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CHEMICAL-INDUCED CHANGES IN INTRACELLULAR REDOX STATE AND IN APOPTOSIS

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Abstract. Necrosis and apoptosis are two ways by which cells die. A major concept of apoptosis is that it is a controlled process. From this concept it follows that cells contain a molecule or molecules which under specific, regulated circumstances mediate cell death. Recent data confirm that oxygen free radicals can be mediators of apoptosis. Chemicals could induce apoptosis due to reactive oxygen species production and changes in the intracellular redox state. Therefore, a complete understanding of the processes involved in apoptosis, and mechanisms of its manipulation, could provide novel strategies to the control of xenobiotic toxicity and give an impetus to design new therapeutic interventions.

INTRODUCTION

For many years cell death has been considered as a degenerative process dependent on toxic injury that can occur through the effect of some chemical, physical or biological (infectious) agents. However, a non-toxic mode of cell death is known to occur. "Necrosis" as pathological cell death, and "apoptosis"* as physiological cell death or programmed cell death, are two ways by which cells die. "Necrosis" has been a traditional term for accidental cell death after massive tissue damage leading to rapid collapse of internal homeostasis of the cell. The earliest morphological changes are swelling of the cytoplasm and organelles. These changes are associated with membrane lysis and inflammation. "Apoptosis" is a form of non-toxic cell death occurring during embryogenesis as well as a form of toxic cell death occurring in many pathological conditions i.e. in the elimination of damaged or neoplastic cells. Apoptosis plays also an important role in the development and regulation of the immune system. In addition, apoptosis is not

* apoptosis – In 1972 Kerr, Wyllie and Currie (18) introduced the term "apoptosis" as opposite to that of "necrosis". Apoptosis is derived from the Greek term and indicates the fall of leaves from trees or the hedding of petals from flowers.

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commonly associated with the inflammatory response that accompanies necrosis. Morphological characteristics of apoptosis include nuclear fragmentation, chromatin condensation and plasma membrane blebbing. Genetic characteristics include the inter-nucleosomal fragmentation of DNA and enhanced expression of a set of genes. Apoptosis seems to be an active mode of cell death requiring active RNA and protein synthesis. This active cell death appears to be supported also by the existence of pro- and anti-apoptotic genes. Much attention has been directed toward the supressory effect of bcl-2 gene family as well as the stimulatory effect of p53 gene (3,9,15,18,31,34,44).

The list of apoptosis inducers is long, and includes over a hundred of different agents. It is convenient to divide the apoptosis initiating stimuli into two groups: physiological, such as alterations in the level of cytokines and steroid hormones, and non-physiological, some of which are: viral infection, physical agents like ionising radiation or temperature changes, or chemical agents: anticancer drugs, calcium ionophores or environmental toxicants (41). Thus, the induction of apoptosis by any given factor offers insight neither into the toxic properties of the given factor nor into the mechanism of apoptosis itself. The fundamental biochemical mechanism of cell death is unknown, but appear to be conserved across phyla and cell types (7,34,41). This lack of knowledge makes it particularly complicated to understand why a cell dies by apoptosis or necrosis.

A major concept of apoptosis is that it is a controlled process. From this concept it follows that cells contain a molecule or molecules that under specific regulated circumstances mediate cell death (3). This suggests that different stimuli require some common intracellular mediator that would elicit the generalized features of apoptosis (34). Therefore, we ought to distinguish between the inducer of apoptosis and the mediator of apoptosis.

MEDIATORS OF APOPTOSIS

In 1994 Sarafian and Bredesen proposed the identification of apoptosis mediator, as opposite to inducer, which would offer some insight into the biochemistry of apoptosis (34). Such a mediator should possess three major "biochemical" properties: 1) the mediator should demonstrate a change (an increase or decrease in activity) during apoptosis, prior to cell death, 2) modulation of the putative mediator should modulate apoptosis accordingly, 3) expression of anti-apoptotic genes should affect the putative mediator. Studies from different laboratories have implicated reactive oxygen species (ROS) as mediators of apoptosis.

OXYGEN FREE RADICALS

A free radical is an atom, molecule or a compound with one or more unpaired electrons. These chemical species are highly electrophilic and active. The reactive radicals are generally short-lived species. Electron acceptors such as molecular oxygen react easily with free radicals to became oxygen free radicals. The reduction by four electrons of molecular oxygen produces reactive species of oxygen (ROS); which include: singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\circ -}$), hydroxyl radical ($^{\circ}OH$) and peroxide anion (O_2^{--}). Free radicals are produced in normal as well as in pathological cell metabolism. ROS are produced in intracellular systems

like the mitochondrial and microsomal electron transport systems, in peroxisomes and through the autoxidation of small soluble molecules; catecholamines, flavins, quinones and thiols. Reactions catalyzed by lipoxygenase and cyclooxygenase in the synthesis pathway of leukotrienes, thromboxanes and prostaglandins also involve oxygen free radical production. A main source of superoxide anion is the "respiratory burst" of activated phagocytic cells. All the cellular components: lipids, proteins, nucleic acids and carbohydrates, may be damaged by reaction with reactive oxygen species. When these species react with non-radicals, new free radicals can be formed which leads to chain reactions i.e. lipid peroxidation (14). The overproduction of these oxygen free radicals can give rise to functional and morphological disturbances in the cell through the oxidative stress. According to Sies "oxidative stress" may be defined as a disturbance in the prooxidant-antioxidant balance in favour of the former (37).

In the cell there are enzymatic systems and many biochemical scavengers able to decrease too high level of oxygen free radicals. Primary defense systems diminish the rate of radical reactions by decreasing free radical concentration; these include: enzymes (superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase) or small molecules (glutathione (GSH), vitamin C, uric acid). Secondary defense systems or chain-breaking systems trap radicals, stopping their harmful effects; these include: enzymes (glutathione transferases, oxidoreductases) or molecules (α-tocopherol, β-carotene, bilirubin) (25). Under physiological conditions ROS are part of normal regulatory circuits and the cellular redox state is tightly controlled by antioxidants. However, oxidative stress, arising either from the increase in production of ROS or from the deficiency of antioxidant defence mechanism, could result in reversible or irreversible tissue injury. The biological conseqences are cytotoxicity, acute inflammation, mutation, chromosomal aberrations, carcinogenesis, cellular degeneration related to aging or cell death (28). It rises the possibility, that reactive oxygen species, via tissue oxidant injury, may directly or indirectly participate in the initiation of apoptotic cell death.

CHEMICAL-INDUCED APOPTOSIS BY OXYGEN FREE RADICALS PRODUCTION

According to Sarafian and Bredesen (34):

I. "m e d i a t o r s h o u l d d e m o n s t r a t e a c h a n g e d u r i n g a p o p t o s i s"

These few findings argue that ROS could be mediators of apoptosis:

1) Exposure to low concentration of hydrogen peroxide induces apoptosis in HL-60 cells, while necrosis occurs if the oxidant is present in millimolar concentrations. Hydrogen peroxide produced by the increased activity of an amine oxidase is thought to be responsible for apoptosis of embryonic cells with trophectodermal potential (19).

2) Exposure to increasing doses of redox-cycling 2,3-dimethoxy-1,4-naphthoquinone in pancreatic RINm5F cells progressively results in cell proliferation, apoptosis and finally necrosis due to oxidative stress (8).

3) Exposure of rat thymocytes to pyrrolidine dithiocarbamate (PDTC) (1–40 μM) induces initially a 2–3 fold increase in the intracellular concentration of oxidised glutathione (GSSG), which is followed several hours later by cell shrinkage and chromatin fragmentation (characteristic of apoptosis) (38).

4) Acetaldehyde, produced by the metabolism of ethanol, induces apoptosis of hepatocytes due to oxygen radicals production (17).

5) Zinc deficiency has been shown to result in apoptosis in the myeloid HL-60 and lymphoid Raji cell lines. Zinc deficiency may result in an increase in oxidative damage due to reduction in Cu/Zn SOD activity. A lack of zinc stabilizing action on biomembranes and an increase in membrane binding of redox – active metals could promote oxidant production (32).

II. "modulation of the putative mediator should modulate apoptosis accordingly"

Much of the evidence in support of this idea comes from the observation, that antioxidants could inhibit or delay the onset of this type of cell death. For example:

1) Thymocytes undergoing glucocorticoid-induced apoptosis have been shown to exhibit an early reduction in mitochondrial membrane potential and antioxidants prevent this change (43). Furthermore, both pre-apoptotic and fully apoptotic thymocytes were found to have a lower glutathione (GSH), protein sulphydryl and α-tocopherol content than non-apoptotic cells (38).

2) In an interleukin 3-dependent pro-B lymphocyte cell line, growth factor withdrawal induces apoptosis, while overexpression of glutathione peroxidase provides significant protection (15).

3) Cultured primary sympathetic neurones die by apoptosis when deprived of nerve growth factor (NGF). Direct injection of the cells with Cu/Zn superoxide dismutase (SOD), within the first few hours of NGF removal, delays their death. In neuronal PC 12 cells NGF has been shown to increase the expression of several enzymes involved in antioxidant defence system such as glutathione peroxidase, catalase and γ-glutamylcysteine synthase (12).

4) Tumor necrosis factor (TNF) induces apoptosis in many neoplastic cell types. Intracellular ROS are induced by TNF, and expression of MnSOD or Bcl-2 (see below) inhibits cell death induced by TNF (6).

5) It has been shown that Trolox, a water-soluble vitamin E analogue, can prevent oxidative stress-induced apoptosis in thymocytes. It has been suggested that trolox may protect membranes from oxidation and a resultant dysfunction of calcium homeostasis. In 1995 Fryer proposed a novel function for the vitamin E and its involvement in the control of apoptosis as a "gate keeper" in the regulation of membrane lipid peroxidation (11).

6) Recent studies indicate that exposure to ascorbic acid (antioxidant) can protect thymocytes from apoptotic DNA fragmentation induced by diverse stimuli (24).

III. "expression of anti-apoptotic genes should affect the putative mediator"

The proto-oncogene bcl-2 was shown to inhibit apoptosis in hematopoietic cell lines induced by the withdrawal of interleukin-3. Bcl-2 inhibits a cellular process that

may result in apoptosis or necrosis. Furthermore, expression of bcl-2 was associated with a marked decrease in the net cellular generation of reactive oxygen species and prevention of lipid peroxidation. Bcl-2 also prevents disturbances of the cellular Ca^{2+} homeostasis and ROS production at the mitochondrial level and Ca^{2+} release from endoplasmic reticulum via inositol triphosphate-insensitive pathway by shifting NAD+ to NADH. Thus, inhibition of ROS generetion by bcl-2 inhibits cell death (31,34,44).

The above findings provide evidence that oxidative stress has the potential to induce apoptosis in a wide variety of cells and chemicals can induce apoptosis due to oxygen free radicals production. Although oxidative modifications of proteins and lipids have been also observed in cells undergoing apoptosis in response to non-oxidative stimuli such as glucocorticoids (3), it is now broadly accepted that oxidative stress is capable of promoting apoptosis via an oxidative shift in the intracellular redox state.

INTRACELLULAR REDOX CHANGES AND APOPTOSIS

A tissue possesses the ability to maintain a normal balance between the reduced and oxidized forms of various compounds (redox balance). The cellular redox status (i.e. glutathione, thiols, pyridine nucleotides) modulates many aspects of cellular function. The intracellular environment is highly reduced mainly due to ratios of reduced to oxidised glutathione ranging from 30:1 to 100:1 (25). The glutathione redox system consists of glutathione (GSH), glutathione peroxidase (GPx) and glutathione reductase.

Recent experiments revealed that apoptosis is accompanied by a depletion of the intracellular reduced glutathione (GSH) (44). Decreasing intracellular stores of SH with buthionine sulfoxamine (BSO) sensitizes cells to apoptosis (10). Slater et al. have reported that GSH levels steadily decline in thymocytes undergoing apoptosis in response to glucocorticoids or etoposide, and this event occurs with kinetics similar to the appearance of internucleosomal DNA fragments. They have observed, in human Jurkat lymphocytes treated with antibodies to the Fas/APO-1 surface receptor, an accelerated efflux of the reduced thiol. It suggests, that a loss of intracellular reducing potential is a common component of the downstream events of apoptosis (38).

Beaver and Waring have shown that treatment of thymocytes with reduced glutathione inhibited dexamethasone-induced apoptosis in these cells. They have suggested that a decrease in GSH or an increase in GSSG, or a change in the ratio of GSH to GSSG, constitutes a trigger for apoptosis (2). Recently, Malorni et al. have reported that N-acetylcysteine, which acts both as the precursor of glutathione and as an anioxidant by itself, inhibits apoptosis in HIV-chronically infected U937 cells (21). In another work, thiol compounds, including N-acetylcysteine and thioredoxin, protected cells against apoptosis induced by tumor necrosis factor (23). However, the oxidation of thiols other than glutathione may also be important for initiating the apoptotic process. Sato et al. have demonstrated that oxidation of cellular sulfhydryl (SH) groups induces apoptosis in Jurkat T-cells and human PBL blasts (35).

Thus, all these observations have suggested, that the loss of intracellular reducing potencial, could then explain why the apoptotic cells often accumulate

oxidatively damaged products even if the cell death is initiated by non-oxidative stimuli, and that intracellular oxidation may be a general feature of the effector phase of apoptosis.

Nowadays, a growing attention is being paid, to an endogenous multifunctional bioregulatory free radical nitric oxide (NO°), and to its relations with other oxygen free radicals.

NITRIC OXIDE AND APOPTOSIS

Nitric oxide is generated from the amino acid arginine by the family of enzymes termed NO synthases (NOS). The NO synthesis has been identified in mammals, fish, birds, inverterbrates, plants and bacteria. In mammals, arginine--derived NO synthesis arises from three distinct genes that encode isoforms of NOS. These NOS genes are composed of either 26 exons (inducible NOS (iNOS) and endothelial NOS (eNOS)) or 29 exons (neuronal NOS (nNOS)). NOS isoforms have been classified as either dependent on calcium and calmodulin i.e. consitutive NOS (cNOS), or independent i.e. inducible NOS (iNOS). NOS can continuously generate NO for prolonged periods of time *in vivo*; cNOS produces low levels of NO over several minutes but iNOS, induced by cytokine or chemical stimulation, produces higher levels of NO for a few days (4,27,40). NO is a messenger that is known to exhibit a variety of biological activities including inhibition of tumor cell growth, neurotoxicity, vasorelaxation and bacteriostasis. Some studies have suggested that NO is a cytotoxic agent while others have demonstrated its protective role in the cell.

Wink et al. have observed, by measuring the time-concentration profiles of NO released from various NO donors compounds, that μM levels of NO are required for protection against the toxicity of reactive species such as superoxide, hydrogen peroxide or alkyl peroxides (42). Kanner et al. have shown that NO can prevent the destruction of hemoproteins by hydrogen peroxide (16). Moreover, some studies have confirmed that NO can act as a chain breaking antioxidant in lipid peroxidation reactions. The presence of NO can, by abating the oxidative damage of DNA, either prevent the formation of oxidants, or scavenge them. Many NO effects in different cell types are independent of the second messenger cGMP. The cGMP-independent responses appear to be regulated through redox mechanisms; interactions with thiol containing proteins or redox metal-containing proteins. Furthermore, NO may act through redox-sensitive pathways in the immune system (40).

Mannick et al. have demonstrated that low level of NO plays an important role in human mononuclear cell biology. These experiments indicated that human B lymphocyte cell lines consitutively express low level of macrophage-type NOS (iNOS) that exerts control over cellular responses. These results also suggest that immune NOS activity in Epstein-Barr virus (EBV)-transformed human B lymphocytes and Burkits' lymphoma cell lines, is involved in maintaining EBV latency. It has also been shown that NO can inhibit apoptosis in B lymphocyte cell lines (22). On the other hand, Fehsel et al. have found that chemically generated NO or NO released from activated macrophages, induces DNA strand breaks in islet cells. Recently, they have shown that NO induces apoptosis in murine thymocytes involving p53 expression (9). It has also been found that NO can induce apoptosis in pancreatic B-cells and in murine peritoneal macrophages (4).

Beauvais et al. have observed that NO donors, which generate NO in enzymatic reaction and release low doses of NO in a relative short time, are able to inhibit, *in vitro*, the programmed cell death of human eosinophils from peripheral blood. In contrast, a high amount of NO from other donors led to eosinophils injury (1).

It is interesting to note, that NO can be either toxic or protective depending on the chemistry it undergoes in a given biological milieu. Direct DNA damage is more likely to occur when NO is produced in high amount in oxidizing environments that both deplete natural thiol defenses and support generation of more reactive i.e. nitrosylating and oxidizing NO (4).

On the other hand, a number of systems are known in which the presence of NO can alter or even protect against the effects of superoxide and superoxide-derived reactive oxygen species (16). It has been suggested that at a low level, NO can protect cells from the deleterious effects of ROS, and the cytotoxic properties of NO are thought to be due to the formation of peroxynitrite and the higher level of NO. For this reason, superoxide scavengers such as SOD and thiols can enhance the biological activity of NO.

In biological systems, NO can react with oxygen, superoxide and transition metals (16). It has been predicted that peroxynitrite ($ONOO^-$) is formed *in vivo* from the reaction of nitric oxide and superoxide (39). The production of this strong oxidant has been demonstrated to be associated with the activation and expression of inducible NO synthase. Peroxynitrite can oxidize a variety of biomolecules including thiols, deoxyribose, lipids and α-1-proteinase inhibitors (26). The cytotoxicity of peroxynitrite has been suggested to be involved in initiation of lipid peroxidation and inactivation of enzymes in the mitochondrial electron transport chain (30). Very recently, Lin et al. have demonstrated that in HL-60 and U-937 cell lines peroxynitrite can induce apoptosis in a time- and concentration-dependent manner, and with increasing concentrations of $ONOO^-$ cells die either by apoptosis or necrosis (20).

NOVEL THERAPEUTIC APPROACHES TO THE CONTROL OF CHEMICAL TOXICITY

Understanding of the processes and mechanisms of cell death is fundamental to our knowledge of toxic cell injury and of injury prevention. It is evident, that cell death can be induced by a variety of agents including many drugs, environmental toxicants and physical agents. In toxicology today, understanding of the mechanism of apoptosis and necrosis is essential to the study of the effects of different agents (e.g., xenobiotics) on living organism.

Nowadays, one of the most important research questions is what factors determine whether a given toxicant induces apoptosis or necrosis. Many observations have strengthened the hypothesis, that different inducers require some common intracellular mediator to elicit generalized features of apoptosis and/or necrosis seen in most cell types. Recent data confirm that oxygen free radicals can be mediators of apoptosis (34). A significant number of chemicals cause cell death due to their effects on the cytochromes P450 and other drug-metabolising enzymes and ROS production (29). This leads to an assumption that oxygen and its derivatives are linked to cell death.

Both induction or inhibition of apoptosis may result in various diseases of the immune system (including AIDS) or tumor development, and in both cases the involvement of oxygen radicals has been reported (21,33,36). Recently, it has been found, that tumor promoters (e.g., oxidants) can inhibit apoptosis of pre-neoplastic hepatocytes in the multi-step process of carcinogenesis (5,36). It is also interesting to note, that mild oxidative stress triggers increased proteolysis and stimulates apoptosis (13). Therefore, chemical toxicants, by ROS production, can interfere with the mechanism which controls apoptosis and can thereby stimulate or prevent cell death.

It may be possible nowadays to maximise the ability of the drug to induce or inhibit the apoptotic response. For example, increasing propensity of cells to activate their apoptotic cell death mechanisms through ROS production, may enhance the effectiveness of toxicants (i.e. anticancer drugs) designed to kill tumor cells. On the other hand, if the drug treatment blocks the suicide response, by Bcl-2 induction or by the protection provided by anti-oxidants, it may prevent the cell from dying at all (7,41). Based on these observations, it can be concluded that, a complete understanding of the processes involved in apoptosis, and mechanisms of its manipulation, could provide novel strategies to the control of xenobiotic toxicity and give an impetus to design new therapeutic or preventive interventions.

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