistance not only to conventional chemotherapy but also to thalidomide [150;151].

The bisphosphonates are other compounds that, although originally used to reduce bone loss in MM due to an anti-osteoclast activity, have also been shown to have a direct effect on MM cells [152]. In fact, zoledronic acid has a direct cytotoxic activity on tumor cells and suppresses angiogenesis [153, 154], inhibits FGF-2- and VEGF-dependent proliferation of endothelial cells and inhibits VEGFR-2 in an autocrine loop [152]. Neridronate exerts its antiangiogenic activity through both a direct effect on endothelial cell proliferative activity and inhibitory effect on the responsivity of the endothelial cells to the proliferative stimuli mediated by angiogenic cytokines [155]. The use of bisphosphonates can cause the osteonecrosis of the jaw (ONJ), a long-lasting disorder that occurs mainly in breast cancer and MM patients treated with intravenous bisphosphonates [156].

Conclusions

The bone marrow microenvironment plays a crucial role in the pathophysiology of MM. It is involved in the crosstalk between plasma cells and bone marrow stromal cells, which increases the survival, proliferation and migration of tumor cells themselves, and represents the substrate for angiogenesis which favours the disease progression. Due to interaction with active microenvironment, MM plasma cells also acquire drug resistance giving less opportunity to therapy response.

Many research studies have tried to better understand the biological mechanisms and the genetic basis of all the interactions between MM cells and bone marrow stromal cells. VEGF, FGF-2, IL-6, macrophages, mast cells, and many others cells and molecules, play the most important role in this process.

Furthermore, several studies have focused their investigation on novel drugs targeting the MM plasma cells and the microenvironment cells. Good results have been already obtained but MM still remains an incurable malignancy, indicating that the role of bone marrow microenvironment is important in MM progression, but its role is still not completely clear.

The future goal for MM therapy may be the simultaneous block of plasma cell proliferation and survival, plasma cells/ bone marrow stromal cells

interaction, and bone marrow stromal cells activity by the combination of biological target drugs.

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Chapter IV

Respiration and Oxidative Metabolism in Spermatozoa

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Abstract

Energetic metabolism is crucial for maintaining the biological function of all cells. Energy sources and redox state depend on metabolism of the oxidative substrates. It is interesting to consider that different cells types have different metabolic patterns. A lot of processes require energy from mitochondrial respiration and cytosol metabolic pathways. Gametes are very special cells and their metabolism and viability allow the fertilization in all species. Mammalian spermatozoa must undergo preparation processes known as capacitation and acrosome reaction that involve biochemical modifications to penetrate the oocyte. Neither molecules nor mechanisms involved in the metabolic pathways leading to sperm capacitation and exocytosis are clearly understood. Capacitation is physiologically dependent on oxidative metabolism where a lot of cytosolic and mitochondrial enzymes are involved. Regarding

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sperm energy sources, lactate dehydrogenase (LDH) shuttle requires an enzyme which is present in both cytosol and the mitochondria matrix and an aminotransferase that can supply substrates for LDH. Alanine aminotransferase and aspartate aminotransferase are present in the majority of mammalian tissues and they have a important role in aminoacid metabolism including spermatozoa. The shuttle of creatine/phosphocreatine is considered to be responsible for the transfer energy from mitochondria. The level of creatine kinase B indicates a normal spermiogenesis and maturation. Creatine kinase activity is related to redox state and cellular energy sources. NADPH level has an important role in sperm function and its interaction with oocyte. Reduced NAD and NADP levels are associated with isocitrate and malate dehydrogenase activities, enzymes that belong to cytosol pathways and Krebs cycle. These reduced coenzymes are involved in the control of tyrosine phosphorylation. Under aerobic conditions, reduction equivalents can be oxidized in the mitochondrion; NADH is unable to cross mitochondrial membrane so shuttle system must be implicated to transfer the reduction equivalents. Shuttles that are involved in this process depend on the cell types. Mitochondria have a key role in the control of reactive oxygen species (ROS) level and energy production in capacitation induction. Sperm processes require equilibrium between oxidative damage susceptibility and ROS level. Sperm respiration is a crucial process in the production of energy and redox state for intracellular signals and the viability of cells. Our purpose is to show the importance of oxidative metabolism on cellular functions. In this case the model is the spermatozoa a haploid cell with the important function of giving a new life in different species.

Introduction

The Spermatozoon

Spermatozoa are haploid cells that can join chromosomes of the female gamete to form a diploid cell, the zygote, which has the potential to develop into a new organism. The mature spermatozoa are divided into two regions the head and the flagellum or tail which is joined at the neck. The head has an acrosome, a nucleus and small amounts of cytoskeleton in a reduced cytosol. The acrosome, originates from Golgi complex, contains hydrolytic enzymes required to penetrate the investments of the oocyte to allow oocyte fertilization. The acrosome varies in form and size in different species [1].

The sperm nucleus contains only one member of each chromosome pair and the chromatin is highly condensed. The flagellum and the head are closely wrapped by the plasma membrane and contains little cytoplasm. There are specific differences in the size and shape of the head and flagellum between species. In mammalian, the flagellum provides the motile force necessary to reach the oocyte surface. The function of the flagellum is associated to the sperm motility and cellular metabolism. In bovine spermatozoa, there are three helices of mitochondria organized into about 64 gyres in the middle piece [2]. The structure of the flagellum is the generator of the form and strength of the wave to move the spermatozoa. Most of invertebrates and nonmammalian vertebrates have spermatozoa that often contain few mitochondria and the flagellum usually has an only axonome [3, 4].

In spermatozoa, the organization and composition of plasma membrane vary between different regions. These domains are dynamic changing during the life of the cell [5]. In the sperm head of most mammals, the major domains of the plasma membrane are acrosomal and postacrosomal region [6].

The spermatozoa are generated by spermatogenesis inside the male gonads via meiotic division [7]. Mammalian spermatozoa undergo morphological, biochemical, and physiological modifications initially in the testis (testicular maturation) and later in the epididymis (epididymal maturation). Several steps of the maturation processes on the sperm plasma membrane are mediated by external enzymes and secretions derived from the epithelium of the genital tract. Degradation of some of the constituent proteins and the elimination of defective spermatozoa are controlled by the degradation/recycling system, the ubiquitin system. Increased sperm ubiquitin was inversely associated with sperm count, motility and % normal morphology, supporting the use of ubiquitin as a biomarker of human semen quality [8].

During epididymal maturation, spermatozoa acquire motility, change nucleus and acrosome, the plasma membrane is modified and cellular metabolism decrease. In bovine, epididymal transit duration is 8-11 days. During maturation, functional changes are produced in the cellular metabolism [9], in the flagellar activity [10] and in the sperm ability to bind to zona pellucida [11]. Changes that occur in the plasma membrane are related to: a) Cell surface charges, b) union with lectins, c) distribution of intramembranous particles, d) the fluidity of the membrane, e) Lipid and protein composition of the membrane and f) union with antibodies during the epididymal transit of the gamete [12]. In the epididymis, there is a very active synthesis of

cholesterol [14] which is transferred to the membrane during sperm maturation [15], indicating that this is a key molecule in the function the membrane [16]. This inclusion of cholesterol allows modifications of the membrane when spermatozoa pass through the female genital tract to fertilize the oocyte.

Some proteins adsorbed or integrated into the membrane of testicular spermatozoa [17] change their location during epididymal maturation and others are altered, masked or replaced by new epididymal proteins [18, 19]. The dynamic changes in the membrane of sperm that are produced through the genital tract allow acquiring fertilizing ability [17]. This haploid cell gives us a model of differential oxidative metabolism and cell organization which helps to achieve its essential role in the reproduction of different species

Sperm capacitation

Epididymal mature spermatozoa are motile but they have not fertilizing capacity. The mammalian spermatozoon has to suffer two important processes to fertilize the oocyte: the capacitation and the acrosomal reaction**.** These events involve biochemical modifications to allow oocyte fertilization. Seminal plasma is composed to secretory fluids from the various glands situated along male genital tract, testicles and epididymus. A lot of polipeptides of seminal plasma that bind to sperm surface have an important role in the fertilizing sperm capacity. Decapacitated factors are proteins secreted by epididymis and accessory glands which have the function of prevent the capacitation induction [20, 21]. It is important to consider that in vivo ejaculated spermatozoa are modified in female tract. During capacitation process, biochemical and ultrastructural changes allow spermatozoa interaction with oocyte, inducing acrosomal reaction [22, 23]. These modifications produce changes in the plasma membrane surface and in the lipid composition. Sperm surface changes observed in several species during capacitation are: the release of negative regulator of capacitation originated in epididymis and/or seminal fluid [24, 22], molecules adsorption to sperm membrane in female genital tract, regionalizing of component of plasma membrane surface [25, 26, 27], and changes in glycoconjugates of the surface [24]. In bovine spermatozoa, *in vitro* heparin capacitation has been characterized by: a) incubation time required in the capacitation [28], b) heparin binding to plasma membrane [29], c) intracellular calcium concentration variation, pH intracelular and AMPc levels [30, 31], d) zona

Pellucida effects [32], e) seminal plasma proteins influences [33] and f) loss of lectins binding to sperm head [34].

In bovine spermatozoa, cryopreservation process provokes premature capacitation [35]. First researchers used different biological fluids (oviductal fluid, follicular fluid and serum) in order to induce *in vitro* capacitation. Spermatozoa of different species require appropriate conditions for the fertility efficiency [23]. The capacitation induction is associated with the modulation of the calcium ATPase activity that is present in the plasma membrane. Quercitin, a specific inhibitor of calcium ATPase, induces sperm capacitation in the mouse, human and bull [36].

Changes in the Sperm Motility

The sperm hyperactivation was first observed in hamster [37]. Hyperactivation or hipermotility are recognized in the hamster, mouse, rat, guinea pig, rabbit, dog, dolphin, sheep, pig, primate, bovine and human spermatozoa [38, 39, 40]. The motility increase facilitates sperm movement through the viscous fluid of the oviduct [41] and covers, especially the zona pellucida of oocyte [42, 39]. One of the requirements for the initiation and maintenance of hipermotility is the presence of calcium, potassium [43] and energy metabolites [44,45,46]. The hipermotility in human spermatozoa is regulated by calcium, calmodulin, calmodulin phosphatase and levels of intracellular cAMP [47].

In the presence of calcium, proteins of oviductal fluid bind to sperm surface increasing motility in cryopreserved bovine spermatozoa [48]. Capacitation induces tyrosine phosphorylation in proteins associated with the hyperactivation of hamster spermatozoa [49]. The physiological role of hipermotility has been demonstrated by its positive correlation with the fertilizing ability of spermatozoa [50, 51].

Acrosome Reaction

The acrosome reaction is a process that involves acrosomal and plasma membrane fusion, a crucial event for oocyte fertilization [39]. The acrosome has hydrolytic enzymes that are released due to the alteration of membrane permeability, causing the destruction of the outer acrosomal membrane and

the plasma membrane adjacent. This process is considered by Bedford a physiological true acrosome reaction when spermatozoa are alive [52].

Barros was the first to demonstrate the acrosome reaction in species as rabbit and hamster [53] which is dependent on the calcium influx during the process [54, 55, 56]. Progesterone and the zona pellucida are physiological initiators of the acrosome reaction that induce intracellular signal cascade that stimulates the calcium influx [57, 58].

Sperm Metabolism

All processes related to cellular life require energy that is contained in ATP or reduction equivalents (NADH, NADPH and FADH2) provided by metabolic substrates which are used by the cells. These compounds are produced by catabolic metabolic pathways as a result of oxidation. Constitutive and adaptive enzymes are involved in these pathways. Regulation of metabolites is usually combined with control of one or two key reactions catalyzed by enzymes. These enzymes catalyze reactions, generally irreversible, usually found at low concentrations, approaching saturation with the substrate and are subject to control mechanisms. Therefore the flux of a metabolic pathway depends on the activity of adaptive enzymes. Due to characteristics mentioned above, these enzymes are considered as key enzymes in metabolic pathway [59, 60]. The study of this type of enzyme allows the relative importance of oxidative metabolism during fertilization.

All mammalian cells can metabolize glucose by glucolysis which produces energy in the form of ATP and derivates metabolites to other metabolic pathways. Glucose can be degraded in the absence of oxygen (anaerobic pathway) giving lactate as final product. In the presence of oxygen (aerobic pathway) pyruvate resulted in the formation of acetyl-CoA, which can enter the Krebs cycle for complete oxidation. Reduced coenzymes that are produced by the cycle yield reduction equivalent to the mitochondrial respiratory chain, generating free energy to be used in the synthesis of ATP (oxidative phosphorylation). Cellular oxygen availability determines the fate of pyruvate formed in glycolysis. If anaerobic conditions prevail, the NADH is re-oxidized when pyruvate is reduced to lactate by the action of lactate dehydrogenase (LDH), and NAD is used again in the glycolysis [61, 62]. The lactate dehydrogenase is found in the cytosol and mitochondria. LDH is a tetramer formed by association of two different polypeptide chains A and B,

resulting in the presence of five isoenzymes with different affinities for their substrates. In spermatozoa has been described a specific isoenzyme which is formed by C subunits named LDH-X [63, 64, 65].

Lactate Dehydrogenase Reaction

$$\text{Piruvate} + \text{NADH} \leftrightarrows \text{Lactate} + \text{NAD}$$

Lactate is one of the oxidative substrates used by the spermatozoa. In spermatocytes and spermatides the production of ATP requires high concentrations of lactate, *in vivo*, this concentration derived from the metabolism of Sertoli cells [66]. Spermatozoa in the absence of exogenous substrates, a time sufficient to induce capacitation [67] are unable to fertilize the oocyte and glucose addition provokes immediately fertilization.

In mouse spermatozoa the acrosome reaction is induced only 10% in the absence of glucose. Bedford suggested that the true acrosome reaction is physiologically dependent on oxidative metabolism compare to the false exocytotic process [52]. Lactate is present in high concentrations in seminal fluid and secretions of the female genital tract and it may be used by the spermatozoa as oxidative metabolite [68]. LDH-X is one of the most abundant enzymes of the mature spermatozoa, which is involved in the metabolism of pyruvate and lactate [69]. The enzyme lactate dehydrogenase is located in the head, middle piece and flagellum, while enzymes such as NAD-dependent malate dehydrogenase is found only in the middle piece in spermatozoa of the ram, boar and buffalo [70]. In bovine spermatozoa, lactate dehydrogenase competes successfully with mitochondrial electron transport to generate NAD in the conversion of pyruvate to lactate [71]. LDH was found in the cytosol and the mitochondria so LDH- shuttle transport reduction equivalents between both compartments of the mouse spermatozoa [72].

The Krebs cycle occurs in mitochondria and is the common fate for the oxidation of metabolites from carbohydrates, lipids and proteins. Reduction equivalents are produced by the activity of specific dehydrogenases during the cycle activity. The reduced coenzymes are re- oxidized by respiratory chain generating ATP. This process requires oxygen as final acceptor of reduction equivalents and its absence determines the cessation of cycle activity in catabolic effect. The final products of some metabolic pathways become intermediaries of the cycle; otherwise they can be precursors for anabolic

pathways so Krebs cycle is considered an amphibole pathway. When the oxygen supply is adequate the activity of the cycle depends on the immediate supply of oxidized coenzymes produced by the respiratory chain activity, the availability of ADP and the rate of ATP utilization. Regulatory cycle enzymes are α-ketoglutarate dehydrogenase, citrate synthase and isocitrate dehydrogenase. However, the isocitrate dehydrogenase (IDH) is considered the main regulatory enzyme, which requires magnesium or manganese for a suitable catalyst. It has been described three isoenzymes, two localized in the mitochondria and one in cytosol, using the mitochondrial NAD and NADP as electron acceptor, unlike the cytosolic one, which only requires NADP. NAD-dependent isoenzyme is regulated by allosteric ADP and AMP in a positive way and by ATP and NADH in the negative form, while the NADP-dependent isoenzymes have not regulatory properties [73, 62].

Isocitrate dehydrogenase reaction

a) Isocitrate dehydrogenase (1.1.1.41)

$$\text{Isocitrate} + \text{NAD} \leftrightarrow \alpha\text{-Ketoglutarate} + \text{NADH}$$

b) Isocitrate dehydrogenase (1.1.1.42)

$$\text{Isocitrate} + \text{NADP} \leftrightarrow \alpha\text{-Ketoglutarate} + \text{NADPH}$$

Enzyme malate dehydrogenase (MDH) is not considered a regulatory enzyme of the cycle, however, has a key role in the transport of compounds across the mitochondrial membrane due to the presence of cytosolic and mitochondrial isoenzymes. The mitochondrial membrane is not permeable to the passage of oxalacetate and NADH, unlike malate, the latter may carry reduction equivalents to the mitochondria [62]. It was noted that the cytosolic isoenzyme is inhibited by high concentrations of malate and the mitochondrial one by high concentrations of oxalacetate [74]. The enzyme MDH-NADP is localized in the cytosol as enzymes of the cycle of pentose phosphate pathway and is an important source of reduced cellular NADP [62].

Malate dehydrogenase reaction

a) Malate dehydrogenase (1.1.1.37)

$$\text{Malate} + \text{NAD} \leftrightarrow \text{Oxalacetate} + \text{NADH}$$

b) Malate dehydrogenase (1.1.1.40) decarboxylating

$$\text{Malate} + \text{NADP} \leftrightarrow \text{Piruvate} + CO_2 + \text{NADPH}$$

Murine spermatozoa have aminotransferases (cytosolic and mitochondrial enzyme location) that can supply substrates for LDH-X and can use the product of this reaction (pyruvate). The LDH-shuttle between cytosol and mitochondria requires: (a) an enzyme LDH-X that catalyses the interconversion between alpha-ketoacids and alpha-hydroxyacids which is present in both cytosol and the mitochondria matrix and (b) an aminotransferase that can supply substrates for LDH-X [75]. Alanine aminotransferase (ALT) and aspartate aminotransferase are present in the majority of mammalian tissues and they have a key role in amino acid metabolism [76]. Bull sperm mitochondria possess biochemical properties closely resembling mitochondria from other tissues, in spite of their greatly modified structures [77].

The mitochondrial activity enables bull spermatozoa to utilize the malate–aspartate shuttle as well as the LDH- shuttle to transfer reduction equivalents produced by glycolysis in the cytosol to the mitochondria for complete oxidation by O2 [78].

Mitochondria from frozen–thawed bovine spermatozoa are capable of generating oxidative energy [14], using pyruvate and lactate as oxidative substrates [80]. Various alpha-ketoacids and alpha-hydroxyacids are substrates for LDH-X (that has a higher affinity for lactate and pyruvate) [81].

In bull spermatozoa heparin and quercetin, inductors of capacitation modify sperm oxidative metabolism. Although heparin capacitation generates a respiratory burst, quercetin induces capacitation but maintains basal cellular respiration [82]. In bovine sperm, citrate accumulates to a concentration significantly higher than that is involved in the Krebs cycle [71]. It has been described three isozymes of isocitrate dehydrogenase, two mitochondrial and cytosol. In bovine spermatozoa, there is a relationship of IDH-NADP activity / NAD ratio of 2:1. The recorded activities of the isozymes of IDH suggests that the high concentration of citrate detected in bovine spermatozoa, would be

used for isocitrate formation in order to produce reducing power, through the cycle that is established between IDH-isoenzymes (mitochondrial- cytosolic enzymes). Although in heparin capacitation the IDH- NADP decreases by 50%, maintaining sufficient activity to provide a redox state in the form of NADPH required for capacitation.

In the study of sperm acrosome reaction with progesterone, the IDH-NADP shows a decrease in its activity, unlike the significant increase in the NAD-dependent enzyme in bovine spermatozoa. The IDH-NAD of the Krebs cycle is stimulated to supply the energy requirements for acrosome reaction [83]. Because the spermatozoon is able to fuse with the oocyte when NADPH is added to the incubation medium to replace glucose, indicates that NADPH required for spermatozoa to achieve fertilization [84], level of activity of IDH-NADP detected in bovine spermatozoa in the presence of progesterone maintains the availability of reduced coenzyme for fertilization of the oocyte [83]. Furthermore the operation of the Krebs cycle stimulates the exocytotic process, confirmed by the activity of enzymes referred. The energy produced would be required by kinases and mechanisms involved in the event, unlike the capacitation increases energy demand due to sperm motility increase [82, 83].

In bovine spermatozoa, there is a pyruvate / lactate translocase and a second translocase dependent on the concentration of malate that only transports pyruvate [85]. Without malate, about 90 % of the piruvate was converted to lactate and acetate or L-acetylcarnitine. Pyruvate is capable of maintaining the active respiratory state when L-carnitine or L-malate is added to the medium [86]. The malate is obtained from pyruvate or lactate. MDH-NADP and IDH-NADP have a higher activities respect to MDH-NAD and IDH-NAD respectively in cryopreserved bovine spermatozoa [83]. The transfer of reduction equivalents from cytosol to the mitochondria occurs mainly by the malate-aspartate shuttle, due to lack of activity detected in the glycerol 3-phosphate dehydrogenase in epididymal spermatozoa and low spermatozoa activity in mature rat [87] (Figure 1). In rat, MDH-NADP is active in mature and epididymal spermatozoa [88]. The high and constant activity of MDH-NADP observed with capacitation inducers (heparin or quercetin), and with progesterone suggests that this enzyme would sustain through pyruvate, the lactate-pyruvate shuttle activity, in order to maintain the reductive power (NADPH and NADH) between the cytosolic and mitochondrial compartments [83].

Reduction equivalents produced by the Krebs cycle or other metabolic pathways are eventually transferred to the mitochondrial respiratory chain, with consequent reduction of oxygen to form water (Figure 2).

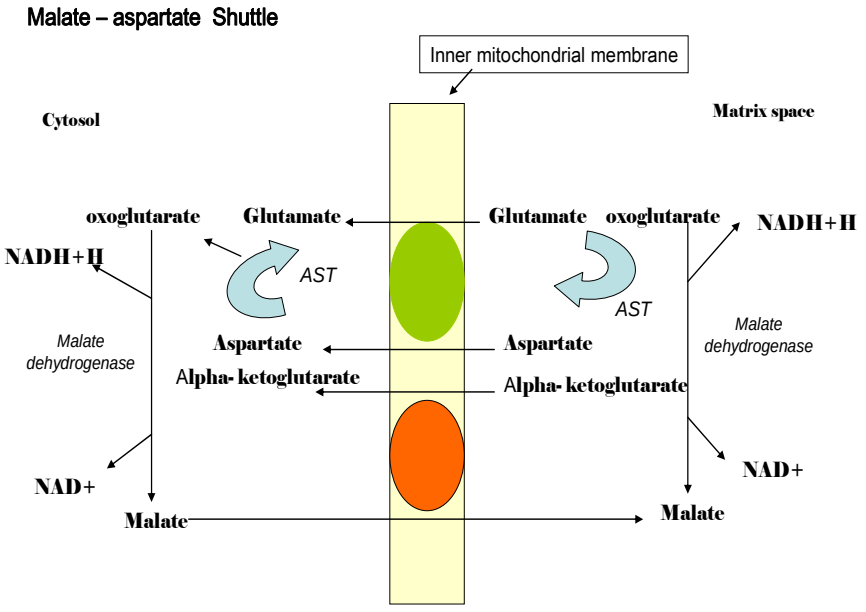

Figure 1. **Malate –aspartate shuttle.**

This shuttle is composed by: a) Malate dehydrogenase in the mitochondrial matrix and intermembrane space, b) Aspartate aminotransferase (AST) in the mitochondrial matrix and Intermembrane space, c) Malate-alpha-Ketoglutarate antiporter in the inner membrane, and d) Glutamate-Aspartate antiporter in the inner membrane. NADH in the cytosol is oxidized to NAD, and NAD in the matrix is reduced to NADH. the NAD in the cytosol can then be reduced again by another round of glycolysis, and the NADH in the matrix can be used to pass electrons to the electron transport chain so that ATP can be synthesized.

The creatine-creatine phosphate shuttle transports energy from mitochondria to the cytosol. Isoenzyme of creatine kinase M (CKM) is located in mitochondria and catalyzes the transfer of phosphate from ATP to creatine. The reaction catalyzed by creatine kinase is reversible and when ATP is required, it can be generated from creatine phosphate.

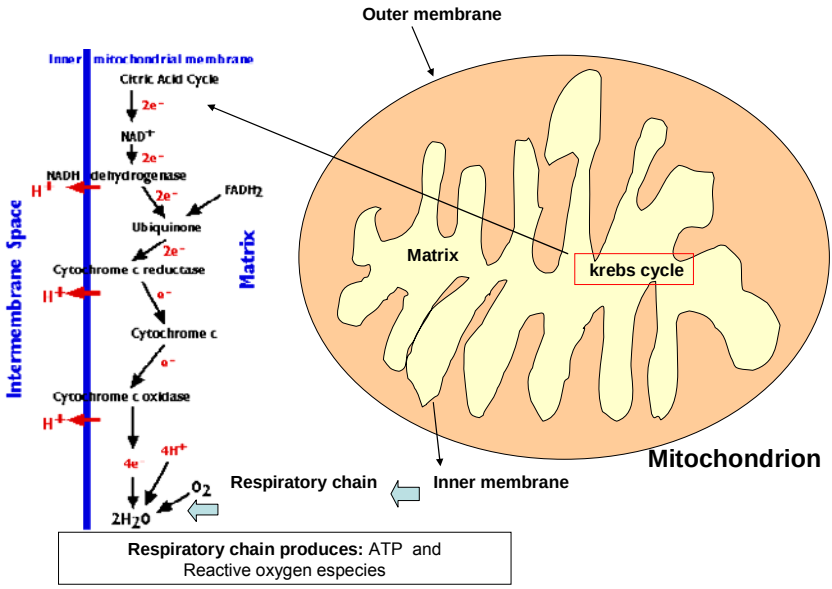

Figure 2. **Krebs cycle and Electron Transport Chain**

The Krebs cycle occurs in mitochondrial matrix and is the common fate for the oxidation of metabolites from carbohydrates, lipids and proteins. Reduction equivalents are produced by the activity of specific dehydrogenases during the cycle activity. Mitochondria are a major site of intracellular ROS formation, which results in disruption of electron transport. In the inner mitochondrial membrane, the coupling of electron transport to oxidative phosphorylation maintains the high mitochondrial membrane potential required for mitochondrial ATP production in somatic and gamete cells

Creatine Kinase Reaction

$$\text{Creatine} + \text{ATP} \leftrightarrow \text{Creatine phosphate} + \text{ADP}$$

Creatine phosphate is transported from mitochondrial matrix to the cytosol through protein pores localized in the mitochondrial membrane, being available for the generation of ATP extramitocondrial. Subsequently CK isoenzymes mediate the transport of high energy phosphate groups. The shuttle creatine -creatine phosphate is composed of different isoenzymes of

creatine kinases and allows rapid transport of energy from the mitochondrial matrix to the cytosol [89].

The metabolism of the spermatozoon increases (oxygen consumption and glycolysis) in the female genital tract or after incubation in capacitation medium [90, 91]. In mouse spermatozoa oxygen consumption was increased up to 2-5 hours after incubation in capacitation medium with albumin and oxidative substrates [92].

Mitochondrial oxidative phosphorylation is needed for production of energy required in sperm motility. Specific inhibitors of ATPase, decrease the rate of respiration of sperm motility, indicating that most of the catabolism of ATP is linked to the dynein ATPase in Psetta maxima, a marine teleost [93]. The motility of carp spermatozoa mainly depends on the ATP produced by mitochondria that is stored during the time prior to sperm activation [94]. In fertilization the fusion of gametes is depended on the oxidative metabolism of glucose [84].

In sea urchin spermatozoa, a shuttle creatine kinase has been localized in cytosol and mitochondrial, this localization is essential for sperm motility [95].

The activity level of the isoform of creatine kinase M reflects a normal maturation, predicting the fertilizing potential in human spermatozoa. The immature spermatozoon is characterized by the retention of cytoplasm and a high activity of CK-M [96]. The creatine phosphate is required for sperm motility that is depended on relation of ADP / creatine phosphate / creatine kinase and ATP / ADP / dynein. In the sea urchin sperm motility increase is accompanied by reduced ratio of creatine phosphate / ATP and creatine phosphate / Pi [97]. The creatine kinase activity and sperm morphology are considered a parameter of sperm quality in subfertile human [98]. Mitochondrial creatine kinase (CK-Mi) is located in the inner membrane of mitochondria in tissues of vertebrates [99]. In human spermatozoa have been detected a mitochondrial CK and a high concentration of CK-MM and CK-BB present in the seminal fluid [100].

In sperm from cryopreserved bovine semen, LDH-X activity has been detected using mitochondrial pyruvate and lactate as oxidative substrates [80]. Inhibition of membrane calcium ATPase by quercitin induces capacitation an increase in intracellular calcium similar to that obtained with heparin [82]. In bovine spermatozoa heparin increases the lateral movements of the head and the frequency of flagellum movements [101] and the consumption of ATP which is due to the activity of the dynein-ATPase [93, 102]. The hipermotility

that occurs during heparin capacitation requires the production of ATP, the initiation and maintenance of hipermotility depends on the presence of calcium, potassium [43] and energy metabolites [44, 46]. The increase of oxygen consumption during capacitation with heparin suggests that sperm mitochondria have an important role in oxidative energy availability. Sperm hyperactivation generated by heparin leads to a state of active mitochondrial respiration (high ADP levels), thereby increasing oxygen consumption. The respiration of heparin or quercetin capacitated spermatozoa is sensitive to cyanide, suggesting that the oxygen consumption detected is generated by the mitochondrial respiratory chain as a main energy source for this process. The motility decrease in the presence of cyanide (with both inducers) indicated that mitochondria supported sperm oxidative energy requirements [82].

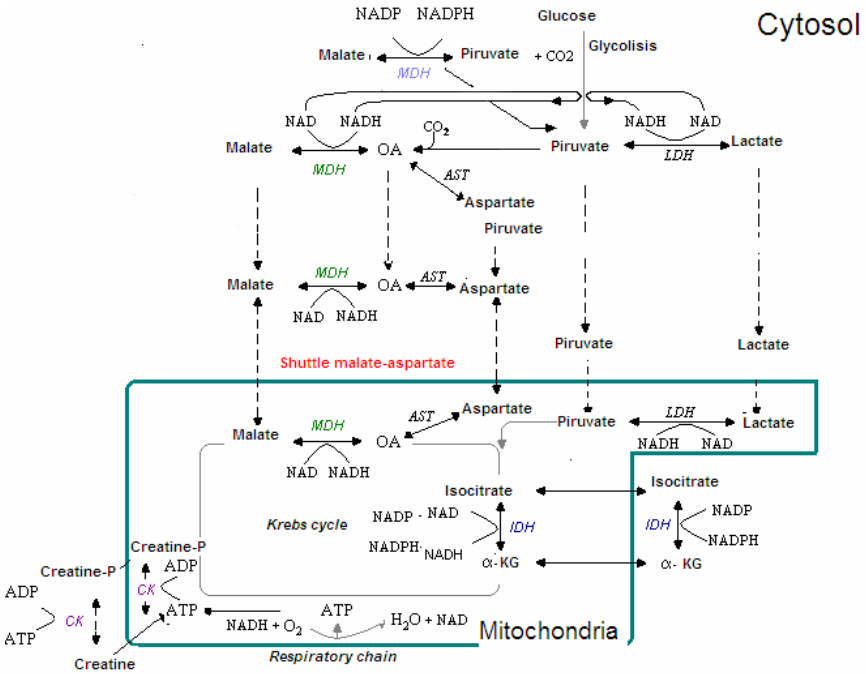

Figure 3. **Sperm oxidative metabolic pathways.** This map represents the metabolic pathways that spermatozoa can use to generate energy and the ROS level required for its function: Malate-aspartate, lactate dehydrogenase, isocitrate dehydrogenase and creatine kinase shuttles are represented. All pathways are related to mitochondria. These metabolic connections indicate that mitochondrial respiration is an important control of the cellular metabolism.

In bovine and mouse spermatozoa, the calcium-dependent ATPase of the plasma membrane induces capacitation [103, 36]. Although both inducers of capacitation (heparin and quercitin) reached the same level of intracellular calcium, treatment with quercitin unchanged oxygen consumption. This difference in oxygen consumption with heparin or quercitin, indicates that intracellular calcium increase may activate different intracellular signals maintaining the mitochondrial coupling required for producing oxidative energy [82].

In acrosome reacted spermatozoa, oxygen consumption induced by progesterone is lower than the increase observed in spermatozoa only capacitated with heparin [58]. These oxygen consumption variations indicate that capacitation requires an active mitochondrial state so this process depends on the cellular respiration efficiency more than acrosome reaction.

The levels of NAD and NADP are associated with the regulation of tyrosine phosphorylation during human sperm capacitation [104]. Furthertmore all the evidences of the shuttle malate-aspartate and isocitrate dehydrogenase activities mentioned, it is important to consider that these shuttles are involved in maintaining levels of reduced coenzyme for capacitation and acrosome reaction [83].

Sea urchin spermatozoa have an active shuttle of creatine kinase that is essential for sperm motility [95]. In rooster and human spermatozoa were found CK isoforms CKBB and MICK located on the flagellum and middle piece, respectively [89]. In bovine spermatozoa, there was a decrease in the activity of creatine kinase B during heparin or quercitin capacitation. The decline in enzyme activity as a member of the shuttle suggests that there is a regulation of the intracellular mechanisms induced by heparin or quercitin to obtain energy through substrate-level phosphorylation [105].

Reactive oxygen species produced a reversible inactivation of creatine kinase activity due to oxidation of SH groups of cysteine 278 and 282 of mitochondrial creatine kinase [106]. During capacitation, superoxide anion ($O_2^{\cdot -}$) is generated by NADP- oxidases localized in the plasma membrane which is required to induce hipermotility and capacitation process [107]. The participation of this oxidase could contribute in part to increase the consumption of oxygen in capacitated spermatozoa [82]. Intracellular calcium concentration regulates the binding of CK to the mitochondrial protein porins of the inner mitochondrial membrane that participates in creatine- creatine phosphate exchange between cytosol and mitochondria [108] (Figure 3).

Reactive oxygen species (ROS) inevitably arise from the oxidative metabolism. ROS are superoxide anions (O_2^{-}), hydroxyl radicals, hydrogen peroxide and various unstable oxidized lipids. A number of theories have been put forward including the involvement of membrane lipids, membrane thiols and protein sulfhydryl-disulfide status; however, the most promising mechanism involves tyrosine phosphorylation of specific sperm proteins. Guthrie & Welch have also observed a significant decrease in mitochondria membrane potential (MMP) below a critical threshold after freezing and thawing of semen [109]. Mitochondria are a major site of intracellular ROS formation, which results in disruption of electron transport [110]. The coupling of electron transport to oxidative phosphorylation maintains the high mitochondrial membrane potential required for mitochondrial ATP production in somatic cells. This process could be disrupted by ROS formation, resulting in a decrease in the sperm MMP and motility, since high sperm MMP is required for mitochondrial ATP production and sperm motility [107, 111]. First damage provoked by ROS, occurs in the sperm membrane, decreasing sperm motility and its ability to fuse with the oocyte. The second damage can alter the sperm DNA, resulting in the passage of defective paternal DNA on to the conceptus.

Conclusion

Our purpose is to show the importance of oxidative metabolism on cellular functions. In this case the model is the spermatozoa a haploid cell with the important function of giving a new life in different species.

The hipermotility, sperm process that occurs in different species during capacitation requires the production of ATP. The increase of oxygen consumption during capacitation with heparin suggests that the sperm mitochondria have an important role in oxidative energy availability. Sperm hyperactivation generated by heparin capacitation leads to a state of active mitochondrial respiration (high ADP levels), thereby increasing oxygen consumption. ADP concentration increase because of ATP is used by hipermotility and regulatory kinases involved in intracellular signals.

Differences in oxygen consumption in the sperm processes indicate that they may activate different intracellular signals maintaining the mitochondrial coupling required for producing oxidative energy.

The shuttle malate-aspartate and isocitrate dehydrogenase activities are important to preserve levels of reduced coenzyme for sperm processes. Cellular respiration controls the metabolism to supply energy for sperm requirements. Cellular shuttles transfer reduction equivalents between the cytosol and mitochondria. The levels of NAD and NADP are associated with the cellular regulation as tyrosine phosphorylation protein in order to allow oocyte fertilization.

Creatine –creatine phosphate shuttle is regulated by intracellular mechanisms to obtain energy through substrate-level phosphorylation.

Spermatozoa must undergo many changes throughout its life in reproductive tract of male and female. This is a challenge for cellular oxidative metabolism in order to do its reproduction function. Ejaculated spermatozoa must undergo the capacitation and acrosome reaction in order to fertilize the oocyte. Each process has differential requirement where metabolic pathways such as the Krebs cycle, respiratory chain, malate- aspartate, lactate dehydrogenase and creatine kinase shuttles are especially involved. Cellular respiration is depended on enzymes activities of the metabolism and the energy requirements that are changing the redox state and ADP concentration.

Oxygen toxicity is an inherent challenge to aerobic life forms altering sperm interaction with oocyte. Sperm damage in membranes, proteins and DNA is related to the modification in intracellular signals mechanisms that affect the fertility. Respiration produces reactive oxygen species that may play different roles in the cell life. Cellular respiration state and the ROS level are factors that control the cell function.

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Chapter V

Plectin Deficiency and Human Hepatocellular Carcinoma

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Abstract

Plectin is a cross-linking protein that organizes the cytoskeleton into a stable meshwork. Intermediate filaments (one kind of cytoskeletal component) are important in building the cellular architecture. Hepatocytes have very simple intermediate filaments composed of cytokeratin 8 (type II) and cytokeratin 18 (type I). We previously reported that cytokeratin 18 is modulated in human hepatocellular carcinoma. Because hepatocellular carcinoma cells are morphologically different from those of normal liver, we speculated that aberrant expression of plectin and the disorganization of intermediate filaments might play some role in the pleomorphism of hepatocellular carcinoma cells. By immunohistochemistry and immunoblotting, we confirmed that human hepatocellular carcinoma tissues are deficient in plectin. In *in vitro* experiments, we noted that the organization of cytokeratin 18 was

altered after plectin knockdown by small interfering RNA (siRNA). In addition, cytokeratin 18 was modulated after plectin degradation in response to staurosporine-induced apoptosis. We hypothesize that plectin deficiency plays an important role in the transformation of cells in human hepatocellular carcinoma.

Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the most common liver primary cancer, comprising approximately 90% of liver cancers, the fifth most common cancer worldwide, and the third leading cause of cancer-related death, exceeded only by cancers of the lung and stomach. The incidence of HCC is increasing and shows marked differences in geographic distribution. More than 80% of HCC cases occur in developing countries, particularly the Far East and Southeast Asia [Kao & Chen, 2005]. Hepatocellular carcinoma is common in Taiwan; the age-adjusted incidence has increased from approximately 15/100,000 in the 1980s to approximately 30/100,000 recently [Chen, 2007].

The etiology of HCC is not clear, but several risk factors have been identified. The major risk factors are viruses (chronic hepatitis B and hepatitis C), toxins (alcohol and aflatoxins), metabolism-related (diabetes, nonalcoholic fatty liver disease, hereditary hemochromatosis), and immune-related (primary biliary cirrhosis and autoimmune hepatitis). Among these risk factors, chronic hepatitis B and C infections are considered to be the main cause of HCC. The geographic variability in the incidence of HCC has been attributed to the changing distribution and natural history of hepatitis B and hepatitis C virus infection [Gomaa et al., 2008]. Before 1990, approximately 80% of patients with HCC in Taiwan were positive for hepatitis B surface-antigen (HBsAg). As a result of a neonatal vaccination program against hepatitis B initiated in 1984, the HBsAg carrier rate and the annual incidence of childhood HCC has decreased in recent years [Chen, 2007].

Grossly, HCC presents as a solitary mass, multiple nodules, or a diffuse pattern. Hemorrhage and necrosis are frequently noted in the tumor mass, but cirrhosis is present in nontumor areas. Microscopically, HCC cells are generally arranged in a trabecular or acinar pattern in a fine fibrovascular stroma. The morphology of hepatoma cells differs from that of normal liver cells; they are pleomorphic. Hepatocyte architecture is maintained by the

cytoskeleton [Feldmann, 1989]. We therefore considered that during the transformation of normal hepatocytes in human HCC, the cytoskeleton may be altered or modulated. That is, the pathogenesis of human HCC may be related to the cytoskeleton.

The Cytoskeleton

The properties of cell shape, internal organization, and movement depend on complex networks of filaments within the cytoplasm, which comprise the cytoskeleton. Three major cytoskeletal protein networks that maintain cellular structure and integrity in eukaryotic cells are microtubules, microfilaments, and intermediate filaments [Arias, 1988]. The cytoskeleton of human liver cells, like that of other animal cells, contains these three components [Feldmann, 1989].

Microtubules (MTs), with an outer diameter of 25 nm, are long, hollow cylinders composed of multiple isotypes of α- and β-tubulin [Nogales, 2000]. MTs are dynamic protein polymers that play essential roles in cell division, maintenance of cell shape, vesicle transport, and motility [Desai & Mitchison, 1997]. MTs serve as tracks for cellular transport, with motor proteins moving along them, carrying specific membrane-bound organelles to specific sites within the cell [Amos & Baker, 1979]. The discovery of signaling molecules that interact with MTs indicates that MTs are critical for the spatial organization of signal transduction [Gundersen & Cook, 1999]. MTs are important for the process of mitosis, making them an important target for anticancer drugs. Microtubule-targeted drugs inhibit MT dynamics, leading to a block of mitosis and to apoptosis of cancer cells [Jordan & Wilson, 2004]. Cell lines with a higher βIII-tubulin level are more resistant to the anticancer agent paclitaxel [Ranganathan et al., 1998].

Microfilaments (MFs) are two-stranded helical polymers composed of actin. With a diameter of 5 nm to 9 nm, they are organized into a variety of linear bundles, two-dimensional networks, and three-dimensional gels [Volkmann et al., 2001]. They are most highly concentrated in the plasma membrane cortex; this actin-rich layer controls the shape and surface movements of cells [Kabsch & Vandekerckhove, 1992]. MFs function in the

maintenance of cell morphology and polarity, in endocytosis, contractility and motility, and cell division [Furukawa & Fechheimer, 1997]. It has recently been reported that actin dynamics play a central role in apoptosis and aging [Gourlay & Ayscough, 2005]. In tumorigenesis, regulation of cancer cell motility via actin reorganization has been reported [Yamazaki et al., 2005]. Molecules that link migratory signals to the actin cytoskeleton are upregulated in invasive and metastatic cancer cells [Yamaguchi & Condeelis, 2007]. Other studies have shown that MFs might be dynamic targets for cancer chemotherapy [Jordan & Wilson, 1998].

Intermediate filaments (IFs), with a diameter of 10 nm, are ropelike fibers that form a basket around the nucleus that extends to the cell periphery. They provide a flexible scaffolding, the function of which is to structure the cytoplasm and resist stresses applied to the cell [Fuchs & Cleveland, 1998]. IFs comprise a large, heterogeneous family and include cytokeratin, desmin, vimentin, glial fibrillary acidic protein, neurofilaments, and nuclear lamin [Lazarides, 1980].

Cytokeratins (CKs), with an equal number of specific paired acidic and neutral or basic subunits, are found primarily in epithelial cells [Moll et al., 1982]. They are by far the most complex class of IF proteins, with at least 19 distinct forms in human epithelia and eight more in the keratins of hair and nail [Cooper et al., 1985]. IFs are formed by CKs in human liver parenchyma cells; these cells have a very simple CK composition and express only one CK pair: CK8 (type II, molecular weight 52 kDa) and CK18 (type I, molecular weight 45 kDa) [Van Eyken & Desmet, 1993]. Cytokeratins are required for the maintenance of hepatocyte integrity [Anne et al., 1997; Loranger et al., 1997].

IFs have been proposed to interact with other cytoskeletal elements, such as MTs and MFs [Svitkina et al., 1996], the plasma membrane and desmosomes [Loranger et al., 2006], the nuclear envelope [Djabali, 1999], mitochondria [Winter et al., 2008], and the Golgi complex [Toivola et al., 2005] in human cells. These attachments may be indirect, via a crosslinking protein, such as plectin or synemin, or direct, by linking IF proteins themselves to protein components of other cellular structures [Bellin et al., 2001]. Several cross-linking proteins, or IF-associated proteins (IFAPs), which mediate interactions between IFs and other cytoskeletal networks have been identified [Fuchs & Cleveland, 1998]. Among these, **plectin** is the most

versatile cytoskeletal-linking protein, expressed in a variety of tissues and mammalian cell types, especially those with a prominent epithelial component [Wiche, 1998; Kazerounian et al., 2002]. In hepatocytes, the main IFAPs are plectin and desmoplakin, which are members of the plakin gene family [Green et al., 2005].

The molecular mass of full-length plectin varies from 507,000 to 527,000 daltons, and the structure is composed of a central ~200-nm α-helical coiled-coil rod domain flanked by globular amino-terminal and carboxy-terminal domains. Plectin has been shown to interact with a variety of cytoskeletal structures and proteins [Wiche, 1998]. The carboxy-terminal domain consists of six highly homologous repeat regions, and subdomains for binding IFs, as well as a protein kinase $p34^{cdc2}$ phosphorylation site, have been identified. The amino-terminal domain contains an actin-binding site. Both amino- and carboxy-terminal domains harbor binding sites for integrin β4 [Janda et al., 2001]. Immunogold electron microscopy shows plectin as comprising thin (2-3 nm), up to 200-nm long filaments bridging vimentin and microtubules and/or actin filaments [Svitkina et al., 1996].

The Cytoskeleton and Plectin in Human Diseases and Neoplasms

The integrity of the cytoskeleton is essential for proper cell function; therefore, changes in the composition of the cytoskeleton may contribute to human diseases including cancer. The formation of MTs is highly dynamic, and their polymerization dynamics are tightly regulated by MT-associated proteins [Amos & Baker, 1979], which have been implicated in the pathology of several neurodegenerative diseases. For example, hyperphosphorylated tau protein is the main component of neurofibrillary tangles found in the brains of patients with Alzheimer disease [Mandelkow et al., 1995]. Disorganization of MF structure may also impede the bile-secreting function of hepatocytes [Tsukada et al., 1995]. Missense mutations of α-cardiac actin have been found to cause hypertrophic as well as dilated cardiomyopathy [Vang et al., 2005]. Altered expression of CK genes is known to be related to liver dysfunction, including chronic hepatitis, increased hepatocyte fragility, and decreased bile secretion [Omary & Ku, 1997]. Recently, the possible role of CKs in

tumorigenesis has been reported; keratins modulate cell adhesion, size, G1/S transition, and protein synthesis in liver cells [Galarneau et al., 2007].

Abnormal regulation of the cytoskeleton and associated proteins has been investigated in several neoplasms. For example, an increased level of the βIVb-tubulin isotype in rat liver cancer [Miller et al., 2008], downregulation of the actin-binding protein gelsolin in human colon cancer [Gay et al., 2008], downregulation of CK18 in human breast cancer [Woelfle et al., 2004], an increased level of CK19 mRNA in human oral squamous cell carcinoma [Zhong et al., 2006], and upregulation of tenascin-C and vimentin in breast cancer [Dandachi et al., 2001] have been reported. The involvement of morphologic alterations in invasion and carcinogenesis by cancer cells has also been raised in these studies. In our opinion, hepatoma cells are morphologically different from normal liver cells; therefore, the cytoskeleton and associated proteins of hepatoma cells may be altered in cell transformation in cancer.

The importance of plectin is evident from studies of plectin gene-knockout mice, which die 2 days after birth as a result of severe skin blistering and show abnormalities in muscle and heart [Andra et al., 1997]. In humans, epidermolysis bullosa simplex with muscular dystrophy is an autosomal recessive disorder caused by mutation of the human plectin gene, which is localized to chromosome 8q24.13-qter [Schroder et al., 2002]. Skin blistering is caused by a lack of plectin, which connects CKs to hemidesmosomes, and muscular disorders may result from deficiencies in desmin attachment to the membrane [McLean et al., 1996; Smith et al., 1996]. In prostate carcinoma, the expression of plectin is variable in the stroma around tumor nests [Nagle et al., 1994].

Plectin possesses binding sites for IF proteins, tubulin, and actin, which makes it an integrator of the cytoskeletal network [Rezniczek et al., 2004]. Deficiency of plectin might affect vimentin network dynamics [Spurny et al., 2008] and has been reported to result in aberrant keratin cytoskeleton organization, owing to a lack of orthogonal IF crosslinking [Osmanagic-Myers et al., 2006]. According to Svitkina et al. [1996], the mean density of plectin sidearms along IFs is 24 ± 12 per μm. This IF-plectin complex comprises an extensive crosslinking of cellular components and provides a structural framework for integration of the cytoplasm. Therefore, plectin deficiency in human liver cells might result in a loss of function as a result of a loss of linking between cytoskeletal elements, causing disorganization of the cytoskeleton.

The expression of plectin in a variety of cell types and tissues, pathologic abnormalities resulting from plectin deficiency, and the large number of different plectin-binding partners, hint at an important role of plectin in cell function and in the pathogenesis of many human diseases, including cell transformation in cancer. Discrimination between plectin in normal and tumor tissues, including human liver and hepatoma, has not been well investigated.

Cytokeratin Modulation in Human Hepatocellular Carcinoma

Patterns of expression of tumor and non-tumor liver CK filaments have been visualized by immunohistochemical and immunofluorescence techniques under light and electron microscopy [Vielkind & Swierenga, 1989; Okanoue et al., 1988; Mori, 1994]. These studies are very interesting but have not determined whether CKs are modulated during tumor transformation. Two hypotheses have been proposed concerning the stability versus modulation of CKs in HCC.

The idea of stability is supported by immunohistochemical findings. One study suggests that most HCCs express an immunohistochemical keratin profile identical to that of nonneoplastic hepatocytes [Fischer et al., 1987]. Another study involving immunohistochemical staining of cell lines derived from human liver tumors showed that cell lines derived from HCC were positive for staining with monoclonal and polyclonal keratin antibodies [Tokiwa et al., 1990, 1992]. However, the idea of modulation is supported by other studies. For example, it has been reported that hepatoma cells express low levels of CK8 and CK18 [Rossel et al., 1992]. The human hepatoma cell lines HA22T/VGH and HA47T/VGH lack CK [Lo et al., 1987]. Hepatocytes with a bile duct CK phenotype appear during the early stages of carcinogenesis [Green et al., 1990].

We started investigating the expression of CK18 in human HCC in 1994. In our earliest experiments involving molecular approaches (immunoprecipitation, Western blotting, peptide mapping by two-dimensional gel electrophoresis), we identified modulation of CK18 in human HCC [Su et al., 1994]. In that study, human liver and HCC tissues were collected, and CK18 was extracted and purified. The obtained molecules were analyzed by gel electrophoresis and Western blotting. The results revealed that in normal

liver, CK18 is present as a 45-kDa molecule; however, in HCC tissues, the expression of CK18 is downregulated.

In our subsequent experiments comparing CK18 mRNA expression in human normal and HCC tissues, we found that modulation of CK18 in human HCC occurred at the protein level but not at the mRNA level [Liu et al., 1997]. We extracted mRNA from each specimen by the acid guanidinium thiocyanate phenol chloroform method, reverse-transcribed mRNA to cDNA, and amplified the products by polymerase chain reaction (PCR). The PCR products were digested by the restriction enzymes *Sma*I and *Bam*HI. The PCR products and digested cDNA fragments of normal liver tissues, HCC tissues, and the human hepatoma cell line PLC/PRF/5 were found to be identical. These results demonstrated that modulation of CK18 in human HCC occurs at the protein level but not at the mRNA level. In that study, modulation of CK18 was also identified in the human hepatoma cell line PLC/PRF/5 as well as in HCC tissues. On the basis of these two studies, we confirmed that CK18 is modulated in human HCC, and we speculated that CK18 is unstable in human HCC.

Factors Affecting CK18 Stability in Human Hepatocellular Carcinoma

After confirming the modulation of CK18 in human HCC, we wanted to determine factors that influence the stability of CK18 in human hepatocytes. Further experiments revealed that the stability of CK18 in human liver cells is affected by chemical and physical factors. **First**, we found that treatment with colchicine (an inhibitor of microtubule polymerization) modulated CK18 in human liver Chang cells [Liu et al., 2001]. The CK18 in these cells was modulated after long-term treatment (24 hours) with colchicine. The breakdown of MTs also caused changes in cell morphology, from polygonal to spindle or round shaped. These results indicated that an intact microtubule network is a stabilizing factor with respect to CK18 in human liver cells.

Second, we found that laser irradiation could influence the expression of plectin and further disturb the organization of CK18 filaments in human hepatoma cell lines HepG2 and J-5 [Liu et al., 2006]. Immunofluorescence experiments showed that typical filamentous IF structure was preserved in nonradiated HepG2 and J-5 cells, with filaments arrayed from the nucleus to

the cell periphery. These filaments maintained the polygonal cell morphology. In response to photoradiation, IF structure collapsed and became fragmented and irregular and formed aggregated lattices concentrated around the nucleus. The cell shape was more irregular, with a nondistinct periphery. Although IF organization was influenced by photoradiation, the expression of CK protein was not altered, according to Western blot results. In addition, the distributions of plectin were altered by photoradiation. We speculated that CK18 stability is related to other cytoskeletal components (eg, MTs) and/or crosslinking proteins (eg, plectin) in human liver cells.

A Role of Plectin Deficiency in Cytokeratin Modulation in Human Hepatocellular Carcinoma

The organization of IFs is important in maintaining the architecture and shape of hepatocytes. The role of plectin is to organize IFs into an integral and stable meshwork. IFs may be nonfunctional or less functional in crosslinking the cytoskeleton in the absence of plectin. Therefore, it is reasonable to suggest that normal liver cells require plectin to form the cytoskeleton into a stable structure to maintain uniform cell size and shape. Because the morphology of hepatoma cells differs from that of normal liver cells, we hypothesized that plectin deficiency would cause disorganization of hepatic IFs and result in pleomorphism of hepatoma cells.

To clarify the relation between plectin deficiency and IF disorganization in liver cells, we knocked down plectin mRNA expression with the use of small interfering RNA (siRNA) targeted to plectin mRNA in Chang liver cells [Cheng et al., 2008]. The results showed that CK18 mRNA expression was not affected by plectin knockdown, but immunoblot analysis showed that plectin knockdown dramatically decreased plectin as well as CK18 protein expression (Figure 1). Immunofluorescence experiments showed that plectin in untreated cells was distributed mainly as a mesh structure in the perinuclear region, extending to the plasma membrane in a granular pattern. The pattern of CK18 was a fine filamentous network within the cytoplasm, also abundant around the perinuclear region; the mesh-like distribution extended toward the plasma membrane.

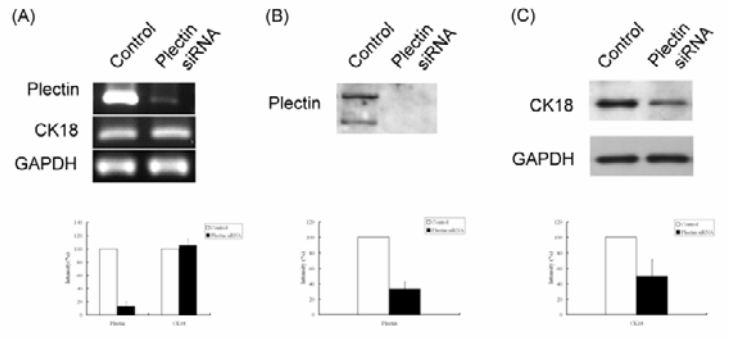

Figure 1. Plectin siRNA interference in Chang liver cell. After RNAi treatment, the mRNA expression by RT-PCR shown the plectin knockdown cells were not affected with CK18 expression (A). In immunoblot analysis, the plectin RNAi treated cells resulted in the plectin knockdown showed that CK18 had dramatically decreased (C). In the plectin RNAi treated cells, the expression of mRNA (A) and protein (B) in plectin was markedly down regulated. GAPDH was compared as an internal control. (Published in: *Journal of Molecular Histology*, 39(Pt 2), 209-216, 2008)

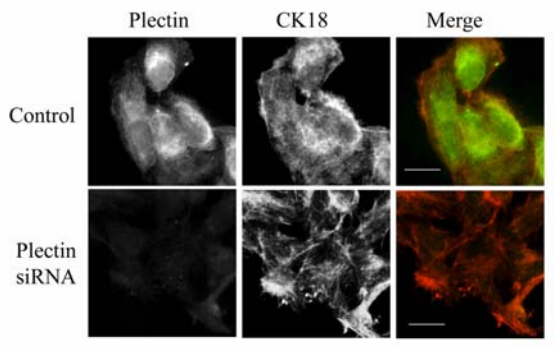

Figure 2. Immunofluorescence assay of plectin and CK18 in Chang liver cells after apoptosis induction. Control untreated cells (upper panel), the plectin was mainly distributed as mesh structure in the perinuclear region and extended to the membrane with a granular pattern; the CK18 exhibited fine filament networks in the cytolasm, abundant in the perinuclear region, the mesh-like distributions extended toward cell membrane; DAPI staining shows uniform shape of DNA; the merged picture displays CK18 colocalized with plectin. Chang liver cells treated with 1 μM STS for 4 hours (lower panel), the plectin was concentrated around the nuclei with a granular pattern; CK18 filament networks were disrupted and reorganized in a granular pattern; DAPI staining shows fragmentation of nuclei and condensation of DNA and the merged picture shows dramatic morphological change with collapse of the plectin and CK18 structures. Scare bar = 20 μm. (Published in: *Journal of Molecular Histology*, 39(Pt 2), 209-216, 2008)

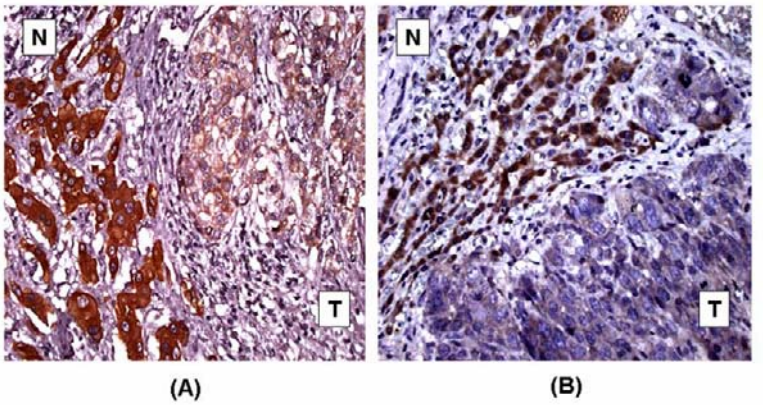

Figure 3. Immunohistochemical staining of CK18 (A) and plectin (B) in human hepatocellular carcinomas (×200). (A) CK18 was strong positive in non-tumor part of HCC (on left half, labeled as N); while in tumor part, CK18 was weak (on right half, labeled as T). (B) Plectin also revealed strong positive in non-tumor part (on left upper half, labeled as N), while hepatoma tissue showed weak staining (on right lower half, labeled as N). (Published in: *Journal of Molecular Histology*, 39(Pt 2), 209-216, 2008

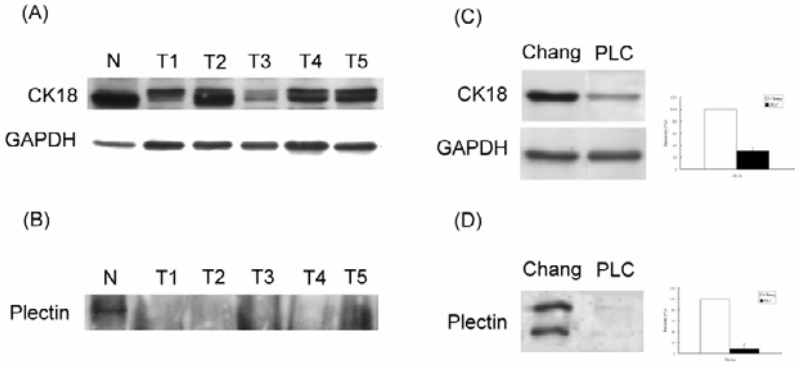

Figure 4. (A) and (B), immunoblot analysis of normal liver and hepatoma tissues. Total proteins extracted from liver (N) and hepatoma (T1 to T5) tissues were analyzed using monoclonal anti-CK18 and anti-plectin antibodies. The expression of plectin was decreased in HCC tissues compared with normal liver tissues (B). The CK18 was modulated and two close bands were revealed in HCC tissues, the normal liver tissue presented only one band (A). (C) and (D), immunoblot analysis of Chang liver cells and PLC/PRF/5 cells. The plectin expression was dramatic different in Chang liver cells comparing with PLC/PRF/5 cells (D). CK18 was modulated in PLC/PRF/5 cells (C). GAPDH was used as internal control. (Published in: *Journal of Molecular Histology*, 39(Pt 2), 209-216, 2008)

In response to plectin knockdown, plectin immunofluorescence was too faint to detect. Shrinkage of CK18 network was revealed, and disorganized filament bundles with a conspicuously tangled and collapsed mesh structure were observed (Figure 2). Thus, we confirmed that a deficiency of plectin resulted in CK18 modulation and unstable IF organization *in vitro*. In that study, immunohistochemistry and Western blotting also confirmed that plectin was downregulated in human HCC *in vivo* (Figures 3 and 4).

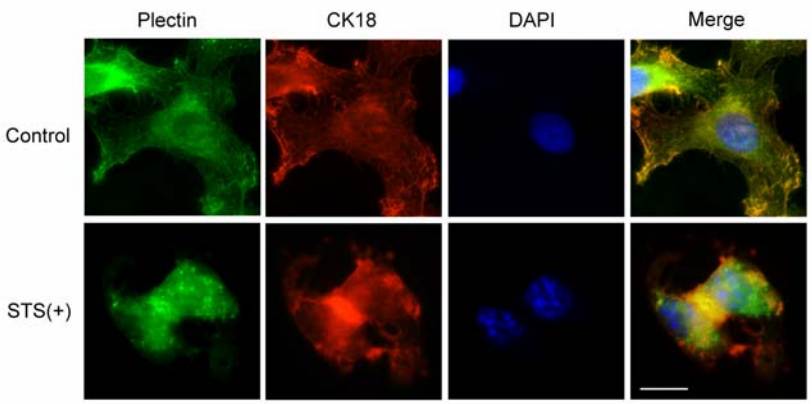

Figure 5. Immunofluorescence assay of plectin and CK18 in Chang liver cells after apoptosis induction. Control untreated cells (upper panel), the plectin was mainly distributed as mesh structure in the perinuclear region and extended to the membrane with a granular pattern; the CK18 exhibited fine filament networks in the cytolasm, abundant in the perinuclear region, the mesh-like distributions extended toward cell membrane; DAPI staining shows uniform shape of DNA; the merged picture displays CK18 colocalized with plectin. Chang liver cells treated with 1 μM STS for 4 hours (lower panel), the plectin was concentrated around the nuclei with a granular pattern; CK18 filament networks were disrupted and reorganized in a granular pattern; DAPI staining shows fragmentation of nuclei and condensation of DNA and the merged picture shows dramatic morphological change with collapse of the plectin and CK18 structures. Scare bar = 20 μm. (Published in: *In Vivo*, 22(5), 543-548, 2008)

Deficiency or downregulation of plectin in cells might abolish linking among cytoskeletal elements, resulting in disorganization of the cytoskeleton and in cellular pleomorphism [Cheng et al., 2008]. A similar phenomenon was also demonstrated in mouse plectin knockout cells [Osmanagic-Myers et al., 2006]. Results of that study showed that in the absence of plectin, keratin networks were less delicate, the mesh size was increased, and individual

filaments appeared bundled and straighter. Results of another study suggested that plectin may play a role in reorganization of the actin cytoskeleton during death receptor-mediated apoptosis [Sonnenberg & Liem, 2007]. A relation between cytoskeletal structure and pleomorphism of cancer cells was identified by electron microscopy of the human prostate cancer cell line DU145, which confirmed that alterations in cell shape were regulated by microfilaments [Chakraborty & Von Stein, 1986]. We also found that, in addition to human HCC, plectin expression was altered in human colon adenoma and adenocarcinoma [Lee et al., 2004]. Immunohistochemical staining of plectin in human colon adenoma and adenocarcinoma cells revealed that plectin was upregulated in colorectal adenocarcinoma as well as in bizarre glands and locally invasive tumor nests in tubular adenoma compared to normal colorectal mucosa. These data suggested that plectin expression in different types of human neoplasms may vary.

Chemical (colchicine), physical (laser irradiation), and biological (siRNA) techniques have proven effective for exploring the role of plectin deficiency in CK18 instability; however, these techniques have created an artificial state. Looking at the endogenous state, we compared plectin expression in HCC and normal liver tissues as well as normal and hepatoma cell lines by immunohistochemistry and Western blotting. We found that plectin expression was downregulated in HCC tissues and in hepatoma cell lines [Cheng et al., 2008]. In addition, apoptosis induced plectin cleavage, resulting in CK18 modulation and unstable IF organization [Liu et al., 2008]. Zymogenic plectin was seen at approximately 300 kDa to 400 kDa in untreated liver cells, whereas degraded plectin appeared at 200 kDa in staurosporine-treated cells. Similarly, zymogenic CK18 was found in untreated liver cells, but two bands, corresponding to zymogenic CK18 and cleaved CK18, appeared after staurosporine treatment. Immunofluorescence showed that plectin in untreated cells was distributed mainly as a mesh structure in the perinuclear region, extending to the plasma membrane in a granular pattern. The pattern of CK18 exhibited a fine filamentous network within the cytoplasm and was also abundant in the perinuclear region, with a mesh-like distribution extending toward the plasma membrane. In response to treatment with staurosporine, the plectin mesh collapsed, concentrated in the perinuclear region, and showed a granular pattern. The CK18 network also displayed a disrupted pattern and granular structure after the induction of apoptosis (Figure 5).

Possible Mechanism of Plectin Deficiency in Human Hepatocellular Carcinoma

In our previous study, we found that apoptosis-induced plectin cleavage resulted in CK18 modulation and unstable IF organization in hepatocytes [Liu et al., 2008]. These results suggest that apoptosis-related protein degradation may play a role in the plectin deficiency observed in human HCC. Cells that fail to undergo appropriate apoptosis are involved in degenerative diseases and cancer. Apoptosis of liver cells has become the focus of many researchers since it became apparent that deregulation of the apoptotic program is involved in the pathophysiology of liver diseases [Thompson et al., 1995; Schuchmann & Galle, 2001]. HCC appears to escape immune surveillance and apoptosis induced by cytotoxic T cells by expressing CD95L [Patel et al., 1999]. Another study showed that during apoptosis, CK8/18 reorganizes into granular structures enriched in phosphorylated CK18, facilitating the rapid collapse of the cytoskeletal architecture [Schutte et al., 2004]. It has been shown that apoptosis plays an important role in the modulation of plectin *in vivo* and that plectin is a substrate for caspase 8 during apoptosis and is required for reorganization of the microfilament system [Stegh et al., 2000]. Recently, the pathway of colchicine-induced apoptosis, which results in cytoskeletal alteration, has been elucidated [Jordà et al., 2005].

Plectin is a substrate for endogenous kinases and proteases. Protein kinase A and protein kinase C regulate the interaction of plectin with lamin B and vimentin; therefore, plectin may be a major effecter of the phosphorylation-dependent regulatory system involved in the spatial organization and anchorage of the cytoskeleton [Foisner et al., 1991]. In rat hepatocytes, plectin is a naringin-sensitive phosphoprotein that plays a role in cytoskeletal disruption and apoptosis induced by algal toxins [Larsen et al., 2002]. Hepatocyte plectin is also a substrate of μ-calpain, and degradation of plectin may be an important event in the destabilization of hepatoma cells [Muenchbach et al., 1998]. In the human breast carcinoma cell line MCF7, plectin is a major early *in vivo* substrate for caspase 8 during CD95- and tumor necrosis factor receptor-mediated apoptosis [Stegh et al., 2000]. These observations confirm the *in vivo* degradation of plectin, consistent with our findings.

Conclusions

Abnormal regulation of the cytoskeleton and associated proteins plays important roles in human diseases, including neoplasms. Hepatocellular carcinoma is highly prevalent and poses critical medical problems in Taiwan. We reported that CK18 is modulated in human HCC and found CK18 to be unstable in HCC cells. Several factors, including disruption of MTs and/or deficiency of plectin, could be involved in the instability of CK18 in HCC. We subsequently reported that plectin is downregulated in human HCC *in vivo*. These data indicate that plectin deficiency might be the cause of CK18 instability in human HCC. In siRNA knockdown experiments of plectin in human hepatocytes, the expression and organization of CK18 was modulated. We suggest that downregulation of plectin results in IF disorganization and in pleomorphism of hepatoma cells. By affecting cytoskeletal organization, plectin deficiency may be important in the transformation of cells in human HCC. With respect to the mechanism of plectin deficiency in human HCC, apoptosis-related protein degradation may be involved.

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Chapter VI

Studies of Toxin Resistant Beta-Cells: Lessons for the Chemotherapy?

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Abstract

Alloxan and streptozotocin are two classic diabetogenic agents which are employed for the induction of insulin-dependent diabetes mellitus (IDDM) or insulin-independent diabetes mellitus (NIDDM) when different dosages were used. In addition, due to the autoimmunity in type 1 diabetes, a major loss of pancreatic beta-cell mass occurred after local production of various cytotoxic cytokines, mainly interleukin-1 β, interferon-γ, and tumor necrosis factor-α. Therefore, understanding the mechanisms of actions of those toxins offers good lessons for the development of a good beta-cell protection strategy. Furthermore, studies of beta-cells which survive from those toxic challenges are also informative in terms of understanding intrinsic mechanisms of cell defense. Interestingly, studies of those toxin resistant cells were also drew the attentions from cancer researchers, especially in the field of chemotherapy. In this article, we would like to review the current understanding of the mechanisms of each beta cell toxins, to summarize reported toxin resistant insulinoma or immortalized beta cells, and to

compare the different nature of those toxin resistant cells. Finally, with the advance of the knowledge for cancer stem cells, the possible involvement of stem cells enrichment after various toxin challenges was also discussed.

Introduction

In the filed of diabetes, destruction of pancreatic beta-cells, primarily via autoimmunity, viral infection, or chemical toxicity, is considered to be responsible for the etiology of type 1 diabetes. As a result, hyperglycemia due to lack of insulin occurs in type 1 diabetes patients (Kawasaki, et al. 2004). To recovery the loss of beta-cell mass, islet transplantation is one of the approaches which can possibly replenish the shortage of insulin-secreting cells. However, monotherapy of islet transplantation may not solve other issues existed in type 1 diabetes, such as autoimmunity, lack of sufficient amount of islets for transplantation, organ rejection, and so on. In addition, insulin secreting beta-cells became very sophisticated endocrine cells which have a special feature that this cell is actually equipped with weak anti-oxidant defense ability (Pipeleers, et al. 2001). Therefore, generation of toxin resistant cells becomes a new strategy to overcome both cell shortage and immunity problem. In addition, elucidating cell defense mechanisms also provides a good lesson for gene therapy and cell based therapy in order to make either endogenous beta cells or transplantable insulin secreting cells stronger and response to extracellular insulin secretogagous properly.

However, from the point of view among chemotherapists, the major objective of studying toxin resistant cells is finding a way of chemotherapy "NOT" to induce multitoxin resistant cells. Unfortunately, in many cancers, the survival of toxin resistant cells after chemotherapy usually means the predestination of the future recurrence (Dizdar and Altundag 2009; Sabatino, et al. 2009). Therefore, by reviewing toxin resistant pancreatic beta-cell studies, we hope to provide some useful information for the future development of chemotherapeutic agents.

The Mechanisms of Beta-Cell Death

Shortage of functional beta-cells due to either a massive destruction or chronic accumulation of apoptosis ratio appeared to be the ultimate consequence for the pathogenesis in both types of diabetes (Mandrup-Poulsen 2003). Understanding mechanisms of toxin induced beta-cell death could facilitate the investigation of cell defense actions in multitoxin resistant cells. In the present chapter, we would describe two models for the explanations of beta-cell death mechanisms.

Model 1- The Okamoto Model

From 1980s to early 1990s, a series of experiments were conducted by Okamoto et. al. to elucidate the mechanisms underlying the pancreatic beta-cell cytotoxicity (Okamoto 1992). Firstly, the general mechanism of action of beta-cell cytotoxins, alloxan and streptozotocin was proposed. This model focused on DNA breaks caused by alloxan and streptozotocin via either generation of reactive oxygen species or direct alkylation, respectively. Secondly, these breaks could lead to the activation of DNA repair cascade including poly(ADP-ribose) synthetase, which now is known to stabilize genome structure prior to repairing DNA breaks with DNA repair enzymes (Satoh and Lindahl 1994). The activation of poly(ADP-ribose) synthetase, which uses cellular NAD as a substrate, was found to down-regulate proinsulin biosynthesis in islet beta-cells. This inhibition of proinsulin biosynthesis by those two beta-cell cytotoxins could be prevented in the presence of poly(ADP-ribose) synthetase inhibitors, such as nicotinamide, picolinamide, and methylxanthines. Administration of alloxan and streptozotocin *in vivo* also inhibited proinsulin biosynthesis and previous injection of poly (ADP-ribose) synthetase inhibitors was also able to prevent the toxin-induced both depletion of NAD and down-regulation of proinsulin biosynthesis. Diabetogenesis in animals treated with beta-cell damaging agents, other than alloxan and streptozotocin, has been shown to be prevented by intervention of the Okamoto cell damage pathway at various points by using immunosuppressors or modulators (cyclosporin and OK-432), antioxidant enzymes (superoxide dismutase and desferrioxamine), or poly(ADP-ribose) synthetase inhibitors (nicotinamide and 3-aminobenzamide). Therefore, in theory, type 1 diabetes could be prevented by

the intervention of this pathway. However, prevention of type 1 diabetes via inhibition of DNA repair mechanism seems to be controversial if the strategy to be carried out in humans. Indeed, by using hamster cells, Yammamoto et.al. further demonstrated that DNA repair of streptozotocin-treated cells in the presence of nicotinamide was abolished (Yamamoto, et al. 1981). In an animal study, 60-100% of rats surviving from the treatment of streptozotocin in the presence of nicotinamide, picolinamide or 3-aminobenzamide, bore beta-cell tumors. In contrast, 25% of surviving rats treated with alloxan in the presence of poly(ADP-ribose) synthetase inhibitors developed beta-cell tumors. Further characterization has shown that such insulinoma contains insulin mRNA as normal beta-cells. In addition, a novel rat insulinoma gene (rig gene) was further found to be responsible for tumorigenesis. Therefore, although inhibition of poly (ADP-ribose) synthesis could preserve cell viability after exposure to beta-cell toxic agents; however, there is a high risk of activating ocogenesis due to the alteration of gene expression.

Model 2- The Activation of Apoptosis Cascades

Net beta-cell mass results from the balance between beta-cell proliferation versus beta-cell death (Mandrup-Poulsen 2003). Loss of beta-cell mass in type 1 or type 2 diabetes reflected such imbalance in human body. Apoptosis or programmed cell death is normal process in nature to maintain the balance of tissue growth. However, the nature of beta-cell is highly sensitive to a number of pro-apoptotic factors. Once the kinetic of the net beta-cell mass shifting towards accelerating apoptosis, the onset of diabetes would occur when the demand for insulin has no longer to be satisfied. Currently, there are many excellent reviews describing the various mechanisms of apoptosis in details (Cnop, et al. 2005; Donath, et al. 2005; Eizirik and Mandrup-Poulsen 2001; Thomas, et al. 2009). Herein, we only want to address that caspase 3 and caspase 12 are two major players in response to extrinsic or intrinsic cell death pathways (Araki, et al. 2003; Thomas et al. 2009). Extrinsic factors include inflammatory cytokines (IFN-γ, IL-1, and TNF-α), Fas ligand (CD95L) which act via the level of receptors. STAT-1, NF-kB, iNOS, and JNK are some key molecules involved in the signal cascades of those cytokines. In contrast, the intrinsic factors are related to toxins, cytotoxic granule constituents perforin and granzymes, or stress conditions like serum/growth factor withdraw and endoplasmic reticulum stress. When the function of mitochondria became

severely damaged under those conditions, the release of cytochrome c will lead to downstream caspase activation wherease program cell death reaches the point of no return.

Studies of Multitoxin Resistant Cells

Due to the shortage of beta-cells or islets for transplantation and the highly susceptible to destruction nature of beta-cells, selection strategies based on iteratively continuous culture beta cell line with chosen toxin(s) to generate multitoxin resistant cells became a very interesting and attractive idea for treatment of type 1 diabetes (Bloch and Vardi 2005; Hohmeier, et al. 2003). Theoretically, via a proper encapsulation, multitoxin resistant cells can avoid direct contact with self-intolerant immune system therefore the only concern is to resist toxic challenges from small molecules including toxins and cytokines. Currently, we and others have established various multitoxin resistant cell lines via selection strategies. Characteristics of those cell lines were summarized in Table 1.

Group 1- Alloxan / Ninhydrin / Hydrogen Peroxide

Alloxan (2,4,5,6-tetraoxohexahydropyrimidine) was made as early as in 1818 by Brugnatelli according to the review of Rerup (1970) (Rerup 1970). However, it was reproducibly synthesized by Wohler and Liebig in 1838 and has had a great impact in later diabetes research. This chemical is soluble in water with a PKa1 = 6.63. The stability in aqueous solution depends on temperature and pH value. At pH < 3, alloxan is quite stable at room temperature. However, alloxan in solution at pH 7 should be kept below 4 °C in order to avoid rapid formation of alloxanic acid which is irreversible isomeric transformation. According to the review of Lenzen, injection of alloxan to cause mass destruction of pancreatic beta-cells in animals occurs rapidly after injection as first described by Dunn et. al. in 1943. Although the diabetogenic feature of alloxan was widely recognized, the association of cytotoxic action with reactive oxygen species generation was reported by Deamer et. al. in 1971 (Lenzen and Panten 1988). An auto redox cycle

between alloxan and dialuric acid has been proposed as the mechanism for generation of reactive oxygen species with alloxan. The idea that generation of reactive oxygen species by alloxan results in induction of DNA strand breaks was further developed by Okamoto and collegues as a model of beta-cell cytotoxic action (Okamoto 1992). On the other hand, with the characteristics of high affinity thiol groups in alloxan, glucokinase and hexokinase has also been proposed to be responsible for part of the alloxan-mediated inhibitory effects on glucose-induced insulin secretion (Lenzen, et al. 1990; Lenzen, et al. 1987).

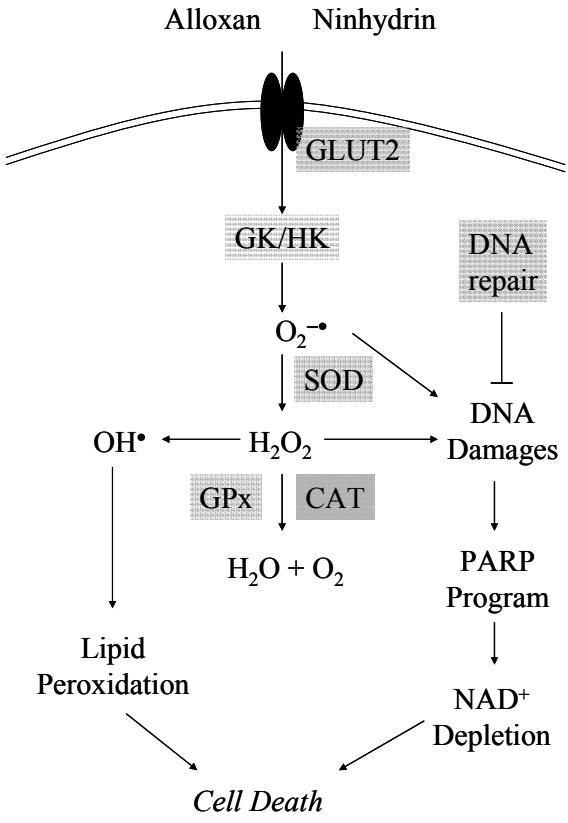

Figure 1. Mechanisms of alloxan/ninhydrin cytotoxicity. It should be noted that potential cell defense actions observed from toxin resistant cells were put in gray-colored boxes.

Table 1. Summary of current multitoxin resistant beta-cells generated by selection strategies.

Source of cells	Toxin employed	Selection strategies	Multidrug Resistance	Drug withdrawing effects	References
RINmS	Streptozotocin	Repeated acute exposure (1h) with STZ (10 mM)	STZ; Alloxan	Nondetermined	(Bloch et al. 2000)
RINmA	Alloxan	Repeated acute exposure (1h) with Alloxan (20 mM)	Alloxan; STZ	Nondetermined	(Bloch et al. 2000)
RINmHP	H_2O_2	Repeated acute exposure (2h) with H_2O_2 (100 and 200 uM)	H_2O_2; Con-A activated splenocytes	Nondetermined	(Bloch et al. 2003)
INS-1_{res}	IL-1β+IFN-γ	Increase of IL-1β + IFN-γ from 0.5 ng/ml+ 5U/ml to 10ng/ml+100U/ml	IL-1β; IFN-γ; IL-β+IFN-γ; Supernatants from activated PBMC	Loss of IFN-γ resistance	(Chen et al. 2000)
INS-1_{res} with bcl-2 overexpression	IL-1β+IFN-γ	Increase of IL-1β + IFN-γ cytokine mix from 0.5 ng/ml+ 5 U/ml to 10 ng/ml+100 U/ml	IL-1β; IFN-γ; IL-β+IFN-γ; H_2O_2 ; SNAP ; SIN-1 ; STZ ; Supernatants from activated PBMC	Nondetermined	(Tran, et al. 2003)
BRINst	Sterptozotocin	Repeated acute exposure (1h) with STZ (20 mM)	STZ; H_2O_2; Ninhydrin	Loss of ninhydrin resistance	(Liu et al. 2007)
BRINnt	Ninhydrin	Repeated acute exposure (1h) with ninhydrin (0.05 mM)	Ninhydrin; H_2O_2	Loss of ninhydrin and H_2O_2 resistance	(Liu et al. 2008)

In contrast, ninhydrin is a stable form of alloxan, a known hydroxyl radical generator (Grankvist, et al. 1986). Ninhydrin, only at low concentration, and alloxan were both able to selectively destroyed pancreatic islets *in vitro* (Jorns, et al. 1997). In addition, both toxins can inhibit glucokinase and hexokinase activities at thiol groups of the sugar-binding site of the enzymes (Lenzen, et al. 1988). However, difference of redox effeciency in ninhydrin and alloxan coupled with glucokinase suggested the different potency in beta-cell cytotoxicity (Lenzen and Munday 1991). Mitochondria functions could be also affected by exposure to ninhydrin and alloxan because both chemicals could inhibit aconitase activity and induce calcium transport (Lenzen, et al. 1992; Lenzen and Mirzaie-Petri 1992). A summarized mechanism of these two toxins was shown in Figure 1.

The Story of Alloxan/Ninhydrin/Hydrogen Peroxide Resistant Cells: RINmA/ BRINnt/RINmHP Cells

By taking advantages of the fact that RINm cells were actually constitute with heterogeneous population, Bloch K et al has employed alloxan to generate toxin resistant cells by repeated acute (1h) exposure of alloxan (20 mM) for two passages (Bloch, et al. 2000). The relative resistance towards the alloxan (10 mM) in comparing with parental cells could be 2 folds more resistant judging by MTT assay. In addition, cell proliferation activity measured by [^{3}H] thymidine incorporation in the presence of alloxan (10 mM) could be up to 10-fold higher than that in parental cells. Reduction of glucose transporter 2 (GLUT2) was suggested to be important mechanism of toxin resistance in such cells although addition of GLUT2 competitor, 3-O-methly glucose, in the presence of alloxan was unable to provide fully protection for unselected cells.

We have also applied similar strategy to generate ninhydrin, a stable analogue of alloxan, resistant BRIN-BD11 cells, named BRINnt cells (Liu, et al. 2008). Our procedures were involved in iteratively acute exposure of high dose of toxin which can destroy most of cells. Survival cells were collected and cultured for later toxin challenges up to 10 rounds. During the selection process, a 4-fold increase of ninhydrin resistance in BRIN-BD11 cells could be observed between 1st and 2nd acute (1h) ninhydrin exposure. However, the resistance could be significantly enhanced when the number of exposures increased. As a result, when we have completed 10 time exposure, the

resistance of BRIN-BD11 cells towards ninhydrin increased 70 folds. After further characterization of BRINnt cells, we have found that BRINnt cells exhibited resistance toward ninhydrin and hydrogen peroxide but not STZ. Both total superoxide dismutase (SOD) and catalase enzyme activities of BRINnt cells were significantly enhanced, and ninhydrin-induced DNA damage was decreased. Moreover, such cells also exhibited enhanced DNA repair efficiency. However, unlike RINmA cells, this was accompanied by loss of secretagogue-induced insulin release, decreased cellular insulin content, and deficits in insulin and GLUT 2 gene expression. We have also carried out a withdrawn experiment to show that prolonged culture of BRINnt cells in the absence of ninhydrin reversed the degenerated function of BRINnt cells but restored ninhydrin susceptibility.

Finally, by directly providing extracellular hydrogen peroxide, Bloch K et al have generated RINmHP cells which were resistant to hydrogen peroxide and conditioned medium produced from activated rat splenocytes (Bloch, et al. 2003). The level of expression and activity of catalase was significantly enhanced in such cells. It was suggested that hydrogen peroxide supplementation might simply result in selection of pre-existing sub-population with higher catalase activity and expression.

When comparing among those multidrug resistance cells, it was interesting to see that RINmA cells could ameliorated both STZ and alloxan mediated cytotoxicity but not hydrogen peroxide. In contrast, BRINnt cells became resistant to both ninhydrin and HP but not STZ. The possible explanations might be due to the way of cells responding those toxic challenges. In RINmA cells, reducing toxin uptake appeared to be very important way to intervene toxin action of alloxan. In contrast, BRINnt cells took more complicated actions including reducing expression of GLUT2 (for toxin uptake), enhancing ROS scavenging cascades (MnSOD and catalase cascade), and DNA repair. Therefore, even though toxins shared with similar characteristics, application of those toxins on different cell lines could end up with promoting different defense mechanisms.

Group 2- Streptozotocin

Streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-D-glucopyranose), in contrast to alloxan, is a diabetogenic agent associated with multiple

cytotoxic actions (Rerup 1970; Weiss 1982). First of all, due to the nature of streptozotocin as a glucose analog, the association of streptozotocin with the glucose transporter 2 was suggested. By over-expression of GLUT2 in insulinoma, RIN cells, the sensitivity of the transfected cells towards streptozotocin was significantly increased compared with either non-transfected partners or cells transfected with glucokinase(Elsner, et al. 2000; Schnedl, et al. 1994). Also, when compared with other alkylating agents which produce similar DNA damage, GLUT2 transfected cells only showed an increase in sensitivity toward streptozotocin; showing that the relative importance of GLUT2 in the diabetogenic properties of streptozotocin. Although superoxide generation by streptozotocin has not been proven (Asayama, et al. 1984), generation of hydrogen peroxide and nitric oxide from STZ has been suggested as being responsible for oxidative and nitrosative stress leading to DNA damage and apoptosis in pancreatic beta-cells (Kaneto, et al. 1995; Kroncke, et al. 1995; Takasu, et al. 1991). Furthermore, streptozotocin also possesses akylating ability to modify guanine at position O6 and N7 (Kokkinakis, et al. 1997). Such an action may also contribute to streptozotocin-induced beta-cell death (Murata, et al. 1999). Mechanisms of STZ cytotoxicity were summarized in Figure 2.

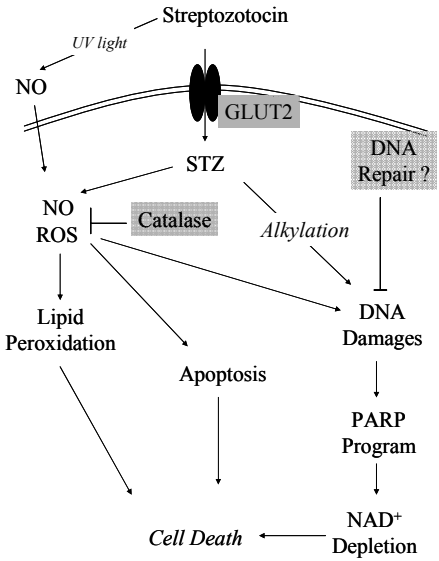

Figure 2. Mechanisms of streptozotocin cytotoxicity. It should be noted that potential cell defense actions observed from toxin resistant cells were put in gray-colored boxes.

The Story of Streptozotocin Resistant RINmS and BRINst Cells

We and Bloch K et al have generated streptozotocin resistant cell lines from either BRIN-BD11 cells or RINm cells. Similar with RINmA cells, RINmS cells became resistant to both STZ and alloxan but not hydrogen peroxide (Bloch et al. 2000). In addition, reduction of GLUT2 was also observed in such cells and was considered to be the action of cell defense. In contrast, BRINst cells became resistant to STZ, ninhydrin, and hydrpgen peroxide (Liu, et al. 2007). Increased catalase activity observed in BRINst cells appeared to be one of the defense mechanisms which are consistent with previous finding that STZ could generate hydrogen peroxide.

Although the selection procedures and cell defense actions in both STZ resistant cells were different, the most striking result from both toxin resistant cell lines was the enhancement of insulin secretory responsiveness after selection (Bloch and Vardi 2001; Liu et al. 2007). Increase of cellular Insulin content has been also observed both in BRINst and RINmS cells. Therefore, Bloch et al has made a suggestion that STZ selection strategy could be useful because such process is accompanied by induction of tumor cell differentiation (Bloch and Vardi 2001). However, it should be noted that resistant cells has to be going to a complete differentiation program. Such concern was described by Prados et al, when differentiation therapy by employing actinomycin D was not fully successful in targeting cells, multidrug resistant human rhabdomyosarcoma cell line (Prados, et al. 1998). However, in the case of streptozotocin resistant cells, both RINmS or BRINst cells appeared to be better differentiated in terms of insulin secretory functions and glucose responsiveness. Moreover, the growth curve is much slower in resistant cells than parental cells. Therefore, such approach in cancer therapy might be a drug and tissue-sensitive case. Nevertheless, more investigation should be carried out to fully address this issue.

Group 3- Cytokines

In type 1 diabetes, it was shown that approximately 70%-80% of β-cells were lost at the time of diagnosis. It was also known that such destruction of β cells was associated with local exposure of high concentration of cytokines produced from infiltrated T-cells and macrophages (Hohmeier et al. 2003).

Major inflammatory agents produced from those cells are interferon-gamma (INF-γ), interleukin-1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α) as well as reactive oxygen/nitrogen species (ROS/RNS) (Rabinovitch 1998). However, the pathological consequence of pancreatic β cells exposed to those inflammatory agents is not promptly occurred but through a progressive nature over years (Srikanta, et al. 1983). Similar situation happened in prediabetic patients could be mimicked *in vitro* exposure of insulin-secreting cells to either IL-1β or IL-1β+IFN-γ resulting in loss of glucose responsiveness, increasing the ratio of proinsulin/insulin, and decreasing the trafficking of insulin granules (Hostens, et al. 1999; Ohara-Imaizumi, et al. 2004). In contrast, prolonged exposure to IL-1β+IFN-γ but not either cytokine alone could actually lead to β-cell death.

Cytokines induced β-cell death is very complicated process indicated by microarray experiments whereas there are about 700 genes were transcriptionally regulated (Cardozo, et al. 2001; Cnop et al. 2005). As shown in Figure 3 summarized the work of Eizirik et al., although IL-1β activates some defense/repair mechanism via transcriptional factor nuclear factor-kappa B (NF-κB); however, other clusters of genes involving in endoplasmic reticulum stress (ER stress), β cell dysfunction, cell death signaling, insulitis, and so on, were also stimulated in the presence of IFN-γ. Therefore, the pathway of β cell death remained to be favored in the presence of cytokines. Apart from signaling and transcriptional regulation, nitric oxide is also highly produced via the induction of inducible nitric oxide synthase (iNOS) expression. In the collaborative effect with ROS production from macrophages, the combination of superoxide ions with nitric oxide could generate highly toxic peroxynitrite. Even when superoxide dismutase actively converts superoxide ions into hydrogen peroxide, without the efficient eliminating process by catalse, hydrogen peroxide could still significantly damage cells via lipid peroxidation by accumulating hydroxyl radicals within the cells (Hohmeier et al. 2003). As shown in Figure 3, the potential mechanisms of cytokine-mediated β-cell death were summarized. The intervention strategies were then intensively evaluated based on these findings.

The Story of Cytokine Resistant Cell line: INSres Cells

IL-1β and IFNγ mediated β cell death could be thought as a very representative model for studies of "soluble molecules" from immuno attack. Chen G et al. has established cytokine resistant INS-1 cell line, called INSres cells (Chen, et al. 2000). The selection strategy was to let native cells exposed to IL-β+IFN-γ at low concentration initially. The dosage of such combination was iteratively increased up to a very high level (10 ng/ml IL-1β + 100 U/ml IFN-γ). At the end of the selection process, the viability of INS-1 cells in the presence of cytokines increased from 15% viability to 100% viability after 48h incubation period. Moreover, inhibition of IL-β activity or even mitogen-stimulated peripheral blood mononuclear cells induced NO production by INSres cells was also clearly demonstrated. Chen G et al also tested the stability of such resistance and found out that most of the cytokine resistance in INSres cells could be maintained for 2 month cell culture period in the absence of cytokines. Loss of IFN-γ resistance is the major observation but regaining the IFN-γ resistance could be obtained by re-stimulation.

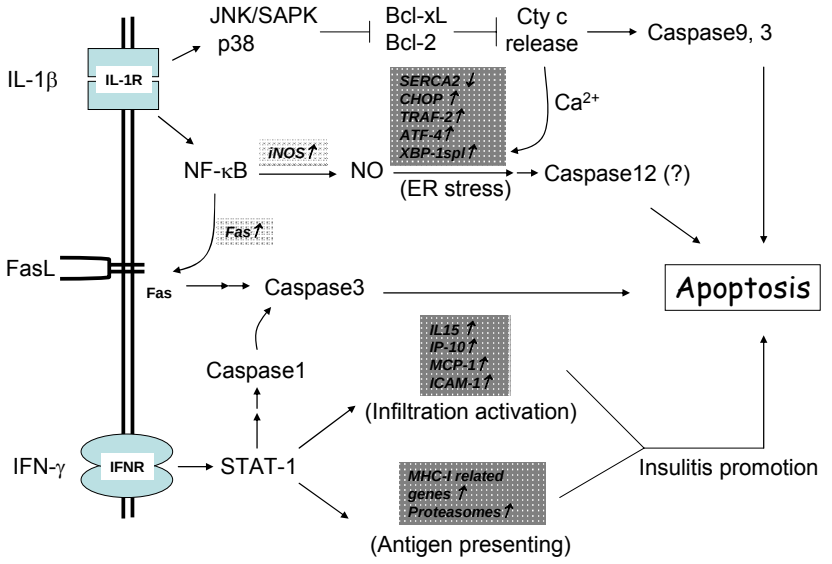

Figure 3. Summarised the activation of signaling cascades and regulation of gene clusters associated with cytotoxicity in the presence of cytokines (Cardozo, et al. 2001; Cnop et al. 2005).

In terms of the mechanisms of cytokine resistance underlying INSres, the inhibition of NO production in INSres cells was associated with a reduction of inducible nitric oxide synthase (iNOS) and NF-κB transactivation was also compromised. However, such blockage appeared to be at the distal stage because degradation of regulatory unit of NF-κB, IκB, was not different from unselected cells (Hohmeier et al. 2003). Interestingly, although the IFN-γ stimulated the phosphorylation of signal transducer and activator of transcription-1α (STAT-1α) was unchanged in both INSres and unselected cells, the protein level of STAT-1α in INSres was actually overexpressed in INSres (Chen, et al. 2001). The importance of such observation was concluded by ectopic expression of STAT-1α in INS-1 cells via adenoviral infection. As a result, the resistance of infected unselected INS-1 cells towards cytokine mix increased 5 folds. It should be recalled that IFN-γ resistance is transcient and re-inducible. Although IL-1β mediated the inhibition of IFN-γ stimulated STAT-1α expression was abolished in INSres cells; however, the association between IL-β signal blockage and IFN-γ resistance in INSres cells is still loose considering the fact that IL-β resistance was maintained at the stage that IFN-γ resistance is lost in INSres cells. Therefore, independent resistance mechanisms to each cytokines might be induced in INSres cells.

"Stemness" of Those Toxin Resistant Cells

Regulation of toxin transportation and efflux could significantly change the sensitivity of target cells towards toxins. Delaying or preventing toxin uptake could be considered the easiest way to prevent the damages of toxins therefore it could be a very important and economic drug resistant mechanism in the biological system. Supporting evidence was derived from several aspects. For examples, glucose-transporter 2 (GLUT2) plays a very important role in the sensitivity of cells towards alloxan or streptozotocin. Firstly, STZ sensitive beta-cells or species had higher level of GLUT-2 expression. Secondly, administration of unmetablolized sugar, 3-methyl glucose, actually improved the resistance of insulinoma. Finally, lower expression of GLUT2 protein was measured in RINmS or RINmA in comparison with parental cells. In the case of BRINst, interestingly, lower expression of GLUT2 also occurred in high passage of BRINst cells when STZ was withdrawn from the medium for 10 more passages. The STZ resistance in those cells was also preserved.

The other way to reduce the potential hazard effects from toxins is to "pump out" the uptake toxins as fast as possible. As matter of fact, high level expression of ATP-binding cassette transporters, which are responsible for toxin transport, are one of the most intriguing characteristics of normal/cancer stem cells (Dean, et al. 2005).

ABC-transporter superfamily constitutes promiscuous transporters pumping out both hydrophobic and hydrophilic compounds (Dean, et al. 2001; Gottesman, et al. 2002). P-glycoprotein (PGP/MRD1 gene product), transcribed from ABCB1, is one of the ATP-binding cassette transporters involved in drug resistance in stem cells (Dean et al. 2005). A high level of PGP expression in insulinmoma cells reported by Sugawara et al indicated the possible existence of "cancer-like stem cells" (Sugawara 1990). Furthermore, Nestin-positive side population cells with the expression of ABCG2 (BCRP1) ATP-binding cassette transporter has been observed in pancreatic islet progenitors (Lechner, et al. 2002). Although there is no report about the endogenous ABCG2 expression in insulinoma, the result that ectopic expression of ABCG2 in INS-1 cells did enhance the efflux of hoechst33342 suggested the potential multidrug resistant cells could be derived from the enrichment of the cancer-like stem cell population with high efficiency for toxin efflux. It would be very interesting to see whether cancer-stem like cells could be enriched via those toxins discussed above.

Conclusion

Multitoxin resistant beta-cell research is interested by both diabetes and cancer investigators because it provides very useful information for both sides. In one hand, those resistant cells become a potential resource for cell based therapy for type 1 diabetes. In addition, it revealed some secrets for making beta cell stronger while insulin secretory function could be preserved. On the other hand, it probably raised many questions and cautions for cancer therapists to be aware of the existence of mutidrug resistant cells after chemotherapy and what kind resistant cells might be selected in response to corresponding drugs. Based on current information, multitoxin resistance cells appeared to be inducible or selectable with all kinds of beta-cell toxins. However, the resistance appeared to be non-permanent and the loss of toxin resistance could be expected once toxin is withdrawn from the system. Similar

observations were also observed in BRINnt, BRINst and INSres cells although the toxin resistance of later two cell lines appeared to be more stable. Although the possibility that cancer stem-like cells with high efficient "toxin pump" were enriched via toxin selection remained to be investigated, promoting or preserving differentiation of beta cells seems to be a valuable approach for cancer chemotherapists. In the case of ninhydrin, the maintenance of beta-cell phenotype might have favorable outcome for preserving ninhydrin toxicity which has a negative association with insulin secretory functions and gene expression. Therefore, utilization of differentiation enhancer like glucagons-like polypeptides-1 (GLP-1) might actually improve the efficacy of such toxin. Findings from STZ resistant cells also indicated that the development of differentiation therapy for beta cell tumors seems to be considerable. The potential advantages might be recovering of normal cell homeostasis, reducing the stemness of the toxin resistant cells, improving the efficacy of alloxan type of drugs, and so on. Finally, the existence of the species variations (i.e. human vs rodents) in terms of β cell sensitivity towards β cell toxins indicates that the "activities" of each mechanisms describing in this chapter might be different in human (Eizirik 1994; Welsh 1999). Nonetheless, with the accumulation of more knowledge into the nature of toxin resistant cells and mechanisms of cell defense, we hope that such effort would ultimately lead to better anti-cancer therapies in the future.

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Chapter VII

"Fatty Acid Metabolism in Cancer Cell Survival: A New Anti-Cancer Target"

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Abstract

There is a renewed interest in the study of the function of fatty acid synthase (FASN) and fatty-acid synthesis in cancer pathogenesis. With the recent discovery that human cancer cells express high levels of fatty acid synthase and undergo significant endogenous fatty-acid synthesis, our understanding of the role of fatty acids in cancer biology is

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expanding. In spite of an anabolic energy-storage pathway, lipogenesis is now associated with clinically aggressive tumor behaviour and tumor-cell growth and survival and has become a druggable target in many human carcinomas. This review outlines the role of FASN in cancer development and stands out the preclinical development of FASN inhibitors and their antitumor effects.

Key words: Cancer; Fatty acid metabolism; Fatty Acid Synthase; New antitumor drugs.

Introduction

It was in the early twentieth century that the first evidence appeared showing that cells in tumor tissues had the pathway for *de novo* synthesis of fatty acids activated. It is now known that the over-expression and activation of the enzyme called fatty acid synthase (FASN) is a common phenotype in most human carcinomas [1-6]. In addition, the FASN enzyme is found in high levels in the blood of cancer patients, it is an indicator of poor prognosis in breast and prostate cancer and its inhibition is selectively cytotoxic to human tumor cells. For these reasons, research into the role of FASN and fatty acid metabolism has become of major interest due to their potential value in the diagnosis and treatment of cancer.

FASN (EC 2.3.1.85) is a very well structured multi-enzymatic complex responsible for *de novo* synthesis of long chain fatty acids (mainly palmitate) from the NADPH-dependent condensation of acetyl-coA and malonyl-CoA [7] (Figure 1). In tumor tissue, FASN is activated and mainly synthesizes palmitate, as it does normally in liver tissue and other lipogenic tissues. However, the amount of palmitate differs significantly between tumor cells and the cells of healthy lipogenic tissues (adipose and liver tissue). Under normal conditions the purpose of fatty acid synthesis in lipogenic tissue is to store energy in the form of triglycerides. When fasting, the expression and activation of FASN is inhibited, malonyl-CoA values fall and oxidation of fatty acids is activated. During lipogenesis, the malonyl-CoA acts by inhibiting the enzymatic complex carnitine palmitoyltransferase-1 [8] (CPT1), which shuttles long chain fatty acids from the cytoplasm into the mitochondrial matrix, where beta-oxidation takes place (Figure 1). In contrast, in tumor tissues, the endogenously synthesized fatty acids are not esterified

and stored as triacylglycerides but, instead, as phospholipids, a fact which indicates that inhibiting FASN will reduce the source of structural membrane phospholipids in tumor cells. In addition, fatty acid synthesis in tumor cells is regulated by transcription factors (hormones, growth factors) or oncoproteins in connection with the kinase-dependent signaling pathways, whereas in normal cells it is regulated mainly by dietary factors.

The consequences of FASN inhibition are also different between normal and tumor cells. Inhibition of FASN activity by drugs induces apoptosis in human cancer cells, both in vitro and in vivo [9-12]. This highlights the importance of FASN expression and fatty acid synthesis in the survival of tumor cells. In this review we will focus primarily on the mechanisms by which FASN is over-expressed in cancer and the scientific-technological advances being made in the development of new FASN-inhibiting antitumor agents.

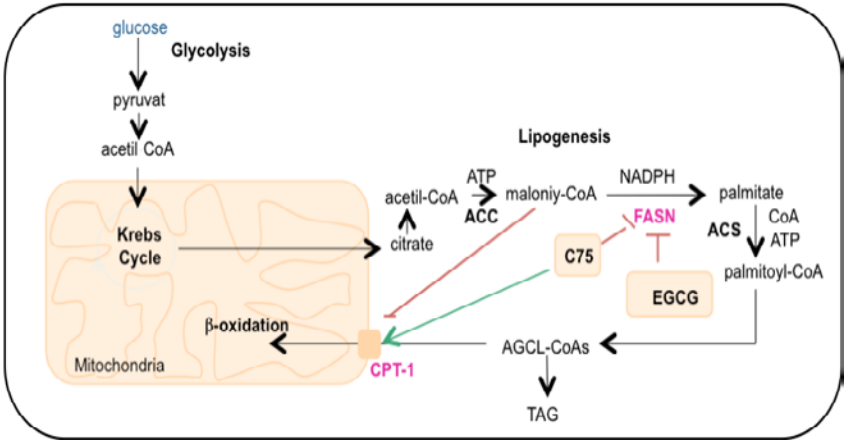
FASN: Regulation and Expression in Cancer

The first data on FASN expression in cancer cell lines dates back to 1987 when Chalbos *et al.* [13] first described an increased expression and activity of FASN in human cells of breast cancer treated with progestins. Subsequently, an increase in FASN expression in prostate cancer cell lines after stimulation with androgens was described [14]. Other studies show a decrease in FASN expression with androgen ablation and a subsequent increase upon acquisition of androgen resistance [15, 16]. Clinical studies show that the serum of patients with breast, prostate, colon and ovary cancer had higher-than-normal FASN values [17]. There is, therefore, plentiful scientific evidence demonstrating the importance of FASN in cancer.

FASN expression is low in most human tissues and only increases in situations that require *de novo* synthesis of fatty acids. The *de novo* synthesis of fatty acids in physiological situations occurs in the liver and adipose tissue, and is regulated by the type of diet and fatty acid requirements (e.g. during pregnancy FASN expression increases in the endometrium). However, unlike the nutritional control of FASN mediated by insulin, glucagon and other hormones related to metabolism [18], FASN expression in cancer is mainly regulated by kinase-dependent signaling pathways (Figure 2): phosphatidylinositol 3 kinase / protein kinase B (PI3K/Akt) and mitogen-

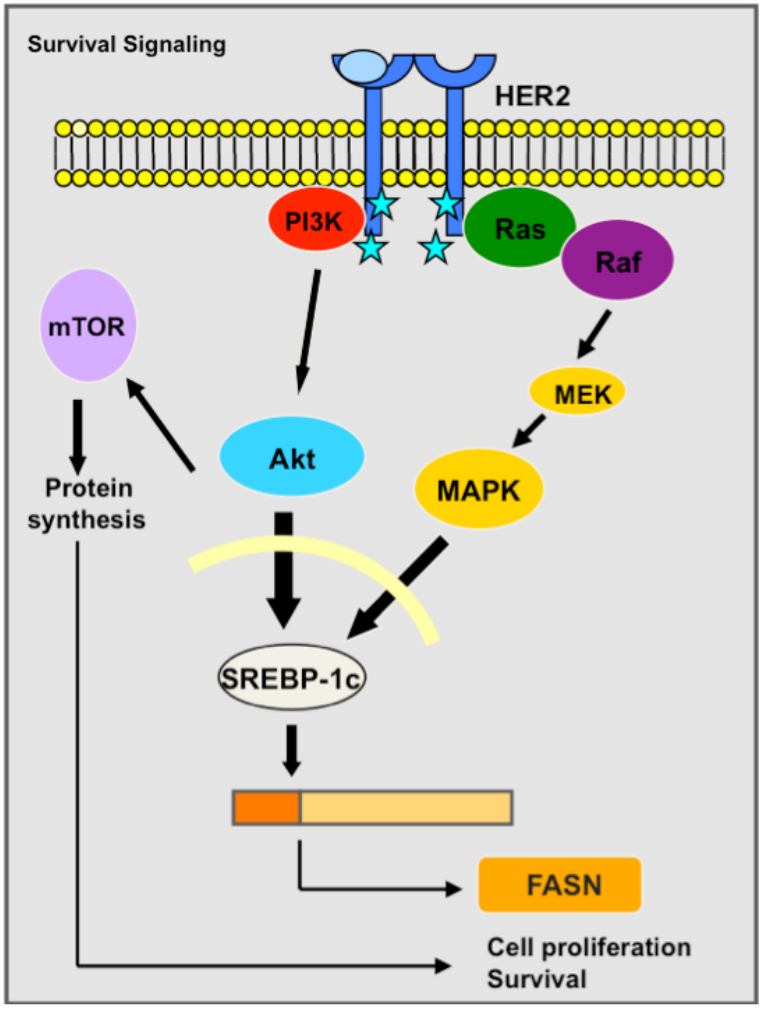
activated protein kinase (MAPK) through the transcription factor, SREBP-1c (sterol regulatory element binding protein-1c) [19, 20].

Studies with inhibitors of MAPK and PI3K in cell lines of breast cancer (MCF-7) and colon cancer (HCT116) showed a decrease in levels of SREBP-1c, FASN transcription and fatty acid synthesis. Moreover, in normal epithelial breast cells transformed with the oncogene H-ras, there was a rise in levels of both FASN and fatty acids [19]. These results were confirmed by immunohistochemistry in tumor tissues of patients with breast cancer, which showed a coordinated expression of FASN and the transcription factor SREBP-1c [21]. Our research group has demonstrated the direct involvement of the oncogene HER2 (epidermal growth factor receptor) in regulating FASN in breast cancer, possibly via kinase-dependent signaling pathways: PI3K/Akt and MAPK [12, 20-22].



Key: ACC: acetyl-coA carboxylase, ATP: adenosine triphosphate; ACS: acyl-CoA synthetase; LCFA: long-chain fatty acids, CoA: coenzyme A; EGCG: epigallocatechin gallate; NADPH: reduced nicotine adenine dinucleotide phosphate; TAG: Triacylglycerides.

Figure 1. Fatty acid metabolism. Glucose is transported from mitochondria to cytoplasm in the form of citrate, which is converted into acetyl-coA. This is metabolized to malonyl-CoA which together with the acetyl-coA and NADPH are substrates of fatty acid synthase (FASN) for the formation of the fatty acid (FA) palmitate. The malonyl-CoA inhibits carnitine palmitoyltransferase 1 (CPT1), by blocking beta-oxidation synthesized FAs. C75 inhibits FASN activity and stimulates the activity of CPT1. EGCG only inhibits the activity of FASN.



Key: FASN: fatty acid synthase; HER2: type 2 epidermal growth factor receptor; PI3K: Phosphatidylinositol 3-Kinase; MAPK: mitogen-activated protein kinase; MEK: extra-cellular signal regulated kinase (activated by Raf and Ras, binding proteins for GTP/GDP); mTOR (mammalian Target of Rapamycin): serine threonine kinase, protein synthesis regulator; SREBP-1c sterol regulatory element binding protein 1c.

Figure 2. Proposed model for genetic over-expression of FASN induced by HER2 in human breast cancer cells. The over-expression by the FASN gene in a subtype of breast cancer cell appears to be modulated by the transcription factor SREBP-1c as a result of activation of kinase-dependent signaling pathways (PI3K/Akt and MAPK) which are related to the hyperactivity of the HER2 oncogene.

A recent study suggests that HER2 regulation of FASN could also be mediated by the signaling pathway of the mTOR (mammalian target of rapamycin) kinase. This is a serine-threonine kinase regulating protein synthesis (Figure 2). In this sense, via the treatment of prostate cancer cells that suffered a deletion of PTEN (the gene suppressor of the PI3K signaling pathway) with a PI3K inhibitor (LY294002), the values for FASN expression were significantly reduced [23]. In the same study, the tumor cells were transfected with the Akt gene and FASN concentrations were restored, indicating the role of the PI3K/Akt signaling pathway in regulating FASN expression and in the development of prostate cancer. These results were subsequently confirmed in human tissues of prostate cancer, in which FASN expression increased with increased PI3K/Akt expression whereas it decreased with increased PTEN expression [24].

FASN: A Prognosis Marker of Cancer

Fatty acid synthesis is a common process in most neoplasias, since fatty acids form part of the structural lipids of the membranes. It is now accepted that fatty acid synthesis must provide some advantage to tumor cells, since it is an anabolic process that consumes energy. This hypothesis has been accepted mainly in the cases of breast and prostate cancer, as there are studies that show a positive association between FASN expression and cancer prognosis. Thus, in stage I breast cancer, high values of FASN expression in tumor cells can increase the risk of dying from the disease by a factor of four compared with low values [25]. In another more recent study, FASN expression is associated with the HER2 expression in tumors with poor prognosis [26]. In prostate cancer, FASN expression leads to a higher Gleason score and a higher risk of death from the disease, which increases by 12 when high FASN values are associated with a deletion of PTEN [23, 24].

It has been observed that FASN expression also confers a worse prognosis in patients with stage I non-small cell lung cancer [4, 27], endometrial carcinoma [28], malign melanoma [29, 30] and soft tissue sarcoma [31] (Table 1). Studies of serum from patients with breast, prostate, colon and ovary cancer have shown high levels of FASN in comparison with healthy subjects [32]. In another clinical study of patients with breast cancer, FASN values increased with the stage of the tumor [33]. However, more studies are needed

to determine the prognostic and / or predictive role of FASN values in the serum of patients with cancer.

The clinical-pathological data does indicate that high levels of FASN expression facilitates growth and proliferation of malignant cells via *de novo* synthesis of fatty acids that make up part of the plasma membrane of proliferating tumor cells. Another hypothesis to explain the relationship between over-expression of FASN and cancer concerns the high acid and hypoxic microenvironment generated in tumors, since FASN may confer a selective advantage in situations of minimal oxygen, acidity and low levels of nutrients.

In conclusion, it appears that a knowledge of FASN expression levels would allow us to predict the prognosis for the disease—including cases at the same stage—and determine which therapeutical approach to take. In this sense, there are known natural and synthetic compounds capable of inhibiting the FASN activity, most of them already in the process of preclinical research.

Table 1. Clinical studies in which FASN values have been determined in cancer-affected patients

Clinical Trial	Neoplasic tissue	Serum
Wang Y. *et al*. Clin. Chim. Acta. (2001)		Breast
Wang Y. *et al*. Cancer Letter (2001)		Ovary
Wang Y. *et al*. Clin. Chim. Acta. (2001)		Prostate
Wang Y. *et al*. Clin. Chim. Acta. (2001)		Colon
Alo P.L. *et al*. Cancer (1996) Milgraum L.Z. *et al*. Clin. Can. Res. (1997)	Breast	
Shurbaji M.S. *et al*. Hum. Pathol. (1996) Epstein J.I. *et al*. Urology (1995)	Prostate	
Visca P. *et al*. Anticancer Res. (2004) Wang Y. *et al*. Zonghua Zhong Liu Za Zhi (2004)	Lung Carcinoma	
Kapur P. *et al*. Mol. Pathol. (2005) Innocenzi D. *et al*. J. Cutan. Pathol. (2003)	Melanoma	
Takahiro T. *et al*. Clin. Cancer Res. (2003)	Soft tissue Sarcoma	
Pizer E.S. *et al*. Cancer (1998)	Endometrium	
Rashid A. *et al*. Am. J. Pathol (1997)	Colon	
Gansler T.S. *et al*. Hum Pathol (1997)	Ovary	

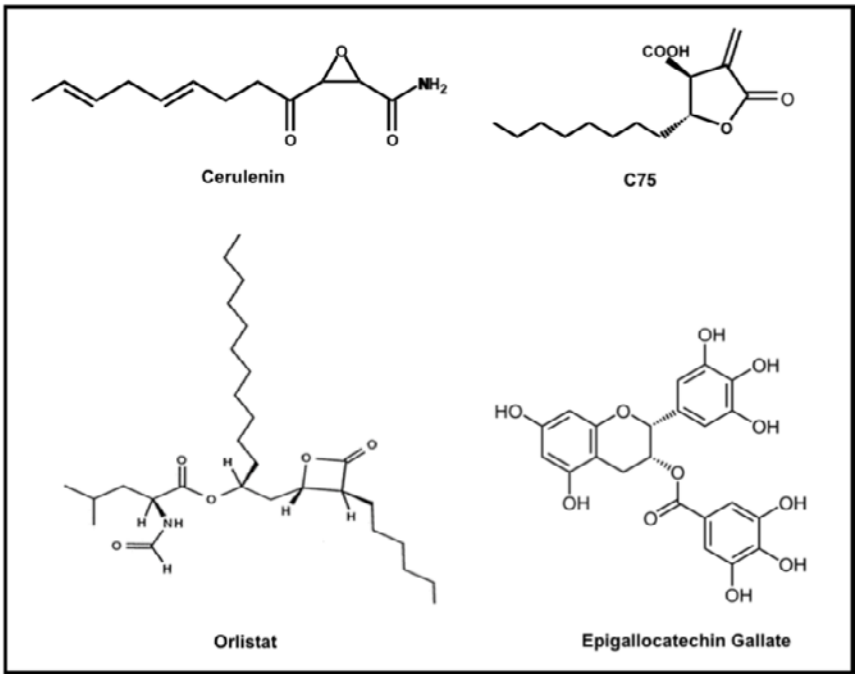


Figure 3. Structure of inhibitors of fatty acid synthase.

Table 2. Effect of EGCG, C75 and cerulenin on FASN inhibition and human breast cancer cell growth

Drug	CI_{50} (µmol)			FASN inhibition activity (%)
	SK-Br3	MCF-7	MDA-MB-231	
	FASN expression values			
	+++	+	+/-	
EGCG	149 (20)	205 (7)	197 (15)	59 (12)
C75	30 (2)	64 (3)	88 (1)	43 (4)
Cerulenin	17 (2)	30 (1)	33 (2)	65 (2)

CI_{50}: minimum inhibitory concentration; EGCG: epigallocatechin gallate; FASN: fatty acid synthase. The mean values are expressed (standard deviation) (n =3). $p < 0.05$.

FASN: An Antitumor Target for the Design of New Antineoplastic Agents

The differential FASN expression between normal cells and tumor cells, the high rates of FASN expression in most recurrent solid tumors and its relation to poor prognosis all lend support to the role of FASN as a potential target for therapeutic intervention in cancer treatment. These findings have led to the development of FASN inhibitors as antitumor drugs (Figure 3). The first inhibitor of FASN activity with cytotoxic effects on tumor cells tested in vitro and *in vivo* in xenotransplantation animal models was cerulenin, a natural product of the fungus *Cephalosporium* caerulens [34]. Then came a synthetic analogue of cerulenin, C75 (alpha-methylene-gamma-butyrolactone)—much more chemically stable and yet with the same antitumoral capacity *in vitro* and *in vivo* (xenotransplantation of human tumor cells of breast, prostate and mesothelioma) [6, 9, 11, 35, 36]. Other pharmacological inhibitors of FASN include the beta lactone orlistat and the antibiotic triclosan (5 chloro 2 (2.4 dichlorophenoxy) phenol).

Despite the fact that C75 provided the first evidence of tumor shrinkage *in vivo* through the inhibition of FASN, treatment with C75 had to be suspended both because of the high weight loss and the anorexic effects suffered by the animals [37, 38]. After studying the mechanism of action by which C75 induced weight loss, our research group has shown that apart from inhibiting FASN, C75 *in vitro* also stimulates another enzyme, carnitine palmitoyltransferase 1 (CPT1), and consequently activates fatty acid oxidation (Figure 1), and *in vivo*, in mice, produces a 20% weight loss within 24 hours of C75 treatment [39].

Numerous epidemiological studies indicate that consumption of green tea (*Camellia sinensis*) brings cardiovascular benefits and has preventive and therapeutic effects on different types of neoplasm (breast, prostate, lung, skin, liver and bladder) [40, 41]. It is also known that the molecules of green tea that produce this biological activity are polyphenolic catechins: (-) epicatechin (EC), (-) epigallocatechin (EGC), (-) epicatechin-3-gallate (ECG) and (-) epigallocatechin-3-gallate (EGCG). Several studies, including ours, show that EGCG, which is the most abundant catechin in green tea, is the most active antitumor compound. Recently, our research group, along with other groups, has shown that EGCG inhibits FASN activity and induces apoptosis in several human tumor cell lines [42-46] (Table 2). Moreover, we found that EGCG had comparable effects to those of C75 in terms of inhibiting FASN activity and

inducing apoptosis in tumor cells and that it also produces a significant reduction of the active forms of the oncoprotein HER2 and the kinases MAPK and Akt. However, unlike C75, we demonstrated that EGCG has no effect on CPT1 activity, fatty acid oxidation or weight loss *in vivo* (Figure 1).

Despite its specificity, EGCG has two characteristics that may limit its therapeutic use: the high dosage required for antitumor activity (Table 2) and its low chemical stability under physiological conditions (neutral or alkaline). With the aim of synthesizing a new series of FASN inhibitors structurally related to EGCG, chemically stable and with a potent anti-tumor activity that does not affect fatty acid oxidation or produce weight loss, we designed and developed a family of polyphenolic FASN inhibitors [47]. Two of these polyphenolic FASN inhibiting compounds have the same antitumor properties as EGCG and also produced a significant reduction in the activation of the oncogene HER2 and the HER2-dependent signaling pathways. The preliminary results, which we believe are very promising, show that they enhance the cytotoxic effect of current treatments for breast cancer [48].

Conclusion

The role of over-activation of lipogenesis in neoplastic disease and the fact that pharmacological inhibition of FASN induces apoptosis of tumor cells is an area of ongoing active research. Initial studies focused on exploring the relationship between pharmacological inhibition of FASN and cytotoxicity. These studies indicated that the accumulation of malonyl-CoA—the FASN substrate—and not the depletion of the final product was the cause of death of cancer cells. Recent work suggests that the metabolic changes associated with FASN inhibition also seem to be involved in the cytotoxicity of cancer cells.

There are a variety of signaling pathways that modulate the cytotoxicity of FASN. It has been shown, for example, that apoptosis induced by FASN inhibition is more effective in cells that have non-functional p53 protein. The involvement of the over-expression of the oncogene HER2 in the induction of apoptosis mediated by FASN inhibition has also been described. It has been shown, both *in vitro* and *in vivo* in breast and ovarian tumor models, that FASN inhibition decreases Akt activation, resulting in the induction of apoptosis. However, more studies are needed to explore the signaling pathways that modulate apoptosis mediated by inhibition of FASN activity.

Inhibition of FASN has been studied in preclinical models of breast, ovarian, colon, lung and prostate cancer. In all of these, treatment with pharmacological inhibitors of FASN results in a dose-dependent reduction in both tumor volume and FASN enzyme activity. What remains to be seen is the development of highly specific pharmacological inhibitors of FASN that may constitute new therapeutic alternatives for cancer treatment.

In conclusion, research on FASN and fatty acid metabolism is of great interest in terms of its applications in the diagnosis, prognosis, treatment and prevention of cancer. Taken together, these findings suggest that FASN is a new target for antitumor drugs and therefore the development of new FASN inhibitors promises to be a valid antitumoral strategy for most solid tumors.

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Chapter VIII

Impaired Mitochondrial Respiration as a Causative Factor in Parkinson's Disease

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Abstract

Parkinson disease (PD) is a neurodegenerative disorder characterized by a progressive loss of the nigrostriatal dopaminergic neurons. Several clinical and experimental studies have suggested the roles of oxidative stress and impaired mitochondrial respiration in the pathogenesis of PD. It is believed that oxidative modification of the proteins of mitochondrial respiratory chain alters their normal function leading to the disruption of electron transport and consequently the impairment of oxidative phosphorylation, culminating in the state of energy crisis in neurons. Systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mouse is the most extensively used experimental model of PD. MPTP-induced neurotoxicity is accompanied by the blockade of electron flow from NADH dehydrogenase to coenzyme Q at or near the same site as do the rotenone (another parkinsonian toxin). Coenzyme Q is an electron acceptor bridging mitochondrial complexes I and II/III and also serves as a potent antioxidant that has been shown to partially recover the function of dopaminergic neurons. Moreover, the infusion of

the ketone bodies in mice confers protection against MPTP-induced dopaminergic neurodegeneration and motor deficits by improving mitochondrial respiration and ATP production. It is highly imperative that novel drug regimens targeting the restoration of mitochondrial respiration, improvement of dopaminergic neurotransmission and alleviation of oxidative stress would offer beneficial effects for the management of PD.

Introduction

Parkinson's disease (PD) is one of the most frequently occurring neurodegenerative diseases in humans and is characterized by resting tremors, hypokinesia, cogwheel rigidity and loss of postural reflexes. This disease predominantly affects the nigrostriatal dopaminergic systems and associated with a progressive loss of dopaminergic neurons in the substantia nigra and massive depletion of dopamine (DA) in striatum. While the underlying cause of PD is not clearly understood, both genetic and environmental factors are thought to be involved in the development of this disease. Epidemiological studies have suggested a possible link between pesticide exposure and PD [Gorell et al 1998; Menegon et al 1998]. Recent evidences point towards a putative role of mitochondrial dysfunction and oxidative stress in the pathogenesis of PD [Mortiboys et al 2007, Greenamyre et al 2001]. The mitochondrial respiratory chain is located in the mitochondrial inner membrane and composed of five complexes including complex I (NADH:ubiquinone oxidoreductase), complex II (succinate:ubiquinone oxidoreductase), complex III (ubiquinol:ferricytochrome c oxidoreductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase). The two electron carriers, coenzyme Q10 (ubiquinone) and cytochrome c are involved in the transfer of electrons between the respective complexes. Loss of mitochondrial complex I catalytic activity in the electron transport chain has been observed in PD patients as well as in the animals models of this disease. Keeney et al [2006] have suggested that reduced complex I function in PD brain mitochondria arises from the oxidation of its catalytic subunits from internal processes, not from external oxidative stress. This complex I auto-oxidation may derive from abnormalities in mitochondrial or nuclear encoded subunits, complex I assembly factors, exposure to complex I toxins, or their synergistic combination [Keeney et al 2006]. Moreover, altered expressions of

the parkin and α-synuclein genes have also been associated with impaired mitochondrial function suggesting an important role of these genes in PD [Palacino et al 2004; Müftüoglu et al 2003; Hsu et al 2000]. This chapter summarized the current research implicating the impairment of mitochondrial respiratory chain in the pathogenesis of PD.

Free Radical Generation in Mitochondria

The mitochondrial respiratory chain is the major site of superoxide free radical production, wherein the complexes I and III are considered to be the major sites of superoxide generation [Liu et al 2002]. Mitochondrial free radical production is promoted by the inhibition of electron transport at any point distal to the sites of superoxide production [Chen et al 2003]. Neurotoxins that induce parkinsonian neuropathology stimulate superoxide production at complex I of the electron transport chain [Takeshige 1994] and also stimulate free radical production at proximal redox sites including mitochondrial matrix dehydrogenases [Fiskum et al 2003]. The free radical generation may result directly from inhibition of the mitochondrial respiratory chain or indirectly during the apoptotic process itself [Seaton et al 1997]. Mitochondria play a pivotal in controlling apoptosis; alterations in energy (ATP) production by mitochondria can induce apoptosis in neurons or increase their sensitivity to apoptosis [Gorman et al 2000]. When complex I is partially inhibited, mitochondria in nerve terminals become more vulnerable to oxidative stress; this mechanism could be crucial in the development of bioenergetic failure in PD [Chinopoulos and Adam-Vizi 2001].

Dopamine: A Key Neurotransmitter Involved in PD

Disruption of dopaminergic neurotransmission is a key feature in PD. Since PD is a slowly progressive disease, it is believed that by the time the first clinical symptoms of PD manifest, approximately 80% of the dopaminergic neurons of the substantia nigra have already degenerated and the disease entered in an advanced stage. Dopamine metabolites, DA quinone and reactive oxygen species (ROS) can directly alter protein function by

oxidative modifications. Several mitochondrial proteins may be the targets of this oxidative damage with possible implication in neurodegenerative conditions including PD [Berman and Hastings 1999]. Nunes et al [2005] have examined the combined effects of nitric oxide (NO) and dihydroxyphenylacetic acid (DOPAC), a major mitochondria-associated DA metabolite on the respiratory chain of isolated rat brain mitochondria. Although DA or DOPAC induced no measurable effects on the mitochondrial respiration rate, a mixture of NO with DOPAC inhibited the rate in a way stronger than that exerted by NO alone; this selective inhibition occurred at the level of complex IV. These investigators suggest that the NO/DOPAC-dependent inhibition of O_2 uptake at cytochrome oxidase may involve nitroxyl anion that may have an implication for mitochondrial dysfunction in PD [Nunes et al 2005]. In another study, DA failed to elicit damage of mitochondria in PC12 cells however it significantly potentiated the effects of NO at or near the threshold level leading to irreversible impairment of mitochondrial respiration [Antunes et al 2002]. In the early stages of PD, NO production increases until reaching a point near the threshold level that induces neuronal damage. Dopamine stored in dopaminergic cells may cause these cells to be more susceptible to the deleterious effects of NO, which involve irreversible impairment of mitochondrial respiration [Antunes et al 2002]. Moreover, an in-vitro study has shown the inhibition of mitochondrial respiration by DA and its metabolites by four distinct MAO-dependent and independent mechanisms [Gluck and Zeevalk 2004]. Choi et al [2006] have shown that tetrahydrobiopterin (BH_4), an obligatory cofactor for the DA synthesis enzyme, tyrosine hydroxylase, inhibits of activities of mitochondrial complexes I and IV leading to selective dopaminergic neurodegeneration both in vitro and in vivo via inducing apoptosis, suggesting the possible relevance of this endogenous molecule to the pathogenesis of PD. Liang et al [2007] have shown that that mitochondria size and mass are not the same for all neurons, and the nigral DA neurons have relatively low mitochondria mass that may contribute to the selective vulnerability of these neurons in the rodent models of PD.

MPTP: A Neurotoxin for the Animal Model of PD

The compound, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a selective neurotoxin of dopaminergic neurons in the substantia nigra and striatum and has been shown to induce Parkinson's disease-like syndrome in humans, primates and rodents [Ballard et al 1985; Langston et al 1984a]. MPTP-treated mice serve as a convenient and extensively used experimental model of PD. Impairment of mitochondrial respiration is one of the main features of MPTP induced deleterious events that ultimately lead to the death of dopaminergic neurons [Nicklas et al 1985; Przedborski and Jackson-Lewis 1998; Przedborski et al 2000]. In brain, MPTP is converted to its toxic metabolite MPP^+ in the presence of enzyme monoamine oxidase B [Chiba et al 1984, Langston et al 1984b]. MPP^+ is actively taken up into nigrostriatal neurons where it inhibits mitochondrial oxidative phosphorylation leading to neuronal death. An in-vitro study has suggested that inhibition of complexes I, III, and IV (but not complex II) by MPP^+ efficiently restrict the transport of electrons down the respiratory chain which ultimately leads to decreased ATP production [Desai et al 1996]. The interaction of MPP^+ with NADH dehydrogenase interferes with the passage of electrons from the iron-sulfur cluster of highest potential to endogenous Q10; this inhibition can be relieved by the addition of a small, water-soluble Q analog [Ramsay et al 1987]. Moreover, overexpression of mutant α-synuclein in the substantia nigra renders transgenic mice more sensitive to MPTP [Song et al 2004] whereas the knockout mice are more resistant to this toxin [Dauer et al 2002].

Zhang et al [2004] have observed the protective effect of dextromethorphan against MPTP-induced neurotoxicity in the wild-type but not in the NADPH oxidase-deficient mice, indicating that NADPH oxidase is a critical mediator of the neuroprotective activity of dextromethorphan. Due to its proven safety record of long-term clinical use in humans, dextromethorphan may be a promising agent for the treatment of PD. Infusion of the ketone body d-beta-hydroxybutyrate has partially protected the mice against dopaminergic neurodegeneration and motor deficits induced by MPTP [Tieu et al 2003]. These effects appear to be mediated by a complex II-dependent mechanism that leads to improved mitochondrial respiration and ATP production. Because of the therapeutic safety record of ketone bodies and their ability to penetrate the blood-brain barrier, d-beta-hydroxybutyrate may

serve a novel neuroprotective therapy for PD [Tieu et al 2003]. Seo et al [2006] have used MPTP-treated mouse model of PD to test the possible therapeutic effect of the rotenone-insensitive NADH-quinone oxidoreductase (NDI1) gene by unilaterally injecting the NDI1-recombinant adeno-associated virus particles (rAAV-NDI1) into the substantia nigra of mice. The substantia nigra neurons on the side used for injection of rAAV-NDI1 retained a high level of tyrosine hydroxylase-positive cells in addition to significantly less denervation in the striatum as well as significantly higher striatal concentrations of DA and its metabolites in the same hemisphere than those of the untreated hemisphere. These findings make the first successful demonstration of complementation of complex I by the NDI1 enzyme in animals offering the resistance to MPTP-induced neuronal injury [Seo et al 2006].

Rotenone: An Environmental Toxin Linked to PD

Rotenone is a commonly used pesticide and a potent inhibitor of mitochondrial complex I. Betarbet et al [2000] have shown that chronic and systemic inhibition of complex I by rotenone causes highly selective nigrostriatal dopaminergic degeneration that is associated behaviorally with hypokinesia and rigidity in rodents suggesting that chronic exposure to this common pesticide can reproduce the anatomical, neurochemical, behavioral and neuropathological features of PD. The primary mechanism of the toxic effects of rotenone on rat brain mitochondria involves significant increase of ROS that causes damage to complex I and complex II, at the level of 4Fe-4S clusters [Panov et al 2005]. It is also presumed that the damage to complexes I and II shifts ROS generation from the CoQ10 sites to more proximal sites, such as flavines, leading to progressive degeneration [Panov et al 2005].

Sharma et al [2006] have shown attenuation of rotenone-induced down-regulation of complex I activity by coenzyme Q10 treatment, suggesting that complex I may be down regulated due to depletion of coenzyme Q10 in the brain. Thus, metallothionein-induced coenzyme Q10 synthesis may provide neuroprotection by augmenting mitochondrial complex I activity in PD. Imamura et al [2006] have investigated the effect of the ketone body, D-beta-hydroxybutyrate (bHB), on rotenone toxicity by using SH-SY5Y

dopaminergic neuroblastoma cells. Rotenone exposure caused the loss of mitochondrial membrane potential, released cytochrome c into the cytosol, and reduced cytochrome c content in mitochondria. Addition of bHB to the medium blocked the toxic effects of rotenone to SH-SY5Y cells suggesting that the neuroprotective effect of bHB is mediated by reversing the inhibition of complex I or II of mitochondrial respiration system [Imamura et al 2006]. Chronic administration of nicotine has been shown to preserve the mitochondrial functions of the rat central nervous system in rotenone-induced Parkinson-like syndrome [Cormier et al 2003]. Using an in-vitro model based on treating human neuroblastoma cells with 5 nm rotenone for 1-4 weeks, it has been suggested that chronic low-grade complex I inhibition caused by rotenone exposure induces accumulation and aggregation of α-synuclein and ubiquitin, progressive oxidative damage, and caspase-dependent death; these mechanisms may be central to PD pathogenesis [Sherer et al 2002]. Another in-vitro study has demonstrated that expression of mutant α-synuclein results in impaired complex I activity and enhances mitochondrial sensitivity to rotenone [Orth et al 2003].

Other Relevant Animal Studies

Lannuzel et al [2003] have examined the neurotoxic potential of another complex I inhibitor, annonacin, the major acetogenin of Annona muricata (soursop), a tropical plant suspected to be the cause of an atypical form of PD in the French West Indies (Guadeloupe). Their findings demonstrate that annonacin promotes dopaminergic neuronal death by impairment of energy production. Takeuchi et al [2005] have suggested the role of N-methyl-d-aspartate (NMDA) receptor signaling in the neurotoxicity of activated microglia. A concordant neuritic beading occurs with a rapid drop in intracellular ATP levels due to the inhibition of mitochondrial respiratory chain complex IV activity downstream of NMDA receptor signaling, leading to neuronal death. Whereas, blockage of NMDA receptors nearly completely abrogates mitochondrial dysfunction and neurotoxicity and this strategy may be an effective therapeutic approach for PD [Takeuchi et al 2005]. The combination of proteomic, genetic and physiological analyses revealed an essential role for parkin gene in the regulation of mitochondrial function and provided a direct evidence of mitochondrial dysfunction and oxidative damage

in the absence of nigral degeneration in a genetic mouse model of PD [Palacino et al 2004]. There was a decreased abundance of a number of proteins involved in mitochondrial function in accordance with marked reductions in respiratory capacity of striatal mitochondria isolated from parkin-/- mice [Palacino et al 2004].

Clinical Studies Implicating Mitochondrial Impairment in PD

Several studies have shown the decreases in the activity and content of complex I in the substantia nigra of PD patients [Schapira et al 1990; Janetzky et al 1994]. Other studies have observed a reduction in a number of complex I and complex IV subunits in idiopathic PD [Hattori et al 1993, Itoh et al 1997]. However, the results from human studies on respiratory chain activity in peripheral tissues including platelets, lymphocytes and skeletal muscle from PD patients and control subjects have been contradictory and inconclusive [reviewed by Mortiboys et al 2007]. Winkler-Stuck et al [2005] have observed a mild mitochondrial defect in skeletal muscle of patients with PD which is accompanied with 1.5 to 2-fold increase of point mutated mtDNA. Parker et al [2008] have observed a tendency for some loss of activity of all electron transport chain complexes in PD patients but this loss was significant only for complex I. They have also assayed this enzyme at various points during the mitochondria purification process and noticed that the specific activity of complex I increased approximately 4 fold during purification; while its activity was lower in PD samples at all stages of purification but reached significance only in purified mitochondria [Parker et al 2008]. The neuroprotective and symptomatic actions of CoQ10 in PD have recently been reported and this agent has been found to be well tolerated as both monotherapy and adjuvant medication in PD patients [Storch 2007]. Storch et al [2007] have determined the safety of nanoparticular CoQ10 in a randomized, double-blind and placebo-controlled clinical trial in 131 PD patients. They observed that nanoparticular CoQ10 at a dosage of 300 mg/d is safe and well-tolerated and leads to plasma levels similar to 1200 mg/d of standard formulations.

Conclusion

The compelling evidences from the animal and clinical studies suggest a prominent role of altered complex I activity in the pathogenesis of PD. It is believed that the state of energy crisis due to impaired electron transport chain function renders the cell highly vulnerable to potentially toxic ROS and other proinflammatory mediators. All these events collectively create a highly destructive cascade leading to the neuronal degeneration. The agents with the ability to block this cascade have been found to be beneficial in attenuation of disease symptoms or inhibiting the progression of PD. However, identification of novel biochemical or molecular markers is important for an early detection of mitochondrial respiratory chain impairment to help screening of high risk individuals for PD so that they could receive the therapies at an early stage for more effective management of this disease.

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Chapter IX

Ecophysiological Look at Organ Respiration in Carnivorous Plants: A Review

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Abstract

On the ecophysiological level, the magnitude of aerobic dark respiration (R_D) of a plant organ is considered a measure of the intensity of metabolism and physiological functions of the organ. In this minireview, properties of R_D are reviewed in different types of organs of carnivorous plants from a functional point of view. Although net photosynthetic rate (P_N) in leaves or traps of terrestrial carnivorous plants is usually several times lower than that in leaves of vascular non-carnivorous plants, R_D in carnivorous plant leaves is relatively high and reaches on average 48 % of P_{Nmax} representing the high metabolic (energetic) cost of carnivory. The metabolic cost of carnivory is markedly high in traps of aquatic carnivorous *Utricularia* species; the traps are structurally quite separate from photosynthetic leaves. In six species, trap R_D was 75-200 % greater than that in leaves but foliar P_N exceeded that in traps 7-10 times. This reflects high metabolic activity of *Utricularia* traps associated with pumping ions and water through the trap walls. However,

it has not yet been explained how the internal trap structures (glands taking part in prey digestion, nutrient absorption, and pumping water) provide the ATP for their demanding functions when complete anoxia occurs in the trap fluid.

R_D values of roots (1.6-5.6 nmol g^{-1}_{FW} s^{-1}) as well as water exudation rates of five carnivorous plant species were comparable with those reported in non-carnivorous plants or even higher. A high proportion of cyanide-resistant respiration (65-89 %) was found in the roots. R_D values of turions (winter buds) of some aquatic carnivorous plant species of the genera *Aldrovanda* and *Utricularia* were about 1.5-4 times lower (on FW basis) than those reported in growing shoots/leaves of these or other aquatic plant species. Contrary to true dormant turions, R_D of non-dormant winter shoot apices of *Aldrovanda* and *Utricularia* was comparable to that in aquatic plant shoots/leaves.

Introduction

On the ecophysiological level, the magnitude of aerobic dark respiration (R_D) of a plant organ is considered a measure of the intensity of metabolism and physiological functions of the organ. Such a concept is accepted also for carnivorous plants (e.g., Adamec 2005, 2006). Carnivorous plants represent an ecological, functional plant group comprising about 650 species of about 16 genera for which it is typical that they capture animal prey by their specialised traps of foliar origin, absorb nutrients from prey carcasses, and utilize them for their growth and development (Juniper et al. 1989). As compared to other processes like rapid movements, prey digestion, enzyme secretion, or nutrient uptake in carnivorous plants, which have always attracted the curiosity of botanists (Juniper et al. 1989, Adamec 1997a), the study of respiration of carnivorous plants has been neglected up to now. Knight (1992) supplemented the classic theory from Givnish et al. (1984) on cost-benefit relationships of carnivory and hypothesised that carnivorous plants invest a greater metabolic (energetic) cost in its traps as an increased R_D but reduced photosynthetic rate (P_N) comparatively to leaves. So far, only several respiration studies have been published for carnivorous plants (see Ellison and Gotelli 2009). They include comparisons of R_D of traps and leaves (Knight 1992; Méndez and Karlsson 1999; Adamec 2006; Pavlovič et al. 2007; Hájek and Adamec 2009) and particulars on R_D of *Utricularia* and *Genlisea* traps (Adamec 2007b), carnivorous plant roots (Adamec 2005), and overwintering buds (turions;

Adamec 2003). In this minireview, properties of R_D in different types of organs of carnivorous plants are reviewed from a functional point of view.

Dark Respiration of Traps and Leaves

As hypothesized by Knight (1992) traps of carnivorous plants as highly specialized organs have higher R_D than leaves/shoots and, simultaneously, due to their lower P_N, represent a great photosynthetic cost. Generally, as compared to leaves of non-carnivorous plants, in which the R_D:P_{Nmax} ratio (as an expression of metabolic cost of leaves) is on average only about 8-17 % (Givnish 1988; Wright et al. 2004), the ratio in carnivorous plant traps is usually much higher and values between 10-162 % (mean about 40-60 %, i.e. 15-25 nmol g^{-1}_{DW} s^{-1}, total range 6-45 nmol g^{-1}_{DW} s^{-1}) have been reported for various species (Méndez and Karlsson 1999; Adamec 2006; Pavlovič et al. 2007; Hájek and Adamec 2009). Although this ratio is surprisingly high in highly specialized aquatic *Utricularia* traps (50-140 %, Adamec 2006), there are no indications that aquatic carnivorous species differ distinctly from terrestrial ones in this parameter (cf. Hájek and Adamec 2009). Rather, very low P_{Nmax} values in *Utricularia* traps are responsible for this ratio. However, even in spite of the scarcity of data, due to highly specialized traps in aquatic carnivorous species, it is obvious that traps of aquatic species represent relatively, per unit DW, much greater metabolic cost (as the trap:leaf R_D ratio) than that of terrestrial species (though it is not possible to compare the proportion of trap R_D to total plant R_D due to a quite different proportion of trap DW in various taxa). As shown by Pavlovič et al. (2007) for two *Nepenthes* species and by Hájek and Adamec (2009) for *Dionaea muscipula* and *Sarracenia purpurea* no consistent difference occurred in DW-based R_D between traps and leaves. However, fresh-weight-based R_D of excised, physiologically highly active tentacles of *Drosera prolifera* was 7.3 times higher than that of the leaf laminae without tentacles (Adamec, unpubl.). Similarly, DW-based trap R_D was by 10 % higher than foliar R_D in *U. macrorhiza* (Knight 1992) and even 1.9-3.3 times higher in six aquatic *Utricularia* species (Adamec 2006) so that the proportion of trap R_D to the total plant respiration amounted to 60-68 % in three aquatic *Utricularia* species (Adamec 2006, 2007a). As it follows from very limited data R_D was changed due to prey addition neither in *Pinguicula vulgaris* leaves (Méndez and Karlsson 1999) nor in two aquatic species (Adamec 2008a) although

P_{Nmax} could be stimulated by this treatment in several species (Adamec 2008a; Farnsworth and Ellison 2008). As carnivorous plants can also take up organic substances from prey carcasses (Juniper et al. 1989, Adamec 1997a) new research should determine generally whether prey addition changes R_D in leaves and traps and the metabolic cost of carnivory, as a possible reason for the increased organ growth.

Particulars of Respiration of *Utricularia* and *Genlisea* Traps

About 50 *Utricularia* (Lentibulariaceae) species are rootless aquatic or amphibian plants which grow in standing, nutrient-poor shallow wetlands in waters with larger concentrations of humic acids and tannins (Juniper at al. 1989). *Utricularia* species capture aquatic prey, such as small crustaceans, mites, rotifers, and protozoa, in their traps (Harms 1999; Jobson and Morris 2001; Richards 2001). Moreover, diverse communities of microorganisms, mainly bacteria, algae, protozoa and rotifers, live inside traps as commensals (Jobson and Morris 2001; Richards 2001). The trap is a water-filled utricle 1-5 mm long with a wall two cell layers thick and a variety of glands and trichomes on both surfaces (Juniper et al. 1989). After an irritation of trigger hairs situated close to the trap door, an organism is sucked in as a result of under-pressure maintained inside the trap (Sydenham and Findlay 1973). During about 30 min after firing, approx. 40 % of the trap water is pumped out and the trap is ready to fire again. Thus, as a consequence of firing, the trap is partly re-filled with the ambient water together with all solutes and particles.

Generally, *Utricularia* traps are physiologically very active organs. Their aerobic DW-based R_D values were 1.9-3.3 times higher (25-43 nmol O_2 g^{-1} s^{-1}) than those of adjacent leaves/shoots (Adamec 2006). Ion and water pumping during the resetting of traps depended markedly on aerobic respiration as inhibitors such as KCN and NaN_3 prevented it when applied internally into traps; outer application was ineffective (Sydenham and Findlay 1975). However, Adamec (2007b) found consistently steady-state zero O_2 concentrations in the trap fluid of traps without prey in six aquatic *Utricularia* species regardless of whether the traps were intact or excised from the shoot or in light or darkness. Thus, under natural conditions, long periods of anoxia inside the traps can be interrupted by periods of 20-100 min after accidental trap firing when a measurable O_2 concentration occurs in the trap fluid. Therefore, captured organisms either die of O_2 deprivation within several

hours and are prey, or are able to tolerate anoxia and are commensals. The fact of the steady-state zero O_2 concentration suggests that internal glands of empty *Utricularia* traps have potentially a very high aerobic R_D when O_2 is available and that they are able to consume all O_2 inside the trap within 20-100 min (Adamec 2007b). It might be the only period when the internal trap structures respire aerobically. The comparison of the 'external' aerobic R_D of *U. reflexa* traps (cut-off traps; 1.03±0.07 nmol g^{-1}_{FW} s^{-1}) with the 'total' aerobic R_D (halved traps; 1.31±0.07 nmol g^{-1}_{FW} s^{-1}) further confirmed that the trap fluid is very isolated from the ambient water outside the traps and that anoxia in the trap fluid is not transferred outdoors. The difference between the 'total' and 'external' aerobic R_D could be attributed to 'internal' aerobic R_D of internal trap structures, mainly glands, and was consistent with the rate of decline of O_2 concentration in the trap fluid measured directly inside the traps.

However, it does not follow from the above facts how the traps really obtain their ATP energy for filling their demanding physiological functions. As there is often a low or even zero O_2 concentration in the ambient water around traps (Guisande et al. 2000, 2004; Adamec 2007a) the period of aerobic respiration after a firing could be markedly shorter than the resetting time needed. It is probable then that the internal glands obtain their metabolic energy from anaerobic fermentation. Thus, further research should determine whether products of anaerobic fermentation (e.g., ethanol, propionic acid) are produced in traps under anoxia. This conclusion does not contradict the hypothesis and findings of Jobson et al. (2004), who suggest a link between faster reaction kinetics of *Utricularia* traps and mutations occurring in the mitochondrial respiratory chain enzyme cytochrome *c* oxidase. Laakkonen et al. (2006) further hypothesize decoupling of mitochondrial proton pumping from electron transfer, which could be a rich source of ATP energy after trap firing under anoxic conditions. Such decoupling would permit traps to optimize power output during times of need. It should be added that traps with digesting prey can have much greater energetic demand than empty traps and that R_D of mainly heterotrophic trap commensals should also be taken into account in all respiration studies in *Utricularia* traps.

Genlisea (Lentibulariaceae) grows in anoxic wet substrates and forms short stems with a rosette of small green leaves and tubular subterranean traps of foliar origin (Juniper et al. 1989). The inverted Y-shaped traps are 3-12 cm long and about 1 mm in diameter. The hollow traps function as 'eel traps'. Digestive glands are abundant in the cavity of the trap. *Genlisea* traps capture fine soil organisms (bacteria, algae, protozoans, nematodes, rotifers, annelids,

crustaceans, and mites (Barthlott et al. 1998; Płachno et al. 2005; Płachno and Wołowski, 2008). The traps are probably passive (Płachno et al. 2008). This explains that, unlike *Utricularia* traps, aerobic R_D in traps of three *Genlisea* species was very low (0.38-1.14 μmol g^{-1}_{FW} s^{-1}) and quite insensitive to either 0.5 mM KCN or 0.2 mM NaN_3 (Adamec 2005). A high proportion of cyanide-resistant respiration (74-87 % of the total R_D) was found in all three species. Trap walls in *Genlisea* (around the vesicle and tubular neck) contain large air spaces connecting them with leaves. Although nearly-saturated O_2 concentrations occurred in the trap walls in *G. hispidula*, the O_2 concentration in the fluid in the central trap cavity in the vesicle and 2 mm below it was strictly zero (Adamec 2007b). This situation is exactly the same like in *Utricularia* traps: abundant digestive glands lining the central trap cavity are able to consume all O_2 diffusing inside the trap from the walls in spite of that the trap is not closed. Again, it is not clear how the digestive glands inside *Genlisea* traps respire under complete anoxia.

Respiration of Carnivorous Plant Roots

Roots of the majority of carnivorous plants grow in bog and fen soils which are usually wet or waterlogged, mostly acidic, and poor in available mineral nutrients (Juniper et al. 1989). A weakly developed root system is common for most species and the root:total biomass ratio lies only between 3.4-23 % (Adamec 1997a). Aerobic R_D values were between 1.6-5.6 nmol O_2 g^{-1}_{FW} s^{-1} in apical root segments in seven carnivorous plant species (Adamec 2005). However, the rate of anaerobic fermentation in roots of two *Drosera* species was only 5-14 % of the aerobic R_D. Neither 0.5 mM KCN nor 0.2 mM NaN_3 influenced root R_D. In roots of five carnivorous species, the proportion of cyanide-resistant respiration was high and between 65-89 % of the total R_D value. Thus, the R_D values found in roots of carnivorous plants are comparable with those reported in roots of non-carnivorous plants in the literature or even higher. Generally, carnivorous plant roots appear to be physiologically very active and well adapted to endure permanent soil anoxia, mainly due to oxygen diffusion through the root intercellulars (Adamec 2005).

Respiration of Turions

Turions are vegetative dormant storage organs produced by perennial aquatic plants. They are modified shoot apices and protect fragile plant shoots from freezing and decaying (Bartley and Spence 1987). Turions of free-floating aquatic carnivorous plants *Aldrovanda* and *Utricularia* spp. break their dormancy at the bottom of an aquatic habitat, but usually germinate at the water surface, in warmer water and at higher irradiance (Adamec 1999, 2003, 2008bBI). *Aldrovanda* turions break off the dying mother shoots, actively sink in autumn and rise in the spring, while *Utricularia* turions are dragged down by decaying shoots (Adamec 1999). The FW-based aerobic R_D of *Aldrovanda vesiculosa* turions at 20 ^oC (0.74-1.5 nmol O_2 g^{-1}_{FW} s^{-1}) was rather low on an absolute scale and reached only about 27-55 % of that of growing shoots of the same species (cf. Adamec 1997b, 2003). It was only 0.22±0.02 nmol g^{-1}_{FW} s^{-1} at 4 oC (Q_{10} 2.78). No significant differences in R_D occurred between innate (autumn) and imposed dormancy (spring) or during two days of breaking imposed dormancy at 20 ^oC (Adamec 2003). However, the anaerobic fermentation rate of *Aldrovanda* turions was only 1.5-7 % of the aerobic R_D and was also constant during the breaking of imposed dormancy. Such a low ratio might reflect both low energy consumption in overwintered turions and their biochemical adaptation to anoxia, saving reserve sugars. It may be suggested that a spring temperature increase causes an increase of turion fermentation or R_D which is further responsible for the evolution of gas in turion lacunae and, thus, for turion rising.

In autumnal (dormant) turions of four aquatic carnivorous plant species (*A. vesiculosa, U. australis, U. ochroleuca, U. bremii*), aerobic R_D values ranged from 0.36-0.71 nmol O_2 g^{-1}_{FW} s^{-1} at 20 ^oC and, except for *U. bremii,* increased by 11-114 % after overwintering (Adamec 2008b). These R_D values of dormant turions represented only 25-73 % of those found in photosynthetic shoots of the same species (cf. Adamec 2006). Thus, turions behave as typical storage, overwintering organs with low R_D. Respiration Q_{10} ranged from 1.75-2.55 in dormant turions and from 2.59-3.39 in non-dormant turions. Except for *Aldrovanda*, turion R_D was not reduced in 0.5 mM KCN (Adamec 2008b). In all turions, the proportion of cyanide-resistant respiration was high and between 50-90 % of the total R_D value. As very similar respiration characteristics were also found in turions of two non-carnivorous species, *Hydrocharis morsus-ranae* and *Caldesia parnassifolia* (Adamec 2008b), it may be concluded that respiration characteristics of turions are not dependent

on plant carnivory. However, contrary to true turions, R_D values in non-dormant winter apices both in Australian *Aldrovanda* populations and temperate *U. radiata* and subtropical *U. purpurea*, and in sprouting turions of temperate *Aldrovanda* were high (2.1-3.1 nmol O_2 g^{-1}_{FW} s^{-1} at 20 °C; Adamec 2008b) and similar to those in aquatic plant leaves or shoots.

Conclusion

As follows from the review study of dark respiration has brought a substantial progress in understanding ecophysiological traits in carnivorous plants. As shown some organs of carnivorous plants (roots, *Utricularia* and *Genlisea* traps, turions) normally face strong hypoxia or anoxia but it is not clear how they do provide ATP energy to ensure their physiological functions under these conditions as their respiration characteristics have mainly been studied under aerobic conditions. R_D has been found to be a distinct criterion between the true, dormant turions and non-dormant winter apices in some aquatic carnivorous plant species.

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