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INVITED COMMENTARY

REDOX REGULATION OF PROGRAMMED CELL DEATH IN LYMPHOCYTES

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A redox imbalance caused by an over-production of prooxidants or a decrease in antioxidants seems to play a role in the programmed cell death that occurs in various developmental programs. Such a physiological function for oxidative stress is particularly applicable to the immune system, wherein individual lymphocytes undergo continuous scrutiny to determine if they should be preserved or programmed to die. Following activation, lymphocytes produced increased levels of reactive oxygen species (ROS) which may serve as intracellular signaling molecules. The ultimate outcome of this increased ROS formation, i.e., lymphocyte proliferation versus programmed cell death, may be dictated by macrophage-derived costimulatory molecules that bolster or diminish lymphocyte antioxidant defenses. HIV-1-infected individuals display multiple symptoms of redox imbalance consistent with their being in oxidative stress, and lymphocytes from such individuals are more prone to undergo apoptosis *in vitro*. It is suggested that oxidative stress is a physiological mediator of programmed cell death in lymphoid cells, and that HIV disease represents an extreme case of what can happen when regulatory safeguards are compromised.

INTRODUCTION

The use of oxygen as a terminal electron acceptor during respiratory energy production endows aerobic organisms with a precarious life style that is dependent upon an appropriate balance of prooxidants and antioxidants. However, this same precarious existence may also endow multicellular organisms with an efficient means for selectively terminating unwanted cells. In many developmental programs, cells which are functionally outmoded or possess undesirable traits are intentionally killed while the viability of neighboring cells is maintained. Such programmed cell death frequently, but not always, occurs via apoptosis.[1,2] The inducer of programmed cell death varies for different cell types, but in some cases either an over-production of reactive oxygen species (ROS)[3,4] or an attenuation of antioxidant defense systems[5] appears to be the ultimate mediator. Similarly, the survival of embryonic cells during development of the *C. elegans* worm is attributable to their expression of the *ced-9* gene product,[6] a functional homologue of the mammalian *bcl-2* gene that protects cells from oxidative stress-mediated apoptosis.[7,8] The programmed death of neurons during development of the vertebrate nervous system may also be redox regulated. Developing neurons are dependent upon neurotrophic factors such as nerve growth factor and brain-derived

neurotrophic factor for survival,[9] both of which can enhance cellular antioxidant levels.[10,11]

The immune system provides another circumstance in which an oxidative equilibrium may initiate programmed cell death. Like the nervous system, the immune system produces a great many more progenitor cells than ultimately survive. Lymphocytes capable of recognizing and imparting immunity to foreign antigens are selectively preserved, while lymphocytes which recognize the host's self antigens are eliminated. For T lymphocytes, most of this selection process occurs within the thymus, wherein >95% of the immature lymphocytes are killed before they undergo final maturation.[12] Mature B lymphocytes appear to undergo a similar selection process within the germinal centers of lymphoid tissues.[13] Thus, both the thymus and germinal centers constitute cellular grave yards containing huge numbers of apoptotic lymphocytes.

REDOX REGULATION OF LYMPHOCYTE PROGRAMMED CELL DEATH

The biochemical pathways which culminate in the programmed death of T and B cells have yet to be completely defined. However, there is a growing body of evidence that is consistent with cellular redox status being at least partly involved: 1) Activation of lymphocytes with stimuli that elicit ROS formation can result in apoptosis;[14-17] 2) Expression of the bcl-2 protein, which as mentioned previously, functions in an antioxidant pathway,[7,8] enables thymocytes and B cells to escape programmed cell death;[18] and 3) The induction of apoptosis in thymocytes can sometimes be inhibited by agents that have antioxidant activity.[19] Furthermore, lymphocytes exhibit several features compatible with their using oxidative stress as a mediator of programmed cell death, one being an inherent susceptibility to killing by ROS. It was recently demonstrated that the CEM human T cell line is capable of growth in serum-free media when the culture density is kept high ($>2\times10^5$ cells per ml), but at lower densities the cells rapidly undergo apoptosis.[20] The survival of CEM cells when cultured at high densities was ultimately attributed to their ability to release catalase into the culture medium, demonstrating that apoptosis following serum deprivation may be mediated by ROS, in particular, hydrogen peroxide. Thus, the multicellular oxidant defense strategy observed with prokaryotic organisms,[21] may also be exhibited by eukaryotic cells.

Despite their apparent vulnerability to oxidants, lymphocytes nevertheless utilize ROS in several functional aspects. Like macrophages and neutrophils, B and T lymphocytes produce increased amounts of ROS as a consequence of cellular activation.[14,15] The increase in ROS formation may constitute a signal transduction pathway[22] to elicit cellular events such as increased expression of cell surface adhesion molecules,[23,24] changes in protein phosphorylation,[25] and activation of nuclear transcription factors.[26] Under normal conditions, ROS production coincides with proliferation,[14,15] but in antioxidant-deficient cells, oxidative stress and cell death may occur. This dichotomous role of ROS formation was recently demonstrated in studies comparing the effects of activation on T cell lines versus T cell hybridomas. Whereas T cell lines resemble mature T lymphocytes by being induced to proliferate following antigen or mitogen exposure, T cell hybridomas more closely resemble immature T cells (thymocytes) in that activation evokes apoptotic cell death.[27] The activation-induced death of the T cell hybrids was blocked by the addition of N-acetyl-L-cysteine or reduced glutathione,[28] neither of which affected proliferation of the T cell lines. Other antioxidants have similarly been shown to block activation-induced death in other T cell systems.[19,29]

Redox-regulated programmed cell death may also occur in the absence of ROS formation. Glutathione represents the major intracellular redox buffer and as well functions in several vital antioxidant pathways.[30] However, owing to their lack of cysteine synthesis and inefficient transport of cystine, the predominant extracellular form of cysteine *in vivo*, lymphocytes appear to be in a compromised position with respect to glutathione availability.[31] As a result, the cells display a requirement for either low oxygen tension, antioxidants or thiol agents such as 2-mercaptoethanol to remain viable *in vitro*.[20,32,33] Thiols may promote the growth of lymphoid cells by scavenging extracellular ROS,[34] reducing oxidized protein sulfhydryls,[35] or facilitating cystine uptake.[31] That extracellular thiols can in fact be a primary determinant of lymphocyte survival is demonstrated by reports that 2-mercaptoethanol-independent, but not -dependent lymphoid cell lines release thiols extracellularly,[36] and that T and B lymphocytes transformed by HTLV-1 or EBV, respectively, utilize thioredoxin as an essential autocrine growth factor.[37,38] During the initial phase of an immune response, one function of macrophages is to take up cystine which in turn is released as reduced cysteine that can be transported and used by neighboring lymphocytes to synthesize glutathione.[31] All three thiol functions provide a means by which accessory cells can enhance lymphocyte reactivity.

Like many other molecules which have antioxidant activity, thiol compounds can sometimes act as prooxidants.[39] In numerous cell systems thiols have been shown to exert a paradoxical low dose toxicity due to the hydrogen peroxide that is formed by the spontaneous oxidation of thiols in the presence of a metal catalyst.[39] The addition of various thiol compounds to serum-free CEM cultures evoked a similar aberrant response wherein at low thiol doses the cells were found to undergo apoptosis.[40] The low-dose toxicity was completely inhibited by the simultaneous addition of catalase, but not superoxide dismutase, clearly implicating hydrogen peroxide as the apoptotic mediator. Additional studies (Sandstrom and Buttke, unpublished observations) indicate that autoxidation of cell-derived thiols is at least partly responsible for the low density-induced apoptosis of CEM cells cultured in serum-free media. Thus, secretion of thiol "ambioxidants" may enable macrophages to either enhance lymphocyte growth or to assist in lymphocyte suicide.[41]

Cumulative evidence suggests that cytotoxic T lymphocytes may also be capable of invoking oxidative stress in target cells to kill them by programmed cell death. Cytotoxic T cells bearing the Fas ligand induce apoptosis in target cells expressing the Fas receptor.[42] The Fas-based mechanism is analogous to tumor necrosis factor-α (TNF-α)-mediated killing in several respects, including structural homologies between the Fas ligand and TNF-α,[43] between the Fas and TNF-α receptors,[44] and the ability of bcl-2 and thiols to block Fas- and TNF-α-mediated cell killing.[45–47] Such similarities, coupled with the involvement of ROS in TNF-α-mediated cytotoxicity,[17,48] suggest that Fas-mediated T cell cytotoxicity likewise invokes a lethal, ligand-receptor initiated, redox imbalance to kill target cells.

LIPID HYDROPEROXIDES AS MEDIATORS OF APOPTOSIS

The ROS produced as a consequence of lymphocyte activation can elicit cellular changes by acting as signaling molecules *per se*, or by oxidatively modifying one or more cellular components. With regard to programmed cell death, a ROS- mediated formation of fatty acid hydroperoxides may be of particular importance. Given their oxidative lifestyles, it is curious that animal and plant cells envelope themselves with

polyunsaturated fatty acids that are exceptionally oxygen sensitive. One possible rationale is that oxidation of the polyenoic fatty acids enables the cell to detect changes in the redox state of their environment. Oxygenated fatty acids are important in *inter*-cellular communication, and it makes teleological sense for them to function as *intra*-cellular signal transductants as well. Preferential liberation of fatty acid hydroperoxides by phospholipase A_2 enzymes,[49] yields a signaling molecule capable of increasing cytoplasmic calcium[50,51] and modulating gene expression.[52] The intracellular signaling role of oxygenated fatty acids is best exemplified in TNF-α-mediated cytotoxicity. Immediately after the binding of TNF-α to target cells, there is a coordinated increase in ROS formation, phospholipase A_2 activity and oxidation of arachidonic acid, all of which are essential for TNF-α-mediated cytolysis to ensue.[16,43,53]

Two recent reports have implicated lipid peroxidation in the onset of apoptosis,[7,8] but a more direct relationship was demonstrated in studies with the A3.01 human T cell line.[54] The addition of 15-HPETE caused an immediate expected rise in cytosolic calcium, and within 2–5 hours gross morphological changes consistent with apoptosis were observed, including chromatin condensation and DNA fragmentation. These nuclear changes occurred in the absence of obvious disruptions of other organelles or the plasma membrane, clearly distinguishing this cell death from necrosis. Pre-loading A3.01 cells with the calcium chelator, BAPTA-AM, provided substantial protection from 15-HPETE-induced death, but over-expression of the *bcl-2* gene in A3.01 cells provided no protection from 15-HPETE-induced apoptosis, despite their being much more resistant to killing by TNF-α.[54] This suggests that while bcl-2 can prevent the onset of lipid peroxidation, it provides no protection against lipid hydroperoxides that are formed extracellularly or at intracellular sites that are inaccessible to bcl-2.

One might reasonably ask whether it makes sense to utilize such potentially toxic molecules as lipid hydroperoxides for intracellular mediators, even of programmed cell death. However, despite their notorious reputation, lipid hydroperoxides do not necessarily initiate a rampant, autocatalytic reaction that culminates in massive cellular damage. In fact, it has been suggested that the controlled, selective destruction of reticulocyte mitochondria during erythrocyte maturation is mediated by lipid hydroperoxides.[55] Furthermore, lipid hydroperoxide-derived break-down products may facilitate the break up of dying cells into apoptotic bodies prior to subsequent phagocytosis. A3.01 cells exposed to lipid hydroperoxides displayed only minimal membrane blebbing, a common feature of apoptosis,[1] but exposure to 4-hydroxynonenal, an end-product of 15-HPETE degradation,[56] caused a virtually complete dissolution of A3.01 cells into small vesicles (T.M. Buttke, unpublished observations). The progression of a full apoptotic response may therefore require a combination of lipid hydroperoxides and reactive aldehydes derived therefrom. Interestingly, both types of molecules readily react with proteins, and by so doing facilitate the recognition and ingestion of oxidatively-damaged erythrocytes and oxidized low density lipoprotein by macrophages.[56,57]

OXIDATIVE STRESS AND PROGRAMMED CELL DEATH IN HIV DISEASE

T cells from HIV-infected individuals undergo apoptosis following incubation *in vitro* more readily than do T cells from uninfected individuals,[58] and exposure of the former to various stimuli results in a significant proportion of the cells undergoing activation-induced apoptosis.[59] In accord with the proposed role of oxidative stress as a mediator

of apoptosis,[60,61] various indicators of redox imbalance including decreased thiol levels, antioxidant deficiencies and evidence of lipid peroxidation, have consistently been observed in HIV-infected individuals.[62-64] Studies with acutely- and chronically-HIV infected T cell lines have additionally demonstrated a correlation between HIV gene expression and decreased levels of antioxidant enzymes such as thioredoxin, superoxide dismutase, catalase and glutathione peroxidase.[65-68] These findings imply that HIV-1 has usurped cellular redox from its normal regulatory control mechanisms, and as a result, T cells are predisposed to die following exposure to ROS which would normally have either no effect or an activating effect. The redox imbalance associated with HIV-1 infection may constitute one mechanism by which the virus promotes its own replication. ROS enhance HIV-1 expression in T lymphocytes and monocytic cells,[69,70] while antioxidants such as ascorbic acid, N-acetyl-L-cysteine, pyrrolidinedithiocarbamate and butylated hydroxyanisole decrease virus expression *in vitro*.[71-74] Enhancement of HIV-1 replication by ROS appears to be manifest primarily through the transcription factor, NF-κB. Diverse stimuli such as TNF-α, IL-1, LPS, and phorbol esters lead to NF-κB activation, and ROS formation seems to be a common theme.[26] Given the dependence of HIV-1 on NF-κB in order to replicate,[75] it is not surprising that the virus can foster activation of the transcription factor.

How can HIV-1 shift a T cell's redox equilibrium? One way, mentioned earlier, may be to suppress the host cell's antioxidant defenses. Flores *et al.* found that the HIV-1 Tat protein inhibits translation of the mitochondrially-located manganese superoxide dismutase (MnSOD), thereby leading to an over-accumulation of the superoxide radical.[76] Superoxide may activate NF-κB directly or indirectly by fueling the production of hydrogen peroxide. Superoxide could also promote the formation of lipid hydroperoxides by releasing iron from ferritin, ultimately leading to viral cytopathicity and/or apoptosis of the infected cell.

The apparent ability of HIV-1 to alter its host cell's redox status to favor viral replication may not be unique. The over-production and utilization of thioredoxin as an autocrine-type growth factor by HTLV-1 infected T cells and Epstein-Barr virus infected B cells was noted earlier,[37,38] and the HTLV-1 *tax* gene has also been shown to induce NF-κB[77] and apoptosis[78] in a manner inhibitable by antioxidants or Bcl-2, respectively. Other viruses, such as Epstein-Barr virus, encode functional homologues of bcl-2,[79,80] the purpose of which may be to "protect" the host cell from oxidative stress-mediated death, thereby prolonging virus replication. HIV-1, on the other hand, may, by shifting the redox balance to an oxidative equilibrium, actually predispose the host cell to programmed cell death. Such a tactic appears flawed since it reduces the overall time available for the virus to replicate, but this disadvantage could be negated by a markedly enhanced rate of viral gene transcription.

CONCLUSION

The concept of oxidative stress serving as a physiological mediator of programmed cell death is relatively new, yet supportive evidence is accumulating rapidly. While additional studies are necessary to determine the extent of cellular redox involvement in some cases of lymphocyte-associated programmed cell death, in other instances sufficient evidence already exists. In those latter cases, modulating a lymphoid cell's level of antioxidants may provide a valuable therapeutic strategy for either inducing or preventing programmed cell death. Confirmation of the proposed role of ROS in Fas-mediated cell death should lead to novel approaches for employing oxidative stress

to eradicate malignant or autoreactive lymphoid cells. Similarly, defining the role of T cell redox status in HIV-1 replication and cytopathicity could result in new therapeutic strategies for the treatment of AIDS and other diseases of viral origin.

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