2001

MECHANISMS OF CD4 + T CELL APOPTOSIS AND THE ROLE OF ETHANOL AS A COFACTOR IN HIV PATHOGENESIS

Qing Dong
University of Kentucky

Right click to open a feedback form in a new tab to let us know how this document benefits you.

Recommended Citation
https://uknowledge.uky.edu/gradschool_diss/398

This Dissertation is brought to you for free and open access by the Graduate School at UKnowledge. It has been accepted for inclusion in University of Kentucky Doctoral Dissertations by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsvaky.edu.
ABSTRACT OF DISSERTATION

Qing Dong

The Graduate School
University of Kentucky
2000
MECHANISMS OF CD4+ T CELL
APOPTOSIS AND THE ROLE OF ETHANOL
AS A COFACTOR IN HIV PATHOGENESIS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Kentucky

By

Qing Dong

Lexington, Kentucky

Director: Dr. Linda Chen, Professor of Nutritional Sciences

Lexington, Kentucky

2000
Acquired Immunodeficiency Syndrome (AIDS) was first reported in the United States in 1981 and has since become a major worldwide epidemic. The typical course of HIV disease begins with a primary infection followed by a relatively long latency phase and finally ends in the advance phase also called AIDS. There are two aspects considered the most important in HIV pathogenesis, namely viral replication and CD4+ T cell depletion.

During the latency phase, tumor necrosis factor α (TNFα) has been shown to play a predominant role in HIV-1 replication and disease progression. Since ethanol is also an important risk factor and has been implicated in HIV-1 replication, we investigate the effects of ethanol on TNFα inducible signaling associated with HIV-1 replication in human CD4+ T cells. We demonstrate that clinically relevant ethanol concentrations significantly potentiate TNFα inducible NFκB. Although ethanol effectively collaborated with TNFα, by itself it does not have a direct effect on NFκB activation. The ethanol dependent potentiation of TNFα inducible NFκB nuclear translocation is observed to involve the enhanced degradation of IκBα. Additionally, the ethanol mediated potentiation of TNFα inducible NFκB activation is abrogated by the known antioxidant pyrrolidinedithiocarbamate (PDTC), suggesting an important mechanistic role for reactive oxygen species (ROS) in this process. In correspondence with its effect on
NFκB, ethanol is also able to significantly enhance HIV-1 long terminal repeat (HIV-1-LTR) dependent transcription induced by TNFα.

Apoptosis has been proposed as a critical mechanism for CD4⁺ T cell depletion in HIV pathogenesis. Ceramide, a sphingolipid metabolite, is a common apoptotic transducer involved in CD4⁺ T cell apoptosis. In the current study, we show that ceramide potently induces CD4⁺ T cell apoptosis through activating caspase 3, which may further increase Fas Ligand expression to amplify the apoptotic signaling. Interestingly, the apoptotic effect of ceramide is completely blocked by pretreatment with zinc and the underneath mechanism is suggested to be a direct inhibition of caspase 3 activity by zinc.

Survival factors are equally important in the regulation of apoptotic process. We demonstrate that PI3-kinase/Akt pathway is indispensable for the survival of CD4⁺ T cells. Further, Akt kinase is significantly inactivated and downregulated in oxidative stress induced CD4⁺ T cell apoptosis. N-acetyl-cysteine (NAC) can rescue CD4⁺ T cell from H₂O₂ induced caspase 3 activation and apoptosis, while depletion of glutathione (GSH) exacerbate it.

Overall, this work identifies several mechanisms underlying CD4⁺ T cell apoptosis and provides molecular basis for the role of ethanol as a cofactor that can adversely affect HIV-1 infection and pathogenesis.

Keywords: Ethanol, HIV, Apoptosis, CD4, ROS, Ceramide

(Student’s signature)

(Date)
RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor’s degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgements.

Extensive copying or publication of the dissertation in whole or in part also requires the consent of the Dean of the Graduate school of the University of Kentucky.

Name

Date

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________
Dissertation

Qing Dong

The Graduate School
University of Kentucky
2000
MECHANISMS OF CD4⁺ T CELL APOPTOSIS AND THE ROLE OF ETHANOL AS A COFACTOR IN HIV PATHOGENESIS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Kentucky

By

Qing Dong
Lexington, Kentucky

Director: Dr. Linda Chen, Professor of Nutritional Sciences
Lexington, Kentucky
2000
ACKNOWLEDGMENTS

Many individuals deserve to be thanked for their contribution toward the ultimate completion of my Ph. D. study. First, I would like to thank Dr. Craig McClain and Dr. Shirish Barve for being great mentors. Dr. Linda Chen deserves a special thank for being a dedicated and responsible chair. Thanks, also to Dr. Bernhard Hennig and Dr. David Kaetzel for serving on my committee. Dr. Swati Joshi-Barve and Sujata Kelkar deserve my gratitude for their help and invaluable discussions in the lab.

Finally, I would like to thank my family who faithfully supported me throughout my life. My parents, Ximing and Ewen Dong, my sister Tong and my lovely wife Nan. I would like to single out two of my family members. My dad, Dr. Ximing Dong, introduces me into the magic world of science. His vision and discipline has always been the guidance and driving force for my success. My wife Nan has been my best friend. Without her love and support, I would not be where I am.
# TABLE OF CONTENTS

Acknowledgments...........................................................................................................iii

List of Figures..................................................................................................................v

List of Files .....................................................................................................................vi

Chapter One: General Introduction and Literature Review
  Disease Course of HIV infection.................................................................1
  Apoptosis................................................................................................. 4
  Apoptosis and necrosis...................................................................... 5
  Disorders caused by increased cell survival................................. 6
  Disorders caused by excess cell death........................................... 9
  Apoptosis and CD4⁺ T cell depletion in HIV pathogenesis........... 10
  NFκB and HIV disease................................................................. 14
  Oxidative stress and HIV............................................................ 15
  Sphingomyelin (SM) pathway and HIV disease......................... 16

Chapter Two: The Role of Ethanol as a Cofactor in HIV Pathogenesis
  Introduction..................................................................................... 18
  Experimental Procedure............................................................. 21
  Results............................................................................................. 25
  Discussion....................................................................................... 36

Chapter Three: Mechanism of Ceramide Induced CD4⁺ T cell Apoptosis
  Introduction................................................................................. 41
  Experimental Procedure............................................................. 49
  Results............................................................................................. 52
  Discussion....................................................................................... 69

Chapter Four: The Role PI3K/Akt Pathway in Oxidative Stress Induced Apoptosis
  Introduction..................................................................................... 73
  Experimental Procedure............................................................. 79
  Results............................................................................................. 83
  Discussion....................................................................................... 101

Chapter Five: Summary.........................................................................................104

Reference....................................................................................................................108

Vita .............................................................................................................................136
LIST OF FIGURES

Figure 1, Schematic course of HIV infection ......................................................... 2
Figure 2, Productively infected CD4^+ T cells induce
 apoptosis of bystander uninfected CD4^+ T cells ........................................... 13
Figure 3, Ethanol potentiates TNFα inducible NFκB activation ............................. 26
Figure 4, Identification of TNFα inducible NFκB complexes affected by ethanol .... 28
Figure 5, Ethanol enhances TNFα dependent IκBα proteolysis ............................ 30
Figure 6, PDTC inhibits NFκB activation effected by ethanol and TNFα ............... 31
Figure 7, CHX does not inhibit the potentiating effect of ethanol on TNFα inducible NFκB activation ................................................................. 33
Figure 8, Ethanol pretreatment potentiates TNFα inducible HIV-1 LTR mediated transcription ................................................................. 34
Figure 9, Sphingomyelin metabolism .................................................................. 42
Figure 10, Dose dependent ceramide induced DNA fragmentation ...................... 53
Figure 11, Effect of ceramide on caspase 3 activity ............................................. 54
Figure 12, Inhibition of ceramide induced DNA fragmentation by zinc ............... 55
Figure 13, Zinc has no effect on ceramide induced caspase 3 processing .......... 57
Figure 14, Zinc inhibits ceramide induced caspase 3 activity ......................... 58
Figure 15, Zinc blocks ceramide induced DNA fragmentation in human PBL ...... 60
Figure 16, Ceramide induced Fas Ligand upregulation ........................................ 61
Figure 17, Ceramide induced DNA fragmentation was inhibited by DEVD-cho ...... 63
Figure 18, Ceramide induced CD95L upregulation is partially blocked by DEVD-cho ..................................................................................... 64
Figure 19, LY sensitized Jurkat cells to ceramide induced cell death .................. 66
Figure 20, Inhibition of Akt/PKB by ceramide may be caspase 3 independent ...... 67
Figure 21, Ceramide, LY294002 and Wortmannin Dose-dependently attenuated the activity of Akt/PKB ................................................................. 68
Figure 22, LY294002 and Wortmannin induced cell death in CD4^+ T cells ....... 84
Figure 23, LY294002 dose-dependently induces caspase 3 activity ................. 85
Figure 24, LY 294002 induces proteolysis of procaspase 3 .............................. 86
Figure 25, LY294002 dose-dependently induces DNA fragmentation ............ 87
Figure 26, Oxidative stress induced cell death in CD4^+ T cells ....................... 89
Figure 27, Oxidative stress induced cell death in CD4^+ T cells ....................... 90
Figure 28, H_2O_2 dose-dependently induces caspase 3 activity ...................... 91
Figure 29, Time course of oxidative stress induced P-Akt downregulation .......... 93
Figure 30, Time course of oxidative stress induced Akt downregulation .......... 94
Figure 31, H_2O_2 induced P-Akt downregulation may be caspase 3 independent ... 95
Figure 32, Depletion of GSH level by BSO ......................................................... 97
Figure 33, BSO sensitizes Jurkat cells to H_2O_2 induced cell death ................. 98
Figure 34, NAC rescues Jurkat cells from H_2O_2 induced cell death ............... 99
Figure 35, NAC inhibits H_2O_2 induced caspase 3 activity ............................ 100
LIST OF FILES

ThesisQD.pdf………………………………………………………………………1.08MB
Chapter One

General Introduction and Literature Review

Disease Course of HIV infection

It has been almost seventeen years since human immunodeficiency virus (HIV) was identified as the cause of acquired Immunodeficiency syndrome (AIDS). However, we still have a long way ahead to fully understand how HIV kills its target, the CD4$^+$ T cell, and how this killing cripples the immune system. AIDS is the end-stage disease of HIV infection. The key to understanding its pathogenesis lies in elucidating the course of infection and the virus-host relation in the years preceding terminal illness (Weiss, 1993). Generally, HIV disease propagation can be categorized into three different stages: the primary HIV infection, the clinical latent period and the advanced stage or AIDS (Pantaleo and Fauci, 1994).

The period of primary HIV infection is characterized by those virological and immunological events that reflect the initial spreading and dissemination of HIV, the emergence of HIV-specific immune responses and the down-regulation of viremia (Graziosi, 1993). Further, the primary HIV infection stage has been divided empirically into two periods. One is the spreading and dissemination of HIV and the other is the partial clearance of virus from peripheral blood. The viral dissemination period corresponds to the first two or three weeks of infection and is highlighted by a peak in viremia (Fig. 1). In the initial establishment stage of infection, the immune activation, which is necessarily associated with the generation of an antigen-specific immune response, may actually work in favor of the virus. A group of cytokines including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α and TNF-β, which are released by certain immunocompetent cells that are recruited to the primary anatomic site of virus localization (lymphoid organs), may further enhance the virus replication. Also, activated antigen-specific CD4$^+$ T lymphocytes could serve as ideal targets for supporting high levels of viral replication (Reimann, 1994). Following the peak of viremia, all parameters of virus replication are significantly down-regulated in peripheral blood. At the same
Figure 1. Schematic course of HIV infection
time appears the HIV-specific immune response (Clark, 1991). Both cell-mediated and humoral HIV-specific immune responses contribute to the down-regulation of viremia. HIV-specific cytotoxic T cells are primarily involved in the elimination of HIV infected cells, whereas HIV-specific antibodies are mainly responsible for the clearance of viral particles from the circulation (Pantaleo, 1994). The immune response against HIV is extremely powerful and is clearly more effective than any of the therapeutic approaches currently available in controlling virus replication. However, the HIV specific immune responses are unable to completely clear the virus. Reports show that active virus replication occurs in lymph nodes at any given time during HIV infection (Pantaleo, 1991). There are two potential explanations for the failure of the immune response to totally eliminate the infection. First, the HIV specific immune response is inherently inadequate. Second, the immune responses are adequate but HIV is able to escape the immune response. There is some evidence for the second explanation. HIV may potentially escape elimination by the immune system via two possible mechanisms: first, latently HIV infected cells may not be eliminated by HIV specific CTL; secondly, virus particles trapped in the follicular dendritic cell (FDC) network serve as a continuous source of virus for de novo infection of CD4+ T lymphocytes which are migrating through the lymphoid organs.

The predominantly virological phenomenon of the clinically latent period is the dichotomy in viral burden and viral replication between peripheral blood and lymphoid tissue. Sequestration of infected cells and trapping of virions in the FDC network are responsible for this dichotomy (Pantaleo, 1991; Embretson, 1993; Pantaleo, 1994). Importantly, the general state of immune activation is the most striking immunological phenomenon in this stage. Latently HIV infected cells and trapped virions may be crucial for the propagation of infection over time and they are key factors to maintain a persistent stimulation of the immune system. Both T and B cell lineages are stimulated. Chronic stimulation of the immune system creates favorable conditions for HIV replication and virus dissemination. It has been proposed that virus-CD4 interaction relays a negative signal to the target cell, which may eventually induce cellular unresponsiveness (i.e. anergy) (Mittler, 1989; Linette, 1988; Amadori, 1992) or prime the cell to undergo apoptosis (Terai, 1991; Groux, 1992; Laurent-Crawford, 1991; Gougeon, 1991; Meynard,
Defective production of cytokines such as IL-2 is associated with the quantitative and qualitative defects of CD4+ T lymphocytes and may contribute to the impairment of other cells (i.e. CD8+ cells) (Shearer, 1991). In contrast to IL-2, other cytokines such as IL-1, TNF-α, TNF-β and IL-10, which may either potentiate virus replication or inhibit a variety of immune functions, are overproduced (Poli, 1992; Del Prete, 1993; Clerici, 1994). The general state of immune activation may be the major mechanism for the induction of apoptosis. Apoptosis may be readily detected in lymph nodes of HIV infected individuals at different stages of disease and several cell types including CD4+, CD8+ and B cells are involved in this process. The degree of apoptosis detected in the tissue seems to correlate with the state of immune activation rather than viral burden or disease stage (Meyaard, 1994). It is conceivable that the multiple mechanisms mentioned above contribute directly or indirectly to the progressive degeneration (i.e. follicular involution and extensive fibrotic and fatty infiltration) of the lymphoid tissue that is the main reason for the severe immune dysfunction.

In the advanced stage of disease, severe immunosuppression is the final result of the systemic process of HIV disease. This process involves every component of the immune system and leads to its destruction. During this stage there is a re-equilibration of the levels of viral burden and replication between peripheral blood and lymph node (Pantaleo, 1991). This re-equilibration and high levels of viremia reflect both the complete removal of those mechanisms of HIV sequestration in the lymphoid system and the collapse of the HIV specific immune response. This collapse of immune system is not limited to HIV- specific immune response but is generalized and results in widespread failure of immune responses against other invading pathogens. Symptoms frequently seen include prolonged fatigue, fever, swollen gland (lymph nodes), skin rash or lesion, frequent diarrhea and tumor (Kaposi sarcoma) etc.

**Apoptosis**

In multicellular organisms, homeostasis is maintained through a balance between cell proliferation and cell death. Once development is complete, the viability of the organism depends on the maintenance and renewal of the diverse set of differentiated cell
types. Different cell types vary widely in the mechanisms by which they maintain themselves over the life of the organism (Thompson, 1995). For example, blood cells undergo constant renewal from hematopoietic progenitor cells. In contrast, neural cells have at best a limited capacity for self-renewal, and most neurons survive for the life of the organism. Within each cell type, the control of cell number is determined by a balance between cell proliferation and cell death. The differentiated cells of multicellular organisms all appear to share the ability to carry out their own death through an internally encoded death program (Raff, 1992). This characteristic form of cell death called programmed cell death or apoptosis. Apoptosis can be triggered by a variety of intrinsic and extrinsic signals and allows for the elimination of cells that have been produced in excess, that have developed improperly, or that have sustained genetic damages.

**Apoptosis and necrosis**

Apoptotic cell death can be distinguished from necrotic cell death (Wyllie, 1980). Necrosis is a pathologic form of cell death resulting from acute cellular injury, which is typified by rapid cell swelling and lyses. In contrast, apoptosis is characterized by controlled autodigestion of the cell. The activation of endogenous proteases results in cytoskeletal disruption, cell shrinkage and membrane blebbing. Apoptosis is also characterized by the unique changes in the nucleus. The nucleus undergoes condensation as endonucleases are activated and begin to degrade nuclear DNA. In many cell types, DNA is chopped into DNA fragments the size of oligonucleosomes, whereas in others larger DNA fragments are produced. Apoptosis is also accompanied by a loss of mitochondrial function. This has led to speculation that mitochondria may play an important role in the regulation of apoptosis. The dying cell maintains its cell membrane integrity. However, alterations in the plasma membrane of apoptotic cells signal neighboring phagocytic cells to engulf them and thus to complete the degradation process (Ellis, 1991; Hall, 1994). Apoptotic cells which has not been engulfed immediately breakdown into smaller membrane-bound fragments called apoptotic bodies. An important feature of apoptosis is that it results in the elimination of the dying cell without induction of an inflammatory response (Thompson, 1995). In contrast necrotic cell death
is associated with an early loss of cell membrane integrity, resulting in leakage of cytoplasmic contents and the induction of an inflammatory reaction. Necrosis is often induced by agents that damage cell membranes (i.e. heat) or that interfere with oxidative phosphorylation (i.e. ischemia). The complete or virtual absence of normal cellular detail is due to protein denaturation and nuclear dissolution. Unlike apoptosis, necrosis does not require gene expression, and the cells die as a result of cell membrane or ion pump damage (Staunton, 1998). Ultrastructurally, the necrotic cell initially swells because cell membrane volume control is lost. The cell then lyses, organelles disintegrate, and with nuclear dissolution, there is rarely DNA degradation. The cytoplasmic contents of lysed necrotic cells activate an inflammatory response. Eventually, necrotic material may be removed by macrophages, but persistent inflammation and scarring or cavitation cause irreversible local tissue damage.

**Disorders caused by increased cell survival**

Diseases characterized by the accumulation of cells include cancer, autoimmune diseases and certain viral illness. Cell accumulation can result from either increased proliferation or the failure of cells to undergo apoptosis. Cells from a wide variety of human malignancies have a decreased ability to undergo apoptosis in response to at least some physiologic stimuli (Hoffman, 1994). This is most apparent in metastatic tumors. Most normal cells depend on environment specific factors to maintain their viability. This dependence may serve to prevent normal cells from surviving in the nonphysiologic sites. Metastatic tumor cells have detoured from this homeostatic mechanism and can survive at sites distinct from the tissue in which they arose. Apoptosis is modulated by a large number of evolutionarily conserved oncogenes and tumor suppressor genes. The Bcl-2 gene product is a potent inhibitor of apoptosis. Bcl-2 is homologous with the anti-apoptotic gene *ced-9*, which regulates cell death in *C elegans* (Hengartner, 1994). Bcl-2 was first discovered as a result of its location at the site of a translocation between chromosomes 14 and 18 and is present in most human follicular lymphomas (Bakhshi, 1985). It is suggested that B lymphocytes accumulate in follicular lymphoma because of prolonged survival rather than increased proliferation (Vaux, 1988). Bcl-2 is only one
member of a family of genes that control the apoptotic threshold of a cell (Boise, 1993). At least 15 Bcl-2 family members have been identified in mammalian cells. All members possess at least one of the four conserved motifs known as Bcl-2 homology domains (BH1 to BH4). Most pro-survival members, which can inhibit apoptosis induced by a wide variety of cytotoxic stimuli, contain at least BH1 and BH2 or even all four BH domains like Bcl-2 (Adams, 1998). In the pro-apoptotic members of Bcl-2 family, BH3 domain is essential for the function of the “killers” (Kelekar, 1998). Pro- and anti-apoptotic family members can heterodimerize and seemingly titrate one another’s function, implying that their relative concentration may act as a rheostat for the suicide program (Oltvai, 1993). In tumor cell lines, overexpression of Bcl-2 or of the related gene Bcl-x has been found to confer resistance to cell death in response to chemotherapeutic agents such as cytosine arabinoside and cisplatin (Miyashita, 1993). These results are surprising because chemotherapy was previously thought to kill tumor cells by inducing irreversible metabolic damage that results in target cell necrosis. It currently looks like that the primary mechanism by which most chemotherapeutic agents induce cell death is via creating cellular aberrations in cellular physiology that result in the induction of apoptosis. Bcl-2 is considered a downstream regulator of apoptosis because it inhibits apoptosis induced by a variety of stimuli, including gamma irradiation, hyperthermia, chemotherapeutic agents, and corticosteroids. Thus, Bcl-2 expression is one of several well-defined anti-apoptotic forces. Bcl-2 homologs interact closely in the regulation of apoptosis. The Bcl-2/Bax ratio is the critical determinant for the induction and inhibition of apoptosis. When Bax is in excess, the integrated signal favors apoptosis, and while Bcl-2 is dominant, apoptosis is blocked (Oltvai, 1993).

The tumor suppressor gene p53 encodes a transcription factor normally maintained in low levels. The high frequency with which p53 is functionally inactivated in various human cancers attests to its pivotal role as a terminator against expansion of mutated somatic cells. In many cases, p53 itself is mutated or deleted. Normally, DNA damage can induce p53 activation. Two cellular responses to p53 activation are growth arrest and apoptosis. Which of these two prevails depend on cell type, cell environment and other factors as well (Evan, 1998). Substantial evidence suggests that a major part of p53-mediated growth arrest proceeds through induction of the cyclin-dependent kinase
(Cdk) inhibitor p21 (Hansen, 1997). Cells with sustained DNA damage were held in G1 for “DNA repairs”. If the damage is successfully repaired, the growth arrest can be reversed. If DNA damage is irreparable, the cell undergoes apoptosis. This is referred to as (p53-mediated) DNA damage-induced apoptosis. In cells lacking functional p53, there is reduced susceptibility to radiation and cytotoxic drug induced apoptosis (Lowe, 1993). Cells with inactivated p53 might therefore survive and allow further genetic damage to convey and accumulate. Errors in the repair of DNA damage that might otherwise induce apoptosis could contribute to the high mutation rate observed in many human malignancies. The mechanism by which p53 promote apoptosis is still unclear. Some studies suggest multiple p53 targets such as Bcl-2 antagonist Bax (Yin, 1997), the insulin-like growth factor-I (IGF-I) receptor (Prisco, 1997), and components of the renin-angiotensin system (Pierzchalski, 1997).

C-Myc, the protein encoded by the c-myc oncogene, is a transcription factor, which is normally induced following mitogenic stimulation. C-Myc is required for the cell cycle entry and promotes cell proliferation. However, c-Myc induces apoptosis when insulin-like growth factor and platelet-derived growth factor (PDGF), which normally suppress c-Myc-induced apoptosis, are not available (Evan, 1992). It has been proposed that when c-Myc is active, there is simultaneous induction of proliferation and apoptosis. The apparently paradoxical coupling of cell proliferation and apoptosis may serve as a safe-guard against neoplasia. Successful proliferation requires a dual signal: one will override the apoptotic signal and the other will promote growth (Evan, 1996). Interestingly, IGF, PDGF and Bcl-2 are all able to inhibit c-Myc-induced apoptosis independently of cell proliferation.

Physiologic regulation of cell death is essential for the removal of potentially autoreactive lymphocytes during development and for the removal of excess cells after the completion of an immune response. Failure to remove autoimmune cells can result in autoimmune disease. Recent works in animal model system have clearly demonstrated the importance of dysregulated apoptosis in the etiology of autoimmune disease. One molecule, which is critical in lymphocytes, is the cell surface receptor Fas, a member of the tumor necrosis factor (TNF) receptor family. Stimulation of Fas on activated lymphocytes can induce apoptosis. Two forms of hereditary autoimmune disease have
been attributed to alterations in Fas-mediated apoptosis. MRL-lpr mice, which develop fatal systemic lupus erythematosus by 6 month of age, have a mutation in the Fas receptor. In contrast, the GLD mice, which develop a same illness, have a mutation in the Fas ligand (Suda, 1993). In human, a secreted form of Fas has been discovered (Cheng, 1994). Patients with systemic lupus erythematosus have elevated levels of soluble Fas, which may competitively inhibit Fas ligand-Fas interactions. The resulting decrease in Fas-mediated apoptosis may contribute to the accumulation of autoimmune cells in this disorder. A lupuslike autoimmune disease has also been reported in transgenic mice constitutively overexpressing Bcl-2 in their B cells (Strasser, 1991).

**Disorders caused by excess cell death**

Excessive cell death can result from acquired or genetic conditions that enhance the magnitude of the apoptotic signals or reduce the threshold at which such events induce apoptosis. Although increased apoptotic cell death has been reported in a number of diseases, the underlying mechanism has not been fully defined.

A wide variety of neurodegenerative diseases are characterized by the gradual loss of specific sets of neurons (Isacson, 1993). Such disorders include Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis (ALS), retinitis pigmentosa, spinal muscular atrophy and various forms of cerebellar degeneration. In these diseases, cell death results in specific disorders of movement and central nervous system function. The cell loss in these diseases does not involve an inflammatory response, and apoptosis appears to be the mechanism for the cell death. Oxidative stress, calcium toxicity, mitochondria defects and deficiency of survival factors have all been postulated to contribute to the pathogenesis of these diseases (Ziv, 1994). Each of these pathways predisposes neurons to apoptosis both in vivo and in vitro. Overexpression of Bcl-2 decreases the neurotoxicity of each of these potential inducers of cell death (Zhong, 1993). Neurtrophic growth factors and the extracellular matrix also change the apoptotic threshold of neural cells. In summary, these data suggest a model in which the threshold for cell death is dynamically regulated. The apoptotic threshold of a cell is thus determined by the combined effects of external and internal survival factors.
AIDS, which is induced by the human immunodeficiency virus (HIV), is probably the most dramatic example of virus-associated cell depletion. The development of AIDS has been directly correlated with the depletion of the CD4+ T cells, the cellular targets of viral infection. It has been shown that CD4 acts as a receptor for viral attachment, thus facilitating HIV infection of CD4+ T cells. Surprisingly, most T cells that die during HIV infection do not appear to be infected with HIV. One important point is that why the virus would develop a mechanism to selectively deplete its host cell. The possible explanation may be that CD4+ T cells have a crucial function in establishing protective immune responses against the viral infection. The establishment of a chronic HIV infection may therefore depend on virally mediated depletion of CD4+ T cells and the concomitant loss of the protective immunity. Viral replication itself may not be limited by this form of CD4-dependent cell death, because the virus may specifically down-regulates the CD4 receptor on infected cells (Littman, 1994). We are going to further talk about the relation between HIV infection and apoptosis in detail in the following section.

Apoptosis and CD4+ T cell depletion in HIV pathogenesis

One intriguing puzzle in HIV pathogenesis is that how HIV kills its target, the CD4+ T cells? It appears that killing its major host cell and viral ‘factory’ may be considered as a non-adaptive survival strategy. So does HIV really kill the cell it has infected? There is no clear-cut answer for this question. But more and more evidence is leaning toward the hypothesis that indirect killing of bystander cells may be the main mechanism accounted for the depletion of CD4+ T cells in HIV pathogenesis. More than 90% of CD4+ T lymphocytes reside in the lymphoid tissues. Even during the so-called clinically latent phase of the disease, when the number of CD4+ T lymphocytes in the peripheral blood remains at a steady level and the amount of HIV in the blood remains low, there is massive and persistent infection in the lymphoid tissues (Veazey, 1998). During the course of infection, there is a steady depletion of CD4+ T lymphocytes and the structure of the lymphoid tissue is progressively destroyed. In the final stage of the disease, the immune system is severely impaired and the lymphoid architecture is totally destroyed with complete loss of cellularity and replacement by fibrotic tissue (Pantaleo,
Another major site of HIV infection is the central nervous system (CNS) where atrophy and other structural damage to the CNS are observed in AIDS dementia patients (Dal Pan, 1992).

Several reports showed that HIV infection, at least in vitro, directly induces apoptosis of the CD4+ T lymphocytes (Scarmato, 1996). Cytotoxic T lymphocytes (CTL) could play an important role in killing of HIV-infected cells during early stages of infection (Borrow, 1994). Other reports suggested that perturbation of host-cell membranes could be the primary mechanism of HIV cytopathology (Cloyd, 1991). Despite the above reports, there is increasing evidence in favor of indirect killing of bystander cells by extracellular or cell surface-associated components of HIV infected cells. This could explain why there is such massive death of CD4+ T lymphocytes during the course of HIV infection when the viral burden is comparatively low (Ascher, 1995; Anderson, 1998). The dramatic decline in the number of CD4+ T cells during the course of HIV infection points to the death of large numbers of uninfected bystander cells. A number of studies reported that the majority of CD4+ cells undergoing apoptosis during HIV infection are uninfected (Finkel, 1995; Su, 1995). In lymph nodes from HIV-infected children, apoptosis occurs predominantly in uninfected bystander cells and not in productively infected cells (Finkel, 1995). Cocultureing of HIV-infected cells with uninfected cells resulted in apoptosis of the uninfected cells prior to syncytium formation or establishment of productive viral infection (Nardelli, 1995). It has recently been shown that chronically HIV-infected monocytic cells secrete a low molecular weight soluble factor that induces apoptosis in cocultured T cells (Chen, 1998). Addition of supernatant from HIV-1 infected dendritic cells to unfractionated thymocytes dramatically decreased their viability suggesting that thymocyte killing could be mediated by a soluble factor released from dendritic cells (Beaulieu, 1996). The HIV envelop protein gp120 in solution or on the surface of HIV-infected cells has been implicated in the induction of cell death and cytotoxicity. Cross-linking of CD4 by gp120 immune complex in vitro leads to T cell apoptosis by activation induced cell death (AICD) (Banda, 1997; Banda, 1992; Oyaizu, 1993). As seen in Fig. 2, the infected cell expresses gp120/gp41 on its surface and is producing virus. Expression of CD4 has been down-regulated on the infected cell. Ligation of CD4 on the uninfected bystander cell by virion or by gp120
delivers an inhibitory signal. Subsequent ligation of T cell receptor (TCR) on the uninfected cell by mitogen (or antigen or superantigen) leads to apoptosis. In contrast, TCR ligation of the infected cells by mitogen leads to increased viral replication and cell division. gp120 has also been implicated in causing death of neurons, CD8+ cells and the hematopoietic progenitor cells. Interestingly, lack of gp120-induced anergy and apoptosis in chimpanzees protects them from progression to AIDS (Banda, 1996). The role of HIV regulatory protein Tat in the induction of apoptosis in bystander cells is also documented (Li, 1995). Fas/FasL interaction may also play an important role in lymphocyte apoptosis in HIV disease (Estaquier, 1996).
Figure 2. Productively infected CD4+ T cells induce apoptosis of bystander uninfected CD4+ T cells. The infected cell expresses gp120/gp41 on its surface and is producing virus. Expression of CD4 has been down-modulated on the infected cell. Ligation of CD4 on the uninfected cell by virion (a) or by gp120 (b) delivers an inhibitory signal. Subsequent ligation of the TCR on the uninfected cell by mitogen (or antigen or superantigen) leads to apoptosis. In contrast, TCR ligation of the infected cell by mitogen leads to increased viral replication and cell division.
While contributing to the bystander effect, HIV could also have devised strategies to evade death of infected cells. Inhibition of apoptosis in HIV-infected cells promotes viral production and facilitates persistent infection. Finkel et al. (Finkel, 1995) showed that in HIV-infected lymph nodes HIV-infected cells were very rarely apoptotic, while there was widespread apoptosis in uninfected cells. Some studies suggested that viral proteins inside infected cells including Tat and Nef might inhibit apoptosis (Giellini, 1995; Guy, 1987).

**NFκB and HIV disease**

HIV infection leads to the progressive loss of CD4 T cells and the near complete destruction of the immune system in the majority of infected individuals. High level of viral gene expression and replication result partially from the activation of NFκB transcription factors, which in addition to orchestrating the host inflammatory response also activate the HIV-1 long terminal repeat (LTR). Transcription of the HIV-1 genome depends on the intracellular environment into which the virus integrates and is regulated by a complex interaction between viral regulatory proteins and cellular transcription factors that interacts with the viral LTR region (Gaynor, 1992). Sequences involved in viral gene expression are contained within U3 and R regions of the HIV-1 LTR and may be subdivided into the core promoter elements, the enhancer, the modulatory and negative regulatory elements, and the Tat responsive element (TAR). The most widely studied element in the modulatory region of the HIV-1 LTR is the enhancer region. Which consists of two 10-bp conserved sequences (-109 to –79) known as NFκB motifs. The role of NFκB in controlling the HIV-1 LTR transcription was first established by demonstrating a direct correlation between increases in NFκB DNA binding activity and HIV-1 LTR-directed transcriptional activity during T cell activation (Nabel, 1987). Mutation of NFκB motifs in HIV-1 LTR chloramphenicol acetyltransferase reporter constructs results in a marked decrease in gene expression following transfection into lymphoid cells, in both presence and absence of the tat gene. Transient transfection studies, with combinations of the HIV-1 LTR and HIV-1 enhancer sequences linked to reporter genes, demonstrate that induction of NFκB DNA binding activity in T cells and
monocytes leads to increased HIV-1 LTR-directed gene expression (Grilli, 1993). The NFκB sites act in synergy with NF-AT sites in phorbol myrisstate acetate (PMA)-stimulated T cells to increase the rate of viral replication in cells that respond to mitogenic stimulation (Du, 1993; Stevenson, 1990). Among the elements identified as positively acting factors in basal HIV-1 transcription, TATA, Sp1 and NFκB elements are the most influential in primary monocytes while NFκB and NF-AT have strong effects in activated T cells (Moses, 1994).

NFκB activation represents a double-edged sword in HIV infected cells. It mediates immune response and also leads to enhanced HIV-1 transcription. NFκB has also been implicated in apoptotic signaling. It promotes apoptosis under some circumstances, but acts as a survival factor under other circumstances. Therefore, activation of NFκB may have impact upon HIV pathogenesis at many levels and their relationship is multi-faceted (Deluca, 1999).

**Oxidative stress and HIV**

Several recent papers have proposed that apoptosis is the cause of immune-cell loss in patients infected with HIV. The process of apoptosis and latent virus activation may be linked to ‘oxidative stress’ in HIV infection. An excessive production of reactive oxygen species (ROS) and a broad deficiency of antioxidant in the course of HIV infection, lend strong support for the premise that ROS exist in HIV infection and AIDS. This warrants a closer examination of the actual mechanisms by which ROS may initiate apoptosis. Moreover, an understanding of how certain antioxidants act to mitigate oxidative stress and interact with apoptotic process may well provide a contributory therapeutic approach.

ROS are normal products of phagocytic activity and cellular respiration. Any antigenic or regulatory stimulus to polymorphonuclear leukocytes, or T cell activity, will increase the expression of ROS (Buttke, 1994). Thus, the high level of antigenic and cytokine activity in HIV/AIDS results in the production of substantial levels of superoxides, hydrogen peroxide and hydroxyl radicals. Furthermore, macrophages and monocytes produce tumor necrosis factor (TNF) in response to free radical activity and
this is found in excess in patients with HIV/AIDS (Johnston, 1978; Tracey, 1989). TNF may play an important role in causing a further increase in the ROS production by: (1) providing an amplification loop that feeds back to excite further production of ROS from macrophages and neutrophiles (Clark, 1988); and (2) reacting with T cells to enhance expression of autocrine cell activators, such as interleukin (IL)-2 and receptors, thereby promoting activation of T cell respiratory activity and an increase in intracellular ROS (Scott-Algara, 1991).

The loss of antioxidant defenses is critical to the development of ROS in patients with HIV/AIDS. It may result directly from the effects that excessive production of ROS overwhelms available antioxidants. Other indirect causes may include compromised gastrointestinal integrity, which results in mal-absorption of nutrients, alternation of systemic amino acids, which results in glutathione deficiency, and loss of electron-transport capacity, which causes the loss of NADPH (Greenspan, 1994).

The mechanisms of oxidant-mediated cell injury may include: (1) membrane-lipid peroxidation and loss of Ca\(^{2+}\) homeostasis. (2) DNA damage (3) Alteration of metabolic pathways/loss of ATP.

**Sphingomyelin (SM) pathway and HIV disease**

The sphingomyelin pathway is a ubiquitous and evolutionarily conserved signaling system. Ceramide, the central molecule in this pathway, serves as a second messenger for a wide range of cellular functions from proliferation and differentiation to growth arrest and apoptosis. In HIV infected individuals, lymphocyte apoptosis may signal T cell depletion and disease progression. It has been shown that circulating populations of CD4\(^{+}\) and CD8\(^{+}\) T cells from HIV-infected patients display large increases in ceramide content and apoptosis as compared with a normal population (Dimarzio, 1997; Cifone, 1997). Also, ceramide levels are markedly elevated in HIV-infected CEM cells (Van Veldhoven, 1992). Treatment of HIV-infected HL-60 cell (Rivas, 1994) or the latently infected myelomonocytic cell line U-111IB and OM-10.1 (Papp, 1994) with ceramide analogues profoundly increased viral production. Alternatively, treatment of HIV-infected patients with L-carnitine, which reduced acid Smase activity, significantly
reduced ceramide levels in peripheral blood mononuclear cells, and correlated with a
decrease in the number of apoptotic CD4\(^+\) T cells (Cifone, 1997). The underlying
mechanism and therapeutic potential need further investigation.
Chapter Two

The Role of Ethanol as a Cofactor in HIV Pathogenesis

Introduction

An important clinical feature of HIV-1 infection is a long period of latency that precedes the development of acquired immunodeficiency syndrome (AIDS) (Melbye, 1986; Fauci, 1988; Embretson, 1993). In latently infected cells, the HIV-1 integrated provirus may exist in an inactive state until the appropriate activation signals cause stimulation of viral transcription. During an HIV-1 infection, a number of cellular factors are induced that can modulate the replication of the latent virus. Among these host cellular factors, proinflammatory cytokines induce virus replication (Folks, 1987; Poli, 1993; Fauci, 1996), while certain other cytokines have an inhibitory or downregulatory effect on HIV-1 replication (Schuitemaker, 1992; Poli, 1991). Of the HIV-1 inducing cytokines, TNFα is the most potent, and is an important regulator of HIV-1 expression in chronically infected T lymphocytes, primary monocytes, and monocytic cell lines (Folks, 1989; Michihiko, 1989; Matsuyama, 1991; Mellors, 1991).

TNFα stimulated HIV-1 expression is mediated by the transcription factor NFκB which binds to the NFκB transcription enhancer motifs in the HIV-1 5’ long terminal repeat (LTR) (Duh 1989). Although other mammalian transcription factors participate in the regulation of the HIV-1 LTR transcriptional activation (Steffy, 1991; Garcia, 1994), NFκB plays a predominant role in TNFα induced LTR-driven transcription (Duh, 1989; Osborn, 1989; Schreck, 1991; Rattner, 1993). NFκB is a member of the NFκB/Rel family of dimeric transcription factors, and it regulates the expression of numerous cellular genes, particularly those involved in the immune and inflammatory responses (Baeuerle, 1994; Baeuerle, 1996; Grilli, 1993; Siebenlist, 1994). The NFκB/Rel family prototype is a heterodimer of a p50 (NF-κB1) and a p65 (RelA) subunit. In unstimulated cells, the p50/p65 (NFκB) complex is sequestered in the cytoplasm by noncovalent association with IκB proteins including IκBα, IκBβ and p105 (Beg, 1993). These IκB molecules mask the nuclear localization signal (NLS) and thus prevent the translocation of NFκB to...
the nucleus (Beg, 1992; Baldwin, 1996). In response to various stimuli, IκB molecules can be ubiquitinated, phosphorylated and then degraded by the ubiquitin-proteasome pathway releasing the NFκB/Rel complexes to the nucleus (Chen, 1995; Finco, 1995). Activation of NFκB by TNFα, mainly involves phosphorylation and degradation of IκBα and subsequent release and nuclear translocation of NFκB (Chen, 1995; Brockman, 1995; Brown, 1995). In addition to various kinases, reactive oxygen species (ROS) have also been implicated as upstream regulators of IκBα phosphorylation as well as second messengers in TNFα signaling leading to NFκB activation (Schreck, 1991; Baeuerle, 1996).

The degree of viral replication during the latency period can significantly alter the onset and progression of the disease (Embretson, 1993; Pantaleo, 1991; Mellors, 1996; O'Brien, 1996). Hence, cofactors that can amplify the TNFα induced activation of HIV-1 expression in cells containing integrated provirus may have major clinical implications. Ability of TNFα to induce HIV-1 expression is amplified by internal host cellular factors, including other cytokines (Poli, 1990; Chun, 1998; Chowdhury, 1993; Bressler, 1993). Similarly, there are several external stimuli, including microbial pathogens and substances of abuse that can affect activation of HIV-1 both individually and in combination with TNFα (Harrison, 1997; Bernier, 1998; Bernier, 1998a; Peterson, 1992; Peterson, 1994; Chao, 1994). Among the different substances of abuse, alcohol is considered an important risk factor/cofactor (Molgaard, 1988; Hulse, 1993; Avins, 1994; Balla, 1994; Mahler, 1994; Zenilman, 1994; Gwati, 1995).

Ethanol has been shown to cause impairment of neutrophil as well as T cell-dependent immune functions (Stoltz, 1999; Waltenbaugh, 1998; Brodie, 1994). In addition to its immunosuppressive effects ethanol may also contribute to the immunopathogenic mechanisms of HIV-1 infection by increasing viral replication (Bagasra, 1993; Bagasra, 1996). Data obtained from clinical as well as experimental studies have established an association with ethanol consumption and acceleration of HIV-1 disease progression (Balla, 1994; Fong, 1994; Wang, 1997). Hence, it is relevant to determine whether ethanol directly affects the expression of HIV-1 in cells carrying the provirus and/or modulates the inducibility of the virus in response to HIV-1 infection specific cytokines.
Since latently infected CD4⁺ T lymphocytes play a major role in the persistence of HIV-1 infection (Chun, 1995; Chun, 1997; Wong, 1997), we evaluated the role of ethanol as a possible cofactor in modulating TNFα signaling and subsequent HIV-1 replication using the human CD4⁺ T cell line, Jurkat. Specifically, the effects of ethanol were determined by analyzing TNFα dependent NFκB activation and subsequent induction of HIV-1 LTR transcription. Oxidative stress and ROS, which play an important role in NFκB activation, are induced in cells and tissues that interact with ethanol and TNFα. Hence, the involvement of ROS in the role of ethanol as a cofactor was also examined. Our findings indicate that ethanol, which by itself does not induce NFκB, potentiates TNFα induced NFκB nuclear translocation and HIV-1 LTR directed transcription in human CD4⁺ T cells. Further, the data suggests that the mechanism(s) underlying the ethanol dependent potentiation of NFκB activation entail enhanced degradation of IκBα and formation of ROS. The synergistic effect of ethanol on TNFα inducible signaling and resulting HIV-1-LTR mediated transcription may explain a critical aspect of its role as a cofactor during HIV-1 pathogenesis.
Experimental Procedure

Cell lines and plasmids

The lymphoid T cell line, Jurkat E6-1 was supplied by the AIDS Research and Reference Reagent Program, NIH (Rockville, MD). HIV-1 LTR-CAT was generously provided by Dr.C.Kunsch (Human Genome Sciences, Rockville, Maryland 20850). Jurkat E6-1 cells were cultured in RPMI 1640 medium (GIBCOBRL, Grand Island, NY) supplemented with 5% fetal bovine serum (GIBCOBRL), 100 units/ml penicillin and 100µg/ml streptomycin (GIBCOBRL) at 37°C in a humidified atmosphere with 5% CO₂.

Cell treatments and extracts

Cells were pretreated with different concentrations of ethanol, followed by treatment with TNF-α for different intervals of time. Nuclear and cytosolic extracts were prepared as described elsewhere60. After incubation, cells were washed once with ice cold phosphate-buffered saline (PBS) and pelleted. The cell pellet was resuspended in 250µl lysis buffer (10mM HEPES (pH 7.9), 1.5mM MgCl₂, 10mM KCl, 0.5mM dithiothreitol (DTT), 0.5mM phenylmethylsulfonyl fluoride (PMSF), 1µg/ml leupeptin, 1µg/ml pepstatin, 1µg/ml leucine thiol and 0.1% (v/v) Nonidet P-40) and incubated on ice for 30 min. Nuclei were collected by microcentrifugation (4000rpm, 4°C, 5min). The supernatant (cytosolic extract) was recovered and stored at -80°C. Nuclei were resuspended in 200µl extraction buffer (20mM HEPES (pH 7.8), 25% Glycerol, 520mM NaCl, 1.5mM MgCl₂, 0.1mM EDTA, 0.5mM PMSF, 0.5mM DTT, 0.2% Nonidet P-40, 1µg/ml leupeptin, 1µg/ml pepstatin and 1µg/ml leucine thiol) and incubated on ice for 60 min. Nuclear debris was removed by microcentrifugation (15,000 rpm, 4°C, 20min). Supernatant (nuclear extract) was stored at -80°C in small aliquots until use.
**Electrophoretic mobility shift assay (EMSA)**

The double-strand NFκB oligonucleotide probe containing the consensus NFκB binding site corresponding to the sequence 5’ –AGTTGAGGGGACTTTCCCAGGC- 3’ (Promega, Madison, WI) was labeled with [γ-32P] ATP (ICN, Costa Mesa, CA) by using T4 kinase (GIBCOBRL). Nuclear extracts were prepared as described above. Equal amounts of nuclear proteins (7µg), were incubated for 15 min on ice in reaction buffer [10mM Tris (pH7.5), 100mM NaCl, 1mM DTT, 1mM EDTA, 4%(v/v) glycerol, 80µg/ml sonicated salmon sperm DNA (GIBCOBRL)] followed by the addition of γ-32P-labeled ds NFκB probe (0.5 ng). The reactions were incubated at room temperature for 20min and resolved on a 5% acrylamide/bisacrylamide (30:0.8%) nondenaturing gel in 0.5X TBE [50mM Tris (pH8.0), 45mM boric acid and 0.5mM EDTA]. Competition assays were performed by adding a 25-fold molar excess of either unlabeled ds NFκB probe or a ds mutant NFκB (mtNFκB) probe with a “G” to “C” substitution in the NFκB/Rel binding motif [5’- AGTTGAGGGCGACTTTCCCAGGC- 3’ (Santa Cruz Biotechnology, Santa Cruz, CA)]. Supershift assays were performed by the addition of p65, p50 and c-rel polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), 15 min before the incubation with the γ-32P-labeled NFκB probe. Gels were dried and visualized by autoradiography. Autoradiograms were scanned using Epson ES-1200C imaging system and the NFκB specific bands were analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**Western blot analysis**

Cytosolic extracts were prepared as described above. 15µg of protein was separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membrane in a buffer containing 30mM Tris, 200mM glycine and 20% methanol. The membranes were blocked by incubation in 1XTBS (10mM Tris HCl (PH8.0), 150mM NaCl) with 5% dried milk for 1h at room temperature and then incubated at 4°C overnight with IkB-α polyclonal antibody (Santa Cruz Biotechnology,
Santa Cruz, CA) in 5% milk/TBS at a dilution of 1:1000. After three 20-min washes with 1XTBS, membranes were incubated with a secondary anti-rabbit IgG conjugated with horseradish peroxidase (Transduction Lab, Lexington, KY) at the dilution of 1:1333. The reaction was then visualized with the enhanced chemiluminescence detection system (ECL) as recommended by the manufacturer (Amersham International plc, Buckinghamshire, England).

**Transient Transfection**

Jurkat cells were transiently transfected with the pHIV-LTR-CAT plasmid by using Lipofectamine, as per the instructions supplied by the manufacturer (GIBCOBRL). Briefly, transient transfections were accomplished by using 8 µl of Lipofectamine reagent and 5 µg of plasmid DNA per ml per transfection reaction. Before adding to cells, the Lipofectamine-DNA complexes were allowed to form for 30 min at room temperature in serum-free medium. Cells were prepared for transfection, by washing and resuspending in serum-free medium to obtain a final cell density of 2 x 10^6 cells/ml. Cells were incubated with the Lipofectamine-DNA complexes for 6 h, after which the serum-free medium was replaced with complete medium. Cells were incubated further for 24 h before treatment with ethanol and/or TNFα. To minimize the variations in plasmid transfection efficiencies, transfected cells were pooled 24 h after transfection and were then separated into various treatment groups.

**Chloramphenicol acetyl transferase (CAT) activity assay**

After treatment, the transiently transfected cells were harvested and washed once with 1 X PBS. Cell extracts were prepared by three cycles of freeze-thaw in 0.25M Tris (pH7.8). Protein concentrations were measured by the method of Bradford (Bio-Rad Laboratories, Richmond, CA) and equal amount of protein was analyzed from each sample. First, a 100µl reaction medium was prepared for each sample by mixing 0.4M Tris (pH 7.8), 0.5µCi 14C-chloramphenicol (35 to 55 mCi/mmol, ICN) and 1mM acetyl coenzyme A (Sigma). Next, a 100µl protein/buffer mixture was prepared by adding
20μg of protein from each sample into CAT lysis buffer (250mM Tris-HCl (pH 7.8), 0.5mM EDTA, 2μg/ml Aprotinin). After the addition of reaction medium to protein-buffer mixture, the total reaction mixture(200μl) was vortexed gently and incubated at 37°C for 1h. Samples were extracted with 1ml ethyl acetate, dried and spotted onto a thin layer chromatography (TLC) plate (Fisher). The plate was developed in a mixture of chloroform-methanol (95:5), dried and visualized by autoradiography.
Results

Ethanol synergistically activates TNF-α inducible NFκB activation.

TNFα is the major inflammatory cytokine that activates HIV-1-LTR, primarily through NFκB dependent transcriptional activation. Hence, the effect of ethanol pretreatment on TNFα induced NFκB DNA binding activity was evaluated in Jurkat CD4+ T cells. Cells were treated at 37°C with ethanol alone (25 mM, 2.5 h), TNFα alone (250 U/ml, 1.5 h) or pretreated with ethanol at different concentrations (10 to 100 mM, 1 h) and subsequently treated with TNFα (250 U/ml, 1.5 h). Nuclear extracts from the treated cells were obtained and analyzed by EMSA using a NFκB specific probe. Little to no binding to the NFκB probe was observed in nuclear extracts from cells treated with ethanol alone and in the untreated cells (Fig. 3, lanes 1 and 2). As expected, TNFα treatment induced substantial NFκB DNA binding (Fig. 3, lane 3). Pretreatment with ethanol resulted in a significant enhancement of the TNFα inducible NFκB DNA binding activity. Increase in the NFκB activation was seen at all the concentrations of ethanol with maximal increment observed at 25 mM. Although, prior exposure to ethanol consistently enhanced TNFα-inducible NFκB activation, some variation in the degree of enhancement was seen between experiments ranging from 2.0 to 4.0 fold. Importantly, 25 mM ethanol concentration, which gave maximal enhancement of TNFα-inducible NFκB activation, represents blood alcohol levels corresponding to legal levels of intoxication achieved during social drinking (Werch, 1988). Hence all the subsequent experiments were performed with 25 mM ethanol. The specificity of the NFκB DNA binding activity was established by competition experiments on nuclear extracts obtained from TNFα treated cells. NFκB DNA binding activity was abrogated when 25-fold molar excess of unlabeled NFκB oligonucleotide probe (25X NFκB) was included in the DNA binding reaction mixture. Additionally, competition with the 25-fold molar excess of mutant NFκB (25X mtNFκB) oligonucleotide probe with a “G” to “C” substitution in the NFκB/Rel DNA binding motif did not affect binding to the NFκB oligonucleotide probe.
Figure 3. Ethanol potentiates TNFα inducible NFκB activation. Jurkat cells were treated with TNFα (250 U/ml, 1.5 h) with or without pretreatment with varying concentrations of ethanol for 1 h. Nuclear extracts were prepared and analyzed by EMSA using NFκB specific probe; arrows indicate the NFκB specific binding. Competition assays were performed using the same nuclear extract as in lane 3, by adding either 25-fold molar excess of unlabelled NFκB probe or the mutant NFκB (mtNFκB) oligonucleotide probe.
Identification of TNFα induced NFκB complexes affected by ethanol.

NFκB/Rel transcription factors are homo- or heterodimeric complexes that consist of various combinations of Rel subunits. To identify the subunits in the TNFα inducible NFκB complexes that are affected by ethanol, antibody supershift experiments were performed. Nuclear extracts were obtained from cells treated with TNFα alone (250 U/ml, 1.5 h) and cells that were pretreated with ethanol (25 mM, 1 h) followed by TNFα treatment (250 U/ml, 1.5 h). In both treatments, the p65-specific antibody completely shifted complex C1 and the p50-specific antibody primarily shifted complex C2 and reduced the extent of binding of complex C1 (Fig 4; lanes 2, 3, 5 and 6). Since c-Rel is a component of activated NFκB in some cells, activated NFκB complexes were also tested for the presence of c-Rel. The c-Rel specific antibodies had no effect on either complex, which shows that c-Rel does not participate in this NFκB activation process (Fig 4; lanes 1 and 4). Data obtained with p50- and p65-specific antibodies indicates that the NFκB/Rel complexes induced by TNFα and enhanced by ethanol are the p50/p65 heterodimer (C1) and p50/p50 homodimer (C2).

Potentiation of TNFα inducible NFκB by ethanol involves enhanced IκBα proteolysis.

Under unstimulated conditions cytoplasmic inhibitory proteins collectively referred to as IκB bind to NFκB, and sequester it in the cytoplasm. A variety of stimuli can initiate signaling that leads to the degradation of specific IκB proteins, which results in the release and the nuclear translocation of NFκB (Baldwin, 1996). In Jurkat T cells TNFα signaling that leads to NFκB activation predominantly involves IκBα proteolysis (Chen, 1995; Finco, 1995; Brockman, 1995; Brown, 1995). In order to elucidate the mechanism underlying the enhancement of TNFα inducible NFκB DNA binding activity, the effect of ethanol on TNFα dependent IκBα degradation was investigated. Western blot analysis was performed to examine the levels of IκBα proteins in cytoplasmic extracts that were obtained from cells treated with either ethanol alone or TNFα (with or
Figure 4. Identification of TNFα induced NFκB complexes affected by ethanol. Jurkat cells were pretreated with ethanol (25mM, 1 h) followed by the treatment with TNFα (250U/ml, 1.5 h). Nuclear extracts were prepared and analyzed by EMSA with NFκB specific probe. Supershift assay was performed by using polyclonal antibodies specific for p65 and/or p50. C1 and C2 denote the various dimers of NFκB and NS indicates non-specific binding. S denotes the complexes supershifted by either p65 or p50.
without ethanol pretreatment [25 mM, 1 h]) for 15, 30, 60 and 90 min (Fig 5). Ethanol by itself was unable to cause IκBα degradation (lanes 2, 5, 8 and 11), which concurs with its inability to activate NFκB. As expected, IκBα degradation was observed at 15, and 30 min in cells treated with TNFα (lanes 3 and 6). Pretreatment with ethanol and the subsequent exposure to TNFα caused a greater loss of IκBα protein than did exposure to TNFα alone (compare lanes 3 and 4; 6 and 7). Following degradation, IκBα resynthesis was initiated at 60 min (lanes 9 and 10) and reached at, or greater than, basal levels in 90 min in both treatment arms (lanes 12 and 13). In summary, this data suggests that prior exposure to ethanol modulates the TNFα signaling causing enhanced IκBα proteolysis and subsequent release and translocation of NFκB into the nucleus.

**PDTC Inhibits Potentiation of TNFα Induced NFκB by Ethanol**

Since NFκB activation, is known to be affected by redox processes, we further examined the role of oxidative stress and ROS in the effect of ethanol on TNFα mediated NFκB activation. Ethanol consumption causes elevation in TNFα and induces oxidative stress in adult tissues and is associated with subsequent ethanol related damage (McClain, 1999; Bondy, 1992); additionally, exposure to ethanol, both in vivo and in vitro, results in the production of reactive oxygen species (ROS) (Bondy, 1992; Hill, 1998). PDTC is known to inhibit the formation of ROS and NFκB activation by certain stimuli in a cell type-specific manner (Schreck, 1992) and hence its effect on NFκB enhancement by ethanol was investigated. PDTC pretreatment (100 µM, 1 h), markedly suppressed NFκB activation in response to ethanol and TNFα (Fig 6). The data indicates that reactive oxygen intermediates are probably involved in the ethanol enhancement of TNFα inducible NFκB activation.
<table>
<thead>
<tr>
<th>Lane Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>0</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Ethanol 25mM</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TNFα 250 U/ml</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Lane Number  | 8 | 9 | 10 | 11 | 12 | 13 |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>60</td>
<td>60</td>
<td>60</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Ethanol 25mM</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>TNFα 250 U/ml</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 5. Ethanol enhances TNFα-dependent IκBα proteolysis. Jurkat cells were treated with ethanol alone, TNFα alone, and a one hour ethanol pretreatment followed by TNFα (the treatments and times are indicated). After treatment, cytoplasmic extracts were prepared and western blot analysis was performed using a polyclonal anti-IκBα antibody. The asterisks highlight the synergistic effect of ethanol+TNFα on IκBα degradation.
<table>
<thead>
<tr>
<th>Lane Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα (250 U/ml)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol (25 mM)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>PDTC (100 µM)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Figure 6. PDTC inhibits NFκB activation effected by ethanol and TNFα. Jurkat cells were pretreated with 100 µM PDTC for 1 h and then treated with ethanol for 1 h followed by TNFα for 1.5 h. Nuclear extracts prepared from these cells were examined for NFκB activation by EMSA.
Protein synthesis is not required for the potentiation of TNFα inducible NFκB by ethanol.

Experiments were performed to exclude the possibility that ethanol enhanced TNFα inducible NFκB activation requires increased TNFα receptor synthesis and that the signal transduction molecules that are required for this process are pre-formed in cells. Jurkat T cells were pretreated with the protein synthesis inhibitor cycloheximide (CHX) and then treated with ethanol and TNFα or TNFα alone. Fig. 7 demonstrates that CHX had no effect on the potentiating effect of ethanol on NFκB activation. Additionally, the possibility of increased density of the TNFα receptors contributing to enhanced NFκB activation was addressed by flow cytometry analysis using a TNFα fluorokine (R&D System). After a 24 h exposure of the cells to ethanol pretreatment and TNFα, or TNFα alone, no significant changes in the cell surface receptor density were observed (data not shown). Thus, the potentiation of the TNFα inducible NFκB appears to take place in the absence of protein synthesis and apparently does not require the induction of TNFα receptor expression.

Ethanol enhances TNFα inducible HIV-1 LTR directed transcription.

TNFα induces transcriptional activation of the HIV-1 LTR in cells containing integrated provirus, and it is associated with the induction of NFκB binding to the κB-sites in the LTR (Duh, 1989). The observed ethanol mediated potentiation of the TNFα inducible NFκB DNA binding activity suggested a transcriptional mechanism by which ethanol may contribute to the enhancement of TNFα inducible HIV-1 expression. In order, to further elucidate the possible role of ethanol as a cofactor, its effect on TNFα inducible HIV-1 LTR promoter activity was examined in Jurkat T cells transiently transfected with the full length HIV-1 LTR CAT reporter construct. Ethanol treatment alone had no effect on the HIV-1 LTR expression, while predictably TNFα treatment stimulated LTR-mediated CAT expression (Fig. 8). Importantly, pretreatment with ethanol led to a significant additional increase in TNFα inducible CAT activity as
<table>
<thead>
<tr>
<th>Lane Number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα (250 U/ml)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol (mM)</td>
<td>-</td>
<td>25</td>
<td>10</td>
<td>25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHX (10 µg/ml)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Figure 7. Cycloheximide (CHX) does not inhibit the potentiating effect of ethanol on TNFα inducible NFκB activation. Jurkat cells were pretreated with CHX (10 µg/ml) for 1 h and then treated with TNFα alone or ethanol for 1 h followed by TNFα for 1.5 h. After treatment, nuclear extracts were prepared and analyzed by EMSA using a NFκB specific probe. Arrows represent NFκB complexes.
Figure 8. Ethanol pretreatment potentiates TNFα inducible HIV-1 LTR mediated transcription. Jurkat cells transiently transfected with pHIV-1 LTR CAT were treated with ethanol (25 mM) and TNFα (250U/ml) alone or pretreated with 25 mM ethanol for 1 h followed by TNFα (250U/ml) for 24 h. After 24 h treatments cell extracts were prepared and analyzed for CAT activity. The data shown represent the mean ± S.D. of three independent experiments performed in triplicates.
compared to cells treated with TNFα alone. The observed effect of ethanol on HIV-1 LTR CAT expression is in concordance with its ability to potentiate TNFα inducible NFκB activation. Taken together these data suggest that ethanol at clinically relevant concentrations has the potential to enhance TNFα dependent HIV-1 replication in CD4+ T lymphocytes.
Discussion

HIV-1 infection is characterized by a long period of latency during which a detectable reservoir of latently infected CD4$^+$ T cells persist in HIV-1 infected individuals (Chun, 1995; Chun, 1997; Wong, 1997). Since these latently infected cells have the potential to be induced to produce the virus, internal and external stimuli that affect the activation of HIV-1 replication in these cells can significantly alter the onset and progression, of this disease. Thus, an important aspect of HIV-1 pathogenesis is the possible interaction of external and internal stimuli that may lead to an increase in viral replication.

Among several external stimuli that are implicated in HIV-1 infection and AIDS, alcohol consumption is recognized as an important risk factor (Molgaard, 1988; Hulse, 1993; Zenilman, 1994). Several neurological, physiological and immunological abnormalities observed in HIV-1 infected patients are also found in chronic alcoholics (Tyor, 1999), which have led to the postulate that alcohol consumption can alter the progression of HIV-1 infection. Evidence from the HIV-1 infected cohort studies on the role of alcohol as a cofactor in HIV-1 disease progression is mixed and subject to various interpretations (Tyor, 1999; Cook, 1996). It is important to note that these studies have a number of potentially confounding variables that hamper interpretation (for e.g. pre-infection disorders, licit and illicit drug usage, rates and quantities of alcohol consumed, duration since HIV-seroconversion, absence of appropriate matched controls etc.) (Tyor, 1999; Westerberg, 1992; Fein, 1998) leading to inadequate assessment. Evidence indicating the clinical association of alcohol use with the progression of HIV-1 disease, albeit limited, comes from studies evaluating (i) the effect of alcohol on progression of the neurological effects of HIV-1 infection which show that alcohol abuse exacerbated the neuropathological and CNS abnormalities of the HIV-1 infection (Tyor, 1999; Cook, 1996; Fein, 1998; Deicken, 1991; Meyerhoff, 1995) and (ii) the adverse effect of alcohol on HIV-1 infection induced immunosuppression which show that alcohol withdrawal markedly improved the CD4$^+$ cell count in HIV-1 infected alcoholic patients (Pol, 1996). Animal studies which examine the immunomodulatory effects of ethanol using the murine AIDS model (infection with LP-BM5 retrovirus), as well as the transgenic mouse
model (expressing the HIV-1 tat protein) have yielded results that are consistent with the hypothesis that ethanol exposure may enhance the progression of HIV-1 infection (Wang, 1997; Wang, 1995; Prakash, 1998). Adverse effects of alcohol on HIV-1 disease progression are further supported by in vitro studies showing that (i) peripheral blood mononuclear cells (PBMCs) obtained from alcohol consuming subjects exhibit increased susceptibility to HIV-1, significantly increased HIV-1 replication and decreased ability of lymphocytes to produce interleukin-2 (IL-2) and soluble immune response suppressor activity upon activation, as compared to controls (Bagasra, 1993; Bagasra, 1996), (ii) CD4+ cell line, CEM, when exposed to alcohol in vitro and infected with HIV-1, supports significantly greater HIV-1 replication (Saravolatz, 1990) and (iii) exposure of lymphocytes from AIDS patients to low levels of alcohol (0.1%) significantly reduces their HIV-1 specific proliferative responses induced by the env (HIV-1 gp41 envelope) and gag (HIV-1 core) proteins (Nair, 1994). All these studies suggest a role for alcohol as a cofactor in HIV-1 pathogenesis. However, the effect of alcohol on cellular mechanisms relevant to HIV-1 disease progression remains undetermined. Among the internal stimuli critical for HIV-1 pathogenesis TNFα plays a central role in regulating HIV-1 replication (Folks, 1989; Michihiko, 1989; Matsuyama, 1991; Mellors, 1991) and elevated TNFα levels correlate with several AIDS associated pathophysiological changes (Tyor, 1999). Hence, to determine the molecular basis, if any for the proposed, of alcohol in HIV-1 disease progression, its interaction with TNFα was assessed in the present work.

Since latently infected CD4+ T cells are the critical source for reactivating HIV-1 viral replication, where the transition from latency to active replication is primarily controlled by TNFα-inducible NFκB (Folks, 1989; Duh, 1989; Wu, 1995; Gollapudi, 1998; Traber, 1999), we investigated the role of ethanol in the induction of HIV-1 replication in these cells. We used HIV-1 LTR CAT reporter construct to transiently transfect the human CD4+ T cell line (Jurkat E6-1) to determine if ethanol, by itself or in collaboration with TNFα, affects the signaling involved in HIV-1 transcriptional activation. The concentrations of ethanol used in this study reflect the blood alcohol levels that can be achieved during human alcohol consumption (Werch, 1988).

NFκB plays a critical role in the induction of latent HIV-1 virus, and in the TNFα-mediated enhancement of virus production (Vicenzi, 1994; Wu, 1995; Gollapudi,
Hence, we examined the effect of ethanol alone and its effect on TNFα induced NFκB activation. The data obtained clearly shows that ethanol can strongly potentiate TNFα induced NFκB nuclear translocation (Fig. 3). Importantly, ethanol by itself was unable to stimulate NFκB activation, which suggests that the synergy observed between ethanol and TNFα is fundamentally different from other T cell costimulatory systems where each stimulus independently activates NFκB (Harhaj, 1996). Although, ethanol did not have any effect on NFκB activation in T cells it has been shown to affect NFκB activation in monocytes. In vitro exposure of human monocytes to 25 mM ethanol increased NFκB DNA-binding activity with a preferential induction of the transcriptionally inactive, p50/p50 NFκB/Rel homodimer with no induction of the p65/p50 heterodimer (Mandrekar, 1997). Taken together these observations suggest that ethanol affects NFκB activation in a cell-type specific manner.

The mechanism by which TNFα mediates its effects on NFκB nuclear translocation is predominantly regulated by the targeted phosphorylation and subsequent degradation of IκBα. Data obtained on the effect of ethanol on TNFα induced IκBα proteolysis, and NFκB nuclear translocation, shows that a major component of the potentiation of the NFκB activation is enhanced degradation of IκBα (Fig 5). To further address the possible mechanisms involved in the effect of ethanol on NFκB activation, we considered the role of ROS. Generation of ROS has been implicated in the mechanism of NFκB activation in response to diverse stimuli including TNFα (Baeuerle, 1994). In this regard ROS appear to serve as messengers mediating directly or indirectly the release of the inhibitory subunit IκB from NF-kappa B (Schreck, 1991; Schreck, 1992). Our results with PDTC, an antioxidant which is both a metal chelator and a radical scavenger, showed that enhancement of IκBα degradation and NFκB activation in ethanol and TNFα treated cells was PDTC-sensitive (Fig. 6), thus suggesting an important role for ROS in this mechanism.

Agents that stimulate NFκB nuclear translocation induce the phosphorylation of IκBα on serine 32 and 36, which leads to the ubiquitination and the subsequent proteasome-dependent degradation. Recent studies show that TNFα induced IκBα degradation involves the activation of a kinase cascade. The distinct regulatory
components of this TNFα inducible cascade include the NFκB inducing kinase (NIK) that activates a high molecular mass (500-900 kDa) IκB kinase complex (IKK), which is comprised of two functional subunits IKKα and IKKβ that phosphorylate both serine 32 and 36 in IκBα (Lee, 1997; Regnier, 1997). One or more of the multiple regulatory components involved in the TNFα induced IκBα degradation and subsequent NFκB activation can be affected by ethanol. Hence, it will be our next step to investigate the role of ethanol and ROS in enhancing the TNFα induced catalytic activity of IKKα and IKKβ subunits.

When the effect of CHX on ethanol mediated enhancement of TNFα inducible NFκB activation was analyzed, NFκB levels were found to be unaltered (Fig. 7). These data suggest that this ethanol -TNFα synergy is independent of protein synthesis, and the signal transduction molecules required, are not induced but pre-exist in these cells. The data also showed that this ethanol effect does not require TNFα receptor synthesis. These observations suggest that ethanol modulates the TNFα induced signal transduction pathways resulting in the enhancement of NFκB activation.

To further verify, that ethanol can function as a cofactor in the pathogenesis of HIV-1 infection, we determined whether increased NFκB binding activity corresponded with an increase in NFκB-dependent HIV-1 LTR transcriptional activation. Consistent with its inability to activate NFκB, ethanol alone did not induce HIV-1 LTR activity. However, ethanol enhanced TNFα induced HIV-1 LTR transcriptional activity (Fig 8), commensurate with its ability to potentiate TNFα induced NFκB nuclear translocation. Additionally, the fact that PDTC has been shown to have an inhibitory effect on NFκB and HIV-1 LTR activation (Schreck, 1992), further substantiated the importance of NFκB in this process. These data suggest that clinically relevant concentrations of ethanol can increase the TNFα inducible HIV-1 LTR activity via a NFκB dependant signaling pathway.

In summary, our data showed that the in vitro exposure of CD4+ T cells to ethanol, can enhance TNFα mediated NFκB activation and subsequent induction of HIV-1 LTR transcriptional activity. Additionally, the data also indicated that the effect of ethanol on TNFα signaling involves ROS generation and enhanced degradation of IκBα.
Resting CD4+ T cells harboring latent HIV-1 provirus, reside in lymphoid tissue where endogenous cytokine secretion in response to antigenic stimuli is routinely encountered (Fauci, 1993; Pantaleo, 1991); they also traffic continuously throughout the body (Chun, 1997). This offers extensive opportunity for ethanol-TNFα interaction to occur in HIV-1 infected individuals following ethanol consumption. If such a scenario is encountered during the course of an HIV-1 infection, this interaction could either enhance the expression of NFκB-regulatable proinflammatory cytokines, including TNFα, and/or have a potentiating effect on the reactivation of HIV-1 replication in latently infected CD4+ T cells. Overall, the data provide a possible mechanistic explanation for the role of ethanol as a cofactor that can adversely affect the progression from a latency state to clinical expression of HIV-1 related disease. An understanding of the effects of ethanol on host cellular mechanisms involved in HIV-1 pathogenesis may elucidate the relationship between alcohol consumption and AIDS risks, and thus allow the development of better prevention and intervention strategies.
Chapter Three

Mechanism of Ceramide Induced CD4+ T cell Apoptosis

Introduction

The sphingomyelin pathway is a ubiquitous, evolutionary conserved signaling system. Most, if not all, mammalian cells appear capable of signaling through the sphingomyelin pathway. Sphingomyelin is a phospholipid preferentially concentrated in the plasma membrane of mammalian cells. The catabolic pathway for sphingomyelin is via the action of sphingomyelin-specific forms of phospholipase C (PLC), called sphingomyelinases (SMase), yielding ceramide and phosphorylcholine (Fig. 9).

Ceramide generation in mammalian cells primarily occurs via receptor-mediated hydrolysis of sphingomyelin by the action of a neutral or acidic sphingomyelinase. Thus ceramide is defined as a central element in the metabolic pathway of sphingomyelin, serving both as a precursor and an intermediate product (Haimovitz-Friedman, 1997). Ceramide can also be generated by de novo synthesis involving the enzyme ceramide synthase. Several direct targets of creamide have been identified, including a ceramide-activated protein kinase (CAPK) (Liu, 1994), a ceramide-activated protein phosphotase (CAPP) (Dobrowsky, 1992) and the protein kinase Cζ isoform (Lozano, 1994). In addition, ceramide interacts with several signaling systems, including the mitogen-activated protein kinase (MAPK), c-Jun kinase (JNK), caspase and mitochondrial signaling system. In the following, details are given about the interaction between ceramide and each of the signaling pathways just mentioned.

CAPK

CAPK is stimulated by TNF-α and activated the MAPK pathway via phosphorylation of c-Raf-1 (Yao, 1995). Recently, it is concluded identical to the kinase suppressor of Ras (KSR) (Zhang, 1997). It is defined as a 97 kDa membrane-associated,
Figure 9. Sphingomyelin Metabolism
proline-directed protein kinase. Treatment of cells with TNF-\(\alpha\) or synthetic ceramide analogue enhanced KSR autophosphorylation, and increased its ability to phosphorylate and activate Raf-1 on Thr\(^{269}\).

**PKC\(\zeta\)**

PKC\(\zeta\) is an atypical PKC isoform which is insensitive to Diacetylglcerol(DAG) and phorbol esters, but responsive to ceramide. Treatment of U937 cells or NIH3T3 fibroblasts with TNF-\(\alpha\), SMase or ceramide analogue increased PKC\(\zeta\) phosphorylation and activity. PKC\(\zeta\) may play a role in TNF-induced activation of nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) in some cell types. The recent demonstration that PKC\(\zeta\) moves to the nucleus in response to ceramide elevation suggests that it may regulate the transcriptional events during ceramide signaling (Bertolaso, 1998).

**CAPP**

CAPP belongs to the protein phosphatase 2A family of serine/threonine phosphatases (Dobrowsky, 1993; Dobrowsky, 1993a; Law, 1995). These phosphatases exist as heterotrimers in which subunits A and B are regulatory and subunit C is catalytic. The B subunit is required for activation by ceramide. CAPP mediates an antiproliferative response to ceramide, arresting cells at the G1 phase of the cell cycle. It has been speculated that CAPP plays a role in down-regulation of c-myc expression in mammalian cells (Wolff, 1994). Also, some studies suggest CAPP may act as an antagonistic signal, inhibiting ceramide-mediated apoptosis by reversing effects on JNK.

**Ceramide and MAPK**

The MAPK pathway, also called ERK pathway, usually mediates growth and inflammatory signals (Fanger, 1997). Ceramide was shown to regulate the MAPK pathway in different cell types. In cos-7 cells, synthetic ceramide analogues and exogenous SMase mimicked the effect of TNF-\(\alpha\) via successive activation events
involving KSR/CAPK, c-Raf-1, MEK and MAPK (Yao, 1995). Ceramide also mimicked TNF-α-induced activation of c-raf-1, MEK and MAPK in human vein endothelial cells, leading to a pro-inflammatory response (Modur, 1996). In T cells, stimulation of CD28 engages the SMase signaling system for activation of the MAPK cascade, which is involved in proliferation (Boucher, 1995; Chan, 1995).

**Ceramide and JNKs**

The ERK cascade has been shown to communicate growth factor- and phorbol ester-induced proliferative signals. Recently, stress-activated protein kinase (SAPK), also known as c-Jun kinase (JNK) and p38 cascade has been defined. These cascades, activated by environmental stresses such as inflammatory cytokines, ATP depletion, heat and osmotic shock, ionizing and UV irradiation, and endotoxin have been shown to transmit growth arrest, differentiation, or apoptotic signals (Kyriakis, 1994; Kyriakis, 1994; Cano, 1995).

Recent evidence links ceramide-induced apoptosis to the SAPK/JNK protein kinase cascade in a variety of cell types. It has been reported that ionizing radiation, ultra-violet C radiation, H₂O₂, heat shock and TNF-α induce ceramide generation within seconds in primary cultures of bovine endothelial cells and U937 monoblastic leukemia cells, prior to activating the SAPK/JNK cascade (Verheij, 1996). Disruption of signaling down the SAPK/JNK cascade but not the ERK cascade, by overexpression of dominant negative mutants, abrogated TNF-, stress- and ceramide- induced apoptosis. These studies showed that the SAPK/JNK cascade was a downstream effector system for ceramide induced apoptosis. In contrast, ceramide and stress result in little or no change in ERK pathway activation in cells undergoing apoptosis (Pyne, 1996; Westwick, 1995). In fact, ERK cascade has been demonstrated to be anti-apoptotic. The inactivation of the ERK cascade was required in addition to the activation of the SAPK/JNK and p38 cascade for apoptosis to proceed in some cell types (Xia, 1995). This suggests that a balance between SAPK/JNK and ERK may be critical in affecting the apoptotic outcome.
Ceramide and Caspase

Apoptosis in mammalian systems occurs by recruitment of a cascade of caspase proteases. Caspases, a family of cystein proteases with aspartate-specificity, are divided functionally into initiator caspase, such as caspases 2, caspase 8, caspase 9, caspase 10 and effector caspases such as caspase 3 and caspase 7. Initiator caspase can couple to cytokine receptors of the TNF superfamily and link to effector caspase, which commit cells to the death program (Salvesen, 1997). It is well documented that ceramide functions proximal to effector caspases in mammalian system (Tepper, 1997; Dbaibo, 1997; Waterhouse, 1996). Activation of effector caspases by ceramide is inhibited by Bcl-2 overexpression in numerous systems (Susin, 1996; Farschon, 1997; Martin, 1995). In the case of the TNF receptor, a protein termed TNFR-associated death domain (TRADD) binds via its death domain to the receptor and to another adaptor protein termed Fas-associated death domain (FADD) (Chinnaiyan, 1995; Chinnaiyan, 1996; Hsu, 1995). CD95, however, does not require TRADD and binds FADD directly. In addition to its death domain, FADD contain a region termed as death effector domain (DED), another protein-protein interaction motif, which links to the DED of an ICE-like protease termed FLICE/caspase 8. It has been shown that formation of the ligand-dependent multi-protein complex that links directly to an ICE protease is sufficient to initiate a protease cascade that effects apoptosis directly. However, in some cellular system the death domain adaptor protein mechanism may link to and utilize the sphingomyelin pathway for induction of apoptosis. Deletions and mutations of the death domain region of the TNF receptor (Wiegmann, 1994) and CD95 (Cifone, 1995), which abrogate induction of apoptosis, also block A-SMase activation. Further, Dixit and co-worker reported that overexpression of dominant negative FADD blocks ligand-induced ceramide generation (Chinnaiyan, 1996).

Role of ceramide to radiation and drug induced cell death

Recent investigations have implicated ceramide as a mediator of radiation-induced apoptosis. Cells exposed to therapeutic doses of ionizing radiation were shown to
undergo rapid SM hydrolysis, ceramide generation and apoptosis (Michael, 1997; Haimovitz-Friedman, 1994; Chmura, 1997; Bruno, 1998). Ionizing and UV radiation have direct effects on the cell membrane (Cheng, 1996; Devary, 1992) and are capable of generating ceramide in isolated membrane (Haimovitz-Friedman, 1994), independent of the effect on nuclei. Further, lymphoblast from Niemann-Pick patients, who have an inherited deficiency of acid sphingomyelinase activity, fail to respond to ionizing radiation with ceramide generation and apoptosis. Both events are restorable by retroviral transfer of human A-SMase cDNA (Santana, 1996). Similarly, A-SMase knockout mice also expressed defects in radiation induced ceramide generation and apoptosis in vivo (Santana, 1996).

Ceramide has been implicated in the apoptotic response to several chemotherapeutic drugs. Daunorubicin induced apoptosis is preceded by ceramide generation via activation of ceramide synthase (Bose, 1995). Alternatively, daunorubicin-induced ceramide generation might result from neutral SMase activation (Jaffrezou, 1996).

**Ceramide and ROS generation**

Cytokines such as IL-1β and TNF-α are known to induce the production of reaction oxygen species (ROS), a class of highly diffusible and ubiquitous molecules, which have been suggested to act as second messengers (Tiku, 1990; Lo, 1995; Devary, 1991). ROS include species such as superoxide, hydrogen peroxide and hydroxyl radicals. Recent observations has shown that GSH (glutathione) depletion occurs upstream of N-SMase action and ceramide generation in TNF-α signaling in MCF-7 cells (Liu, 1997). Ceramide also has direct effect on isolated mitochondria, resulting in generation of ROS. The site of ceramide action was shown to be at respiratory complex III (Gudz, 1997; Garcia-Ruiz, 1997).

Apoptosis is a genetically regulated, cellular suicide mechanism that plays a crucial role in development and defense of homeostasis (Cryns, 1998). The biochemical pathways of apoptosis can be initiated by a variety of physiological stimuli and environmental stresses. Inhibition of apoptosis is implicated in autoimmune disorders and
cancer, while increased apoptosis is believed to be associated with degenerative diseases (Fisher, 1994; Thompson, 1995).

Ceramide has long been reported to play an important role as an intracellular mediator of apoptosis induced by inflammatory cytokines, environmental stresses and viral infections including TNFα, CD95/Fas/Apo-1, ionizing radiation, ultraviolet-C and HIV-1 infection (Jarvis, 1996; Hannun, 1996; Spiegel, 1996; Mathias, 1998). However, the exact mechanisms through which they mediate the pleiotropic activities of ceramide remain for the most part unknown. The sphingomyelin (SM) pathway is conserved from yeast to human. Lymphoblasts from Niemann-Pick patients, which have an inherited deficiency of acid sphingomyelinase activity, failed to respond to ionizing radiation with ceramide generation and subsequent induction of apoptosis. Both ceramide generation and apoptosis are restorable by retroviral transfer of human acid sphingomyelinase cDNA. Similarly, acid shingomyelinase knockout mice also expressed defects in radiation induced ceramide generation and apoptosis in vivo (Santana, 1996). Therefore, it appears that ceramide is an evolutionarily conserved signal, which is critical for apoptotic response.

In the context of immune functions, ceramide mediated apoptosis is a major factor in the induction of immunosuppression. Cytokine-, stress- and pathogen-induced apoptosis leads to suppression of lymphoproliferative immune response and cell mediated immunity. Zinc is a crucial nutritional component required for the normal development and maintenance of normal immune functions. Clinical and experimental studies have shown that zinc deficiency leads to the impairment of cell mediated immunity. In regards to the effect of zinc on apoptosis and immunosuppression, it has been observed that (i) zinc deficiency can induce apoptosis in immune cells (McCabe, 1993; Treves, 1994; Martin, 1991) (ii) zinc supplementation can prevent apoptosis (Thomas, 1991; matsushita, 1996) and (iii) intracellular pool of chelatable zinc plays a critical role in the induction of late phase of apoptosis by affecting the activation or activity of calcium- and magnesium-dependent endonucleases (Cohen, 1984).

The known antiapoptotic effect of zinc and the importance of ceramide as a critical mediator of apoptosis and immunosuppression led us to study the effect of zinc on the ceramide induced apoptosis in a human CD4⁺ T cell line (Jurkat E6-1) and peripheral
blood lymphocytes from healthy volunteers. Caspase 3 (CPP32/Yama/Apopain) is a well-documented effector caspase, which commits cells to the death program. Recent studies show that zinc is a potent inhibitor of caspase 3 activity in both a cell-free system consisting of purified PARP and recombinant caspase 3 (Perry, 1997) and geranylgeraniol (GGO) induced apoptosis in HL-60 cells (Aiuchi, 1998). Therefore, it raises the possibility that the prevention of apoptosis by zinc may be attributed to its effect on the early “regulation” phase of apoptotic signaling rather than the late “execution” phase. In the current study, we demonstrate that zinc at 100-1000µM completely inhibited ceramide induced DNA fragmentation and caspase 3 activity. The present report documents for the first time the antiapoptotic function of zinc against a critical mediator of inflammatory cytokine- and environmental stress-induced cell death and identifies its role in regulating the early or upstream apoptotic machinery.

As I have mentioned earlier, ceramide could relay its apoptotic signal through SAPK/JNK cascade, resulting in the activation of transcription factors such as AP-1 and NFκB. The expression of Fas-ligand has been shown to be regulated by those transcription factors. Hence we further investigate the effect of ceramide on Fas ligand expression. We found that the induction of apoptosis in Jurkat CD4+ T lymphocytes by ceramide may involve CD95/Fas Ligand upregulation and this upregulation may be caspase 3 dependent.

Considerable evidence suggests that apoptosis is the consequence of disrupting the balance of antiapoptotic and proapoptotic signaling within the cell (Yin, 1994; Knudson, 1997). Ceramide is primarily an inducer of apoptotic signals. Recently, the PI3-kinase/Akt pathway has emerged to be a critical anti-apoptotic pathway for cell survival. So the role of Akt kinase on ceramide induced apoptosis is also evaluated. Interestingly we found that ceramide strongly induce inactivation and even degradation of Akt kinase and this effect seems to be caspase 3 independent.
Experimental Procedure

Western Blot

Total cell extracts were prepared and 15µg of protein from each sample was separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membrane in a buffer containing 30mM Tris, 200mM glycine and 20% methanol. The membranes were blocked by incubation in 1XTBS (10mM Tris HCl (PH8.0), 150mM NaCl) with 5% dried milk for 1h at room temperature and then incubated at 4°C overnight with primary antibody in 5% milk/TBS. After three 20-min washes with 1XTBS, membranes were incubated with secondary antibody conjugated with horseradish peroxidase. The reaction was then visualized with the enhanced chemiluminescence detection system (ECL) as recommended by the manufacturer (Amersham International plc, Buckinghamshire, England).

DNA fragmentation ELISA Assay

DNA fragmentation was quantitated in the cytoplasmic extracts using the cell death ELISA kit manufactured by Boehringer Manheim (Indianapolis, IN) using a mixture of anti-histone-biotin labeled antibody and anti DNA peroxidase conjugated antibody as per the manufacturer’s instructions. DNA fragmentation was quantified photometrically as a function of nucleosomes released, using ABTS (9,2,2’-Azino-di[3-ethylbenzthiazolin-sulfonate]) as the substrate.

Trypan Blue dye exclusion

To measure viability, cells were stained with trypan blue dye and then the numbers of dead and living cells were counted by a previously described method (Mclowskey, 1998).
**DEVDase like activity assay**

To measure DEVDase like activity, cytoplasmic extracts were prepared and analyzed using the CASPASE 3 Cellular Activity Assay Kit PLUS (BIOMOL Research Laboratories, Inc. 5100 Campus Drive Plymouth Meeting, PA-19462).

**DNA Fragmentation Analysis by Gel Electrophoresis**

To measure DNA fragmentation qualitatively, cells were lysed and DNA isolated via a method that has been described earlier (42). Briefly, the cells were solubilized in a lysis buffer (10mM Tris pH 8.0, 100mM NaCl, 25mM EDTA, 0.5% SDS and 400mg/ml Proteinase K and incubated overnight at 37°C. The samples were then extracted three times with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) followed by a chloroform extraction. The DNA was precipitated in the presence of 0.3M NaOAc (pH 5.2) and 10mM MgCl₂ with three volumes of EtOH. The precipitated DNA was resuspended in dH₂O, treated with RNase A (10 mg/ml) for one hour at 37°C, re-extracted and precipitated. The concentration of DNA was quantitated by UV absorbance at 260 nm. Ten micrograms of DNA were analyzed by gel electrophoresis on a 1.6 % agarose gel, and visualized by staining with 0.4 µg/ml Ethidium Bromide.

**Akt-kinase activity assay**

Stimulated cells were collected and lysed in lysis buffer (PBS pH 7.4, 1% NP-40, 200 µM sodium orthovanadate, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin, 4 µg/ml benzamidine, 50 mM potassium fluoride, 1mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1µM okadaic acid) and centrifuged at 15,000g for 10 min at 4 °C. Supernatants were incubated for 2 hr at 4 °C with 10-20 µg of goat anti-PKB antibody conjugated protein G-agarose. Immunoprecipitates were washed in lysis buffer and three times in assay buffer (20 mM MOPS pH 7, 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM sodium orthovanadate, 1
mM dithiothreitol). The final agarose pellets were resuspended in assay buffer and incubated with Akt substrate in assay buffer. The reaction is started by adding 10 µCi $[^{32}\text{P}]$-ATP diluted with 0.5 mM cold ATP and the samples were incubated for 15 min at 37°C. Equal amount of supernatant was transferred to Pierce Phosphocellulose Unit and washed 3 times by Pierce binding buffer. The membrane portion of the Unit was counted.

**MTT assay**

Cell survival was measured using the MTT (3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) assay as described by Hansen et al. (113). Briefly, the cells were seeded onto 96-well culture plates at a density of 20,000 cells/100 µl medium per well. After treatment of the cells, 25 µl of a 5 mg/ml stock solution of MTT was added to each well, and incubated at 37°C for 2 hrs to allow cells to take up the dye and convert it to the formazan derivative. The formazan was solubilized by the addition of 100 µl of lysis buffer (20% SDS in 50% DMF, pH 4.7), and optical density was measured after a 24 hrs incubation at 37°C using a Dyna Tec-700 plate reader.
Results

Ceramide Induces Caspase 3 Activation and DNA Fragmentation

Since ceramide is an important mediator of cytokine and stress induced apoptosis, we investigated the effect of exogenous cell-permeable short chain ceramide in Jurkat T cell line. Cells were exposed to increasing concentrations of C2 ceramide (1, 10, 30, 50µM) for 5h at 37°C. Then, total chromosomal DNA was isolated and analyzed by agarose gel electrophoresis. Up to 10µM, C2 ceramide did not induce DNA fragmentation (Fig. 10, lane 2 and 3). However, ceramide at or greater than 30µM consistently and significantly induced DNA fragmentation in a dose-dependent pattern (Fig. 10, lane 4 and 5). The cytosolic caspase 3 (cpp32/YAMA/apopain) becomes activated in apoptosis in different cell types (Kyriakis, 1994; Kyriakis, 1995). Hence, we further analyzed the effect of ceramide on the induction of caspase 3 activity. The functional activity of caspase 3 was measured by its ability to hydrolyze a specific fluorogenic substrate, Ac-DEVD-AMC. Similar to the DNA fragmentation data, this experiment showed that C2 ceramide at or greater than 30µM dose-dependently induced caspase 3 activity in Jurkat cells (Fig. 11). The above results confirm that ceramide-induced apoptosis involves caspase 3 activation and subsequent DNA fragmentation.

Zinc Inhibits Ceramide-Induced DNA Fragmentation

To investigate the effect of zinc on ceramide-induced DNA fragmentation, cells were either treated with 30µM C2 ceramide alone or pretreated with different concentrations of zinc for 1h followed by the treatment of 30µM C2 ceramide for 5h. At the high pharmacological concentrations, namely 500 and 1000µM, zinc was able to completely block ceramide-induced DNA fragmentation (Fig. 12, Lane 2,3,4). At low pharmacological concentrations (50 and 100µM), zinc consistently blocked ceramide-induced apoptosis at 100µM (Fig. 12, lane 6), but not at 50µM (Fig. 12, lane 5). Zinc by itself had no effect at all concentrations. These data suggest that pretreatment of zinc
Figure 10. Dose dependent ceramide-induced DNA fragmentation. Jurkat cells were treated with increasing concentrations of short-chain Ceramide for 5 hours. DNA fragmentation was detected by agarose gel electrophoresis.
Figure 11. Effect of ceramide on caspase 3 activity. Jurkat cells were treated with increasing concentrations of short-chain ceramide for 3hrs. The activity of functional caspase 3 was detected by its ability to hydrolyze a specific substrate.
Figure 12. Inhibition of ceramide induced DNA Fragmentation by zinc
Cells were treated with C2 ceramide for 5h with/without zinc pretreatment
for 1h. Total cell extracts were evaluated by agarose gel electrophoresis.
from 100 to 1000µM can effectively protect Jurkat T cells from C2 ceramide-induced DNA fragmentation which is a biochemical hallmark of apoptosis.

**Zinc does not Block Ceramide-Induced Caspase 3 Processing**

Caspase 3 proteases are expressed in the intact cell as the form of 32-kDa zymogens. Exogenous C2 ceramide has been shown to induce the proteolytic cleavage of the 32-kD proform of the protease to two subunits of 20-kD and 12-kD respectively (Cano, 1995). The effect of zinc on caspase3 processing was examined by western blot analysis using an antibody specific to the 32-kD protein. C2 ceramide at 30µM was observed to induce significant caspase 3 activation, which resulted in a substantial loss of the 32-kD zymogen (Fig. 13, lane 2). Zinc at 100 and 250µM, which can effectively block ceramide-induced DNA fragmentation, had no effect on ceramide-induced caspase 3 processing (Fig. 13, lane 3 and 4). Therefore, it suggests that the inhibition of ceramide-induced DNA fragmentation by zinc is not due to the prevention of caspase 3 activation.

**Zinc Directly Inhibits Ceramide-Induced Caspase 3 Activity.**

Since zinc dose not block ceramide-induced caspase 3 processing, we further investigated its effect on caspase 3 function. Corresponding to the DNA fragmentation data, zinc at 50µM had minimal effect on ceramide-induced caspase 3 activity (Fig. 14, lane 3), but zinc at 100µM and higher can significantly inhibit activated caspase 3 function (Fig. 14, Lane 4). Considering the fact that zinc can directly inhibit caspase 3 activity in a cell-free system (Boucher, 1995), our results suggest that the antiapoptotic effect of zinc is due to its ability to inhibit directly the active caspase 3 function, but not caspase 3 activation.
Figure 13. Zinc has no effect on Ceramide-Induced Caspase 3 Processing. Jurkat cells were treated with C2 ceramide for 5h with/without zinc pretreatment for 1h. Total cell lysate was evaluated by western blot using a specific antibody to CPP32/caspase 3.
Figure 14. Zinc inhibits ceramide induces caspase 3 activity. Jurkat cells were pretreated with zinc followed by treatment with short-chain ceramide for 3hrs. The activity of functional caspase 3 was detected.
Zinc Protects Human Peripheral Blood Lymphocytes From Ceramide-Induced Cell Death

To exclude the possibility that the antiapoptotic effect of zinc is cell-line specific and to further investigate its clinical relevance, the effect of zinc on ceramide-induced cell death was studied in human peripheral blood lymphocytes (PBLs) from healthy donors. Cells were pretreated with 100µM zinc, followed by the treatment of 30µM C2 ceramide. Cell death was determined by the inability of dead cells to exclude trypan blue dye and DNA fragmentation was also quantitatively measured. Similar to the results from Jurkat cells, C2 ceramide at 30µM can significantly induce DNA fragmentation and cell death in PBLs, which were strongly attenuated by the pretreatment of zinc at 100µM (Fig. 15A, B). These data support the antiapoptotic effect of zinc on ceramide-induced apoptosis and also imply its potential value in clinical therapeutic intervention.

Ceramide Induces Fas Ligand up-regulation

Fas (APO-1, CD95) is a type I cell surface protein belonging to the tumor necrosis factor/nerve growth factor receptor family that transduces cell death signals upon binding to Fas ligand (FasL). Fas is expressed in a variety of cell types such as activated T cells and B cells, macrophages, and hepatocytes. FasL is a 40-kD type II membrane protein belonging to the tumor necrosis factor family of ligands. Binding of Fas ligand (FasL) or stimulation with agonistic antibodies leads to aggregation of the Fas receptor on the cell membrane and the recruitment of specific intracellular signaling molecules and further initiates cell apoptotic machinery. Since ceramide is also a strong inducer of apoptosis, in this study we investigated if there is any interaction or relation between ceramide and Fas/FasL system. Jurkat cells were treated with short-chain ceramide for 3h. Cell lysate was prepared and evaluated by western blot. Fas ligand protein level was detected with an anti-CD95-specific antibody. From Fig. 16, we found that in untreated cells there is a base-line level of Fas ligand expression. Short-chain ceramide at 30µM
Figure 15. Zinc blocks ceramide induces DNA fragmentation in human peripheral blood lymphocyte. PBLs from healthy donors were exposed to C2 ceramide with/without zinc pretreatment. (a) After the treatment of C2 for 5h, DNA fragmentation is detected by ELISA using a anti-histone Ab. (b) Cell death is evaluated by Trypan Blue exclusion 24h after ceramide treatment.
Figure 16. Ceramide induced Fas Ligand up-regulation. Jurkat cells were treated with short-chain ceramide for 3h. Cell lysate was separated by SDS-PAGE and CD95L was detected with an anti-CD95L-specific antibody.
strongly induced Fas ligand expression (Fig. 16, Lane 5), which correlates with the data of ceramide induced caspase 3 activation and DNA fragmentation.

**Inhibition of ceramide induced DNA fragmentation and Fas ligand upregulation by DEVD-cho.**

Recent studies have provided compelling evidence that a cascade of Asp-directed cysteine proteases, caspases, plays a pivotal role in the transduction of apoptotic signals. Among this group, caspase 3 is one of the most studied and is normally considered as a common executor in the effector phase of the death program induced by a variety of stimuli. Interestingly, several recent reports suggested that caspase 3 could also be involved as a regulator in the early or induction phase of apoptosis. In this study, we investigated the interaction of caspase 3 activation and FasL upregulation in the context of ceramide induced apoptosis in CD4⁺ Jurkat cells. To conduct this study, we used a peptide inhibitor, called Ac-DEVD-cho, which primarily inhibits caspase 3 activity. In Fig. 17, pretreatment of Jurkat cells with DEVD-cho completely protects cell from ceramide induced DNA fragmentation. Then we checked what happened on the protein level of FasL. Again, ceramide at 30µM strongly induced FasL expression (Fig. 18, Lane 5). Noticeably, pretreatment of 100µM DEVD-cho for 1h significantly blocked ceramide induced CD95L upregulation (Fig. 18, Lane6). This result supported that ceramide induced FasL upregulation may be caspase 3 dependent.

**Inhibition of Protein Kinase B/Akt by ceramide**

Generation of the membrane sphingolipid ceramide constitutes an important signaling event activated by cellular stresses. Ceramide is an important second messenger in relaying apoptotic signals. As we have shown above, ceramide was able to induce caspase 3 activity and also potentiate the proapoptotic Fas/FasL pathway by upregulating the expression of CD95 ligand. On the other hand, ceramide may also augment the apoptotic effect by inactivation of anti-apoptotic pathways. Protein kinase B has recently
Figure 17. Ceramide-induced DNA fragmentation was inhibited by DEVD-cho. Cells were treated with increasing concentrations of ceramide for 6h with/without pretreatment of Ac-DEVD-cho for 1h. DNA fragmentation is evaluated by cell death ELISA.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ceramide Concentration</th>
<th>Protein Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2 5µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2 10µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2 20µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-2 30µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEVD+ C-2 30µM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 18. Ceramide-induced CD95L upregulation is partially blocked by DEVD-cho. Jurkat cells were treated with increasing concentration of ceramide with/without pretreatment with DEVD-cho. Protein level of FasL is evaluated by western blot.
emerged as a critical survival factor with its targets including Bad, caspase 9 and the Forkhead family of transcription factors. By knowing all the facts above, we are inspired to study the possible relationship between ceramide and PI3K/Akt pathway. First, we pretreated cells with PI3K-specific inhibitor LY294002, followed by the treatment of C2 ceramide. We found that inhibition of PI3K by LY294002 sensitized Jurkat cells to C2 ceramide induced cell death (Fig. 19). Next, we treated cells with C2 ceramide at 30µM and measured the Akt kinase activity in the cell lysate. The Akt kinase activity in ceramide treated cells was greatly reduced (Fig. 21, lane8, Page 101). This could be considered as a novel mechanism for ceramide to conduct its proapoptotic effect. It is not clear whether ceramide can directly inhibit Akt activity or do so through interacting with another molecule (i.e. PI3K).

**Inhibition of Akt by ceramide may be caspase 3 independent**

Since caspase 3 was involved in ceramide induced FasL upregulation in CD4+ Jurkat T lymphocytes, the next question we asked is whether caspase 3 has anything to do with ceramide induced Akt inactivation. Jurkat cells were treated with C2 ceramide with or without the pretreatment of the peptide inhibitor DEVD-cho. Then total cell lysate was prepared and evaluated by western blot using antibodies specifically recognizing phospho-Akt and unphosphorylated Akt. From Fig. 20, we can see that ceramide treatment completely wiped out phospho-Akt and DEVD-cho pretreatment was unable to block this effect. This result suggests that ceramide induced Akt kinase inactivation may take place in the early phase of apoptosis upstream of caspase 3 activation.
Figure 19. LY sensitized Jurkat cells to C2 ceramide induced cell death. Jurkat E6-1 cells were seeded on 96-well plate with the treatment of increasing concentrations of C2 ceramide (0, 10, 20, 30µM) with the presence of different concentrations of LY 294002 (0, 10, 15, 25, 50µM). Cell viability was measured by MTT assay.
Figure 20. Inhibition of Akt/PKB by ceramide may be caspase 3 independent. Cells were treated with ceramide with/without DEVD-cho preincubation Total cell lysates were evaluated by western blot.
Figure 21. Ceramide, LY294002 and Wortmannin dose-dependently attenuated the activity of Akt/PKB. Jurkat E6-1 cells were treated with different concentrations of LY294002, Wortmannin and C2 ceramide. Cells were harvested and prepared for the measurement of Akt kinase activity.
Discussion

It is well known that the apoptosis program is constitutively expressed in most if not all cells. Apoptosis can be initiated by a variety of physiological and environmental stimuli, which are able to trigger one of several distinct apoptotic pathways (Adriana, 1997). The specific signaling pathway activated depends on different cell types and especially the subcellular factors or targets the stimuli would specifically turned on. The different upstream signaling pathways seem to share or converge downstream to a common final execution mechanism to disposal the dying cells. Dr. Horvitz and co-workers defined the Ced-3 gene of the nematode Caenorhabditis elegans and its human analog, the IL-1β converting enzyme (ICE) family of proteases as the principle executors or effectors to carry out the disposal of the apoptotic cells (Yuan, 1993). The ICE/Ced-3 proteases are synthesized as zymogens and are activated by either autocatalysis, cleavage by other ICE/Ced-3 proteases or the serine protease granzyme B. During apoptosis, ICE/Ced-3 proteases are responsible for the orderly progression of the disassembly of cellular organelles. Several targets of ICE/Ced-3 proteases have been identified. The nuclear enzyme, poly-ADP-ribose-polymerase (PARP), involves in DNA damage repair, and is cleaved by CPP32/Caspase3 (Lazebnik, 1994). Other targets of caspase 3 include topoisomerase, the U1-ribonuclearprotein, the nuclear protein lamin, the cytoplasmic protein kinase Cδ, and the membrane protein fodrin, to list a few (Fraser, 1996).

Immense amount of work has been done to clarify mechanisms by which extracellular signals activate the ICE/Ced-3 proteases to conduct apoptosis. The apoptotic function of cytokine receptors, such as Fas/Apo-1/CD95 were reported to link with the ICE/Ced-3 effector caspase via a receptor-associated ‘death domain’ adaptor protein system. While formation of the death domain adaptor protein complex seems to be independent of the sphingolipid secondary messanger ceramide, emerging data suggest that its ability to confer apoptosis is dependent on coordinated signaling via ceramide. Deletions of the death domain region of TNF receptor (Wiegmann, 1994) and CD95/Fas (Cifone, 1995), as well as overexpression of dominant negative FADD/MORT1 blocked ligand-induced ceramide generation and apoptosis. Further, another report suggested that treatment with ceramide analogs can bypass the antiapoptotic effect of dominant negative
FADD/MORT1 and restore apoptosis (Chinnaiyan, 1996). In the current study, we confirmed that cell-permeable short chain C2 ceramide is a strong inducer of caspase 3 activity (Fig. 11) and subsequent DNA fragmentation (Fig. 10).

The influence of Zn\(^{2+}\) on apoptosis is a well-known phenomenon. In both *in vitro* and *in vivo* studies, Zn\(^{2+}\) supplementation prevents apoptosis induced by a variety of agents (Thomas, 1991). Cells grown under the condition of Zn\(^{2+}\) deficiency will undergo spontaneous apoptosis (McCabe, 1993). Traditionally the inhibitory effect of Zn\(^{2+}\) on apoptosis is attributed to its inhibition of a Ca\(^{2+}\) - and Mg\(^{2+}\) - dependent endonuclease, which is responsible of selectively cutting nuclear DNA and causes DNA fragmentation. However, there has always been unsolved doubts about this. Concentrations of Zn\(^{2+}\) used for inhibition of the purified endonuclease were in millimolar range (Gaido, 1991), which seems unachievable physiologically. Since endonucleases function primarily during the “execution” rather than “regulation” phase of apoptosis, its inhibition may not be enough to rescue cell from apoptosis. Recent studies suggest that Zn\(^{2+}\) may interact with some other apoptotic factor, which is involved in the earlier stage of apoptotic machinery. In a cell-free system consisting of purified PARP as a substrate and an apoptotic extract or recombinant caspase 3 as the PARP protease, Zn\(^{2+}\) inhibited PARP proteolysis in the low micromolar range (Perry, 1997). This raises the possibility that caspase 3 could serve as a novel target for zinc in the inhibition of apoptosis. In this study, we evaluated the effect of zinc on ceramide induced apoptosis in CD4\(^{+}\) Jurkat T lymphocytes. From Fig. 12, we clearly showed that Zn\(^{2+}\) from 100 to 1000 \(\mu\)M completely blocked ceramide induced apoptosis. More importantly, the ceramide induced caspase 3 activity is also abrogated by zinc pretreatment (Fig. 14). The pro-caspase 3 can still be processed (Fig. 13), however the activity of functional caspase 3 is being blocked (Fig. 14). The direct mechanism by which Zn\(^{2+}\) inhibits caspase 3 still awaits further investigation. However, studies on the crystal structure of ICE family members suggested that His-237 and Cys-285 are involved in catalysis and conserved in all the caspase family members (Walker, 1994; Kamens, 1995). Therefore, it is very possible that Zn\(^{2+}\) could inhibit caspase 3 activity by interacting with one or both of the conserved sites.

Caspase 3 has been classified mainly as an “executor” caspase. It is accurate in a way that it is the most important protease in dismantling subcellular organelles. However,
caspase 3 does have some other regulatory function in apoptotic signaling. As we showed here in Fig. 16, ceramide was able to upregulate the expression of a proapoptotic factor Fas ligand. This upregulation seems to be dependent on caspase 3 activity. Pretreatment of caspase 3 inhibitor DEVD-cho significantly attenuated this effect (Fig. 18). One important thing is that DEVD-cho can also interact with other caspases (i.e. caspase 7) to a lesser magnitude. Therefore we cannot rule out the possibility that other DEVDase may also participate in this effect. As mentioned before, Fas receptor ligation can induce endogenous ceramide accumulation and further activate caspase 3. So it seems to me that this upregulation of FasL by ceramide could be a positive feedback to amplify the apoptotic signaling cascade to help further carry out the death program.

Apoptosis is not simply controlled or conveyed by only proapoptotic signaling pathways. It is more likely under tight regulation by both proapoptotic and anti-apoptotic factors. PI3K/Akt has emerged as an important anti-apoptotic pathway. As shown in the next chapter, inhibition of this pathway without further stimuli was enough to induce apoptosis in Jurkat cells. In Fig. 21 Lane 8, the activity of Akt kinase was greatly reduced by ceramide treatment. In western blot analysis, the amount of active form of Akt, phospho-akt, was also significantly decreased (Fig. 20) and DEVD-cho pretreatment cannot restore Akt level (Fig. 20). This result put Akt kinase upstream of caspase 3 activation, which is expectable because caspase 9 and bad, a bcl-2 family member, are well-identified targets of Akt kinase. The exact mechanism of ceramide induced Akt inactivation is still unclear. Some studies suggest that ceramide can inhibit PI3K activity further leading to the inactivation of Akt kinase (Zundel, 1998), while others speculated that ceramide could activate a phosphatase to inactivate Akt by dephosphorylation (Zhou, 1998).

The role of sphingolipid ceramide in apoptosis has been the subject under intense investigation. Ceramide has been reported to play many important roles in cellular responses such as differentiation, proliferation or death (Hannun, 1996). Ceramide has been shown to affect almost every cell or tissue type examined, resulting in a wide range of effects in a cell-type specific manner. In HIV-infected individuals, lymphocyte apoptosis is considered as major cause for T cell depletion and disease progression. It has been reported that CD4\(^+\) and CD8\(^+\) T cell from HIV patients display large increase in
ceramide content and apoptosis as compared to normal people (Dimarzio, 1997; Cifone, 1997). Alternatively, treatment of HIV-infected patients with L-carnitine, which reduced acid SMase activity, significantly reduced ceramide levels in peripheral blood cells and partially restored CD4 count. In the treatment of cancer, suppression of anti-apoptotic signals might increase the sensitivity of target tissues to radiation and chemotherapy. Inhibition of ceramide signaling may provide protection from endotoxic shock, and attenuate ceramide-mediated effects in ischaemia/reperfusion injury, in the development of atherosclerosis and in insulin-resistant diabetes. Better understanding of the mechanisms by which ceramide regulates physiological and pathological events may yield opportunities and potentials to therapeutic intervention.
Chapter Four

The Role PI3K/Akt Pathway in Oxidative Stress Induced Apoptosis

Introduction

Apoptosis is the main cause of CD4+ T-lymphocyte depletion in acquired immune deficiency syndrome (AIDS). The tropism of human immunodeficiency virus (HIV) for CD4+ T cells and its cytopathic effect in vitro suggested that the pathogenesis of AIDS was strictly dependent on the direct virus-mediated destruction of CD4+ lymphocytes (Pantaleo, 1993). Also, the dynamic interaction between HIV replication and the destruction of CD4+ lymphocytes was further supported by the observation that when effective antiretroviral treatments was applied, levels of HIV replication declined sharply over a period of 1 to 2 weeks and the CD4+ lymphocyte count increased correspondingly (Ho, 1995; Wei, 1995; Piatak, 1993). The above reports appear to suggest that the direct infection of the cells with subsequent cytotoxicity is the most obvious explanation for the loss of CD4+ T cells. However, there are a few unexplained points. Particularly, it has been shown that during the clinically latent period only a small amount of CD4+ cells are detectably infected with the virus (Ho, 1989; Bagasra, 1992). The proportion of those cells that actively express HIV virus seems insufficient to account for the continued decrease in total T cell numbers. There is evidence that many other mechanisms, in addition to the direct cytotoxicity effect of the virus, may play important roles in CD4+ cell depletion, for example indirect killing, stem cell infection or failure, autoimmunity, and superantigen effects (De Simone, 1996). Later it became apparent that the loss of CD4+ T cells in HIV infection is associated with incomplete lymphocyte activation. It paradoxically results not in cell proliferation, but rather in cell death by a process that is physiologically crucial for the proper development and homeostasis of many tissues, and is called apoptosis (Gougeon, 1993). The in vitro activation of mature T cells from asymptomatic HIV-infected individuals by polyclonal activator has been shown to induce apoptosis in both CD4+ and CD8+ cells (Ameisen, 1995; Ameisen, 1994). In addition,
specific cell death of CD4+ lymphocytes is observed upon activation of T cells by major histocompatibility complex class-II-dependent bacterial superantigens. Even in the absence of activating agents a high background amount of apoptosis occurs in cultured lymphocytes of HIV-infected patients (Ameisen, 1995; Ameisen, 1994). Early investigation of the pathologic mechanism underlying T-cell death in HIV-infected persons showed that crosslinking of the CD4 molecule by anti-CD4 antibodies or by the HIV-1 envelope protein can trigger apoptosis in human CD4+ T cells (Banda, 1992; Cohen, 1992; Oyaizu, 1993; Laurent-Crawford, 1991; Tian, 1993). Abnormal levels of lymphocyte apoptosis have been shown in HIV-infected patients, and this involves not only CD4+ cells but also CD8+ cells (Gougeon, 1993, Carbonari, 1994). Interestingly, both CD4+ and CD8+ cells from HIV-infected long-term survivors who remain clinically healthy and immunologically normal over an extended period of time, have a low propensity to undergo apoptosis with respect to intermediate or advanced progressors. These latter observations argue strongly in favor of the hypothesis that apoptotic mechanisms contribute to the pathogenesis and progression of HIV infection. However, the progression of HIV infection is markedly characterized by the progressive depletion of CD4+ cells, whereas CD8+ lymphocytes are preserved to a substantial level (Pantaleo, 1993). A possible explanation could be that even though all lymphocyte subsets undergo progressive apoptosis, the renewal of CD4+ cells is selectively impaired in such a situation (Bonyadhi, 1993). The preservation of CD8+ cell counts during the course of HIV infection has been recently regarded as a central mechanism to maintain a constant level of circulating T cells without regard to the phenotype (CD4 or CD8). This is called blind homeostasis (Margolick, 1993; Aldeman, 1993). The net preferential replacement of CD4+ cells by CD8+ cells would eventually lead to low levels of CD4+ lymphocytes and subsequent immune failure. The failure of blind T cell homeostasis, triggered by the decline of CD8+ lymphocytes through apoptotic cell death, has been shown to be a precipitating event in the progression to AIDS (Margolick, 1995). Interestingly, in HIV-infected lymph nodes apoptosis occurs in bystander cells and not in the productively infected cells themselves, even though there is a correlation between the amount of apoptosis and productive infection (Finkel, 1995). These might suggest that while uninfected cells are killed during HIV infection, productively infected cells are relatively
resistant to apoptosis. HIV may not kill the cell it infects but rather use it to produce new viruses continuously. Taken together, these observations appear to support the view that in HIV infection the predominant mechanism of lymphocyte depletion is indirect and through the induction of apoptosis.

Several mechanisms may contribute to the induction of apoptosis in HIV infection, including HIV envelope proteins, impaired accessory cell function, superantigens and so on. Many of the chemical and physical treatments capable of inducing apoptosis are known to evoke oxidative stress. For example, both ionizing and ultra-violet radiation are capable of inducing apoptosis, and both generate reactive oxygen species (ROS). Recently another oxidant, nitric oxide (NO) has been implicated as an inducer of apoptosis in macrophages and monocytes (Albina, 1993). Having a single unpaired electron, NO is in itself considered to be a free radical and it also reacts with molecular O₂ to form O₂ and H₂O₂ (Stamler, 1992). Some apoptosis inducing agents are not free radicals themselves, but may elicit ROS formation. Doxorubicin, cisplatin and ether-linked lipids are anti-neoplastic agents, which induce both apoptosis and oxidative damage in sensitive cells (Benchekroun, 1993; Diomede, 1993; Wagner, 1993; Takagi, 1974). Alternatively, oxidative stress can also be induced by decreasing the ability of a cell to scavenge or detoxify ROS. For example, drugs such as buthionine sulfoxamine deplete intracellular stores of glutathione (GSH), thereby rendering cells more susceptible to oxidative stress-induced apoptosis (Zhong, 1993). Evidence that apoptosis can be induced by oxidative stress is provided by observations in which mediators of apoptosis either induced ROS, or were inhibitable by the treatment of antioxidants. Tumor necrosis α (TNFα) is one example of such a mediator. Stimulation of the TNF receptor results in a rapid increase in the level of intracellular ROS (Larrick, 1990; Matthews, 1987) and in various cell types, TNF-mediated apoptosis can be inhibited either by thioredoxin, an intracellular thiol reductant and free radical scavenger, or N-acetylcysteine (NAC), a thiol antioxidant and GSH precursor (Droge, 1992). Moreover, cellular sensitivity or resistance to TNF is correlated with decreased or increased levels of superoxide dismutase (SOD), respectively (Hirose, 1993). For example, SOD-deficient T cells were known to be more susceptible to killing by TNF, ionizing radiation and hyperthermia (Wong, 1991). Hence, the fate of TNF-sensitive cells
may depend on the extent of ROS formation and the cell’s ability to buffer the oxidative stress. Several reports suggest the participation of ROS in HIV infection (Harakeh 1990; Baruchel, 1992). Virus-infected cells display very low levels of antioxidant defense, such as SOD, vitamin E, selenium and glutathione (Baruchel, 1992; Favier, 1994; Buhl, 1989). On the other side, patients undergoing HIV infection display high serum levels of xanthine oxidase (XOD) (Miesel, 1993) and lipid peroxidation, both markers for oxidative stress conditions. Furthermore, opportunistic infection from Mycoplasma can accelerate AIDS progression by induction of oxidative stress (Blanchard, 1994).

Zidovudine or azidothymidine (AZT) is an antiviral drug that stops virus replication by interfering the viral reverse transcriptase and inhibit DNA synthesis (Gilman, 1990). Interestingly, antioxidants such as N-acetyl cysteine (NAC) and dithiocarbamate enhanced the therapeutic effect of zidovudine (Roederer, 1990; Roederer, 1992; Resinger, 1990). The mechanism of action may be related to the ability of NAC to restore GSH levels and reduce the cytotoxic effect of zidovudine. At a molecular level, the transcription of HIV genes is directed by sequences called long terminal repeats (LTR), located at the extremities of the viral genome. These elements provide binding sites for viral (i.e. tat) and cellular transcription factors (i.e. NFκB). Interestingly, NFκB can be strongly activated by ROS and cytokines (i.e. TNFα). This process can be blocked by the treatment of antioxidants such as vitamin E, NAC and α-lipoic acid (Thanos, 1995; Suzuki, 1992; Suzuki, 1993). HIV-1 acts on the cellular redox system to stimulate its own replication and to generate oxidants (Westendorp, 1995). Furthermore, the low levels of endogenous antioxidant (Buttke, 1994) and the enhanced production of macrophage-derived neopterin (Fust, 1995) commonly found in HIV-infected patients also contribute to this imbalance and render cells more susceptible to injury from ROS. It has also been reported that HIV Tat may induce lymphocyte apoptosis through down-regulating the expression of bcl-2 (De Rossi, 1994). Under this condition the transcription factor NFκB, a key element in TNFα gene expression, is up-regulated (Schulze-Osthoff, 1993; Schreck, 1991). As a consequence, HIV-infected patients have increased production of TNFα (Dezube, 1992), which further induces oxidative stress (Hennet, 1993) and stimulates the HIV gene expression via the activation of NFκB (Hohmann, 1990; Folks, 1989; Mellors, 1991). In addition, TNFα can bind its transmembrane receptors and ultimately leads to
apoptosis (Kolesnick, 1994; Schutze, 1994; Pushkareva, 1995). Hence the shift to oxidant status may enhance the replication of HIV directly and through the induction of TNFα.

The possible interactions between HIV infection, oxidative stress and TNFα in HIV pathogenesis may be depicted as following. During the initial stage, HIV infects cells of the immune system, such as macrophages and CD4$^+$ T cells. Then it activates the expression of cellular genes including tnf gene. TNFα induces NFκB translocation, which acts as a transcription factor to stimulate HIV replication and HIV-Tat production. Tat induces oxidative stress which further enhances the activity of TNFα, and CD4$^+$ cells incapable of repairing the ensuing oxidative stress are primed to undergo apoptosis. A vicious cycle may lead to a steady increase of TNF and Tat and a decrease in antioxidants (Famularo, 1997).

Regulation of ROS levels in mitochondria and or cytoplasm in mammalian cells might be mediated by the bcl-2 family gene products (Hockenbery, 1990). Expression of the bcl-2 protein prevents the induction of apoptosis by a variety of oxidative stress, including ionizing radiation, heat shock or GSH depletion (Zhong, 1993; Hockenbery, 1990). It has been suggested that the bcl-2 gene product inhibits apoptosis by interacting with mitochondria SOD (Itoh, 1993). However, bcl-2 expression can also block apoptosis in cells lacking mitochondria (Jacobson, 1993), implying a broader functional distribution of the protein. It has recently been shown that various antioxidants (i.e. NAC) can substitute for bcl-2 expression in factor-deprived cells from undergoing apoptosis (Hockenbery, 1993). From the above evidence, we can see that bcl-2 protein plays a crucial role in regulation of apoptotic machinery and could be an indicator of the ability of a cell to cope with oxidative stress.

Intracellular GSH level is an important indicator of the redox buffering capacity of the cell. A loss of GSH makes cell more susceptible to oxidative damage. Intracellular GSH levels can be artificially lowered in several ways, including withdrawing cysteine from the cell culture medium, binding free sulphydryl groups with diethyl maleate or inhibiting the glutathione synthase by buthionine sulfoximine (BSO). Normally, lowering GSH level alone is insufficient to induce apoptosis. However cells with very low GSH levels may be very sensitive to additional stimuli.
The PI3-kinase/Akt pathway has recently emerged as an important regulator of apoptotic pathways. Bcl-2 family gene product BAD is one of the well-identified targets of Akt kinase. PI3-kinase is a family of enzymes capable of phosphorylating phosphoinositides in their 3’ position. PI3-kinase consists of a regulatory subunit (p85) that binds to an activated growth factor/cytokine receptor and undergoes phosphorylation, which result in the activation of its catalytic subunit (P110). The product of PI3-kinase, PI (3,4,5) P3 or its metabolite PI (3,4) P2, bind to the pleckstain homology (PH) domain of Akt/PKB, which causes a conformational change allowing PDK1 (phosphoinositide-dependent kinase-1) and PDK2 kinases to phosphorylate and activate the Akt/PKB kinase, a serine/threonine protein kinase previously implicated in apoptosis suppression (Klippel, 1997; Stokoe, 1997; Franke, 1997). A proapoptotic member of Bcl-2 family, BAD, was identified as a target of Akt kinase (Mok, 1999). BAD conduct its proapoptotic effect by interacting with Bcl-xl and Bcl-2. Akt kinase was able to phosphorylate BAD. Phosphorylation of BAD leads to its sequestration by an isoform of 14-3-3 protein in the cytosol. Bad bound to 14-3-3 is inactive since it can no longer bind Bcl-2, resulting in enhanced cell survival (Zha, 1996; Hsu, 1997). Recently, Dr. Reed and coworker identified procaspase 9 as another possible target of Akt kinase (Cardone, 1998), suggesting that Akt kinase can serve as a common antiapoptotic effector and regulate the apoptotic pathway at different levels. Since bcl-2 has been shown to play an important role in oxidative stress induced apoptosis and Akt kinase is a key regulator of bcl-2 levels in cell, it is very worthwhile to investigate the possible role of PI3-kinase/Akt pathway in oxidative stress induced cell death.
**Experimental Procedure**

**Western Blot**

Total cell extracts were prepared and 15µg of protein from each sample was separated by 10% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membrane in a buffer containing 30mM Tris, 200mM glycine and 20% methanol. The membranes were blocked by incubation in 1XTBS (10mM Tris HCl (PH8.0), 150mM NaCl) with 5% dried milk for 1h at room temperature and then incubated at 4°C overnight with primary antibody in 5% milk/TBS. After three 20-min washes with 1XTBS, membranes were incubated with secondary antibody conjugated with horseradish peroxidase. The reaction was then visualized with the enhanced chemiluminescence detection system (ECL) as recommended by the manufacturer (Amersham International plc, Buckinghamshire, England).

**DNA fragmentation ELISA Assay**

DNA fragmentation was quantitated in the cytoplasmic extracts using the cell death ELISA kit manufactured by Boehringer Manheim (Indianapolis, IN) using a mixture of anti-histone-biotin labeled antibody and anti DNA peroxidase conjugated antibody as per the manufacturer’s instructions. DNA fragmentation was quantified photometrically as a function of nucleosomes released, using ABTS (9,2,2’-Azino-di[3-ethylbenzthiazolin-sulfonate]) as the substrate.

**Trypan Blue dye exclusion**

To measure viability, cells were stained with trypan blue dye and then the numbers of dead and living cells were counted by a previously described method (Mclowskey, 1998).
DEVDase like activity assay

To measure DEVDase like activity, cytoplasmic extracts were prepared and analyzed using the CASPASE 3 Cellular Activity Assay Kit PLUS (BIOMOL Research Laboratories, Inc. 5100 Campus Drive Plymouth Meeting, PA-19462).

DNA Fragmentation Analysis by Gel Electrophoresis

To measure DNA fragmentation qualitatively, cells were lysed and DNA isolated via a method that has been described earlier (42). Briefly, the cells were solubilized in a lysis buffer (10mM Tris pH 8.0, 100mM NaCl, 25mM EDTA, 0.5% SDS and 400mg/ml Proteinase K and incubated overnight at 37°C. The samples were then extracted three times with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) followed by a chloroform extraction. The DNA was precipitated in the presence of 0.3M NaOAc (pH 5.2) and 10mM MgCl\(_2\) with three volumes of EtOH. The precipitated DNA was resuspended in dH\(_2\)O, treated with RNAse A (10 mg/ml) for one hour at 37°C, re-extracted and precipitated. The concentration of DNA was quantitated by UV absorbance at 260 nm. Ten micrograms of DNA were analyzed by gel electrophoresis on a 1.6% agarose gel, and visualized by staining with 0.4 µg/ml Ethidium Bromide.

Akt-kinase activity assay

Stimulated cells were collected and lysed in lysis buffer (PBS pH 7.4, 1% NP-40, 200 µM sodium orthovanadate, 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin, 4 µg/ml benzamidine, 50 mM potassium fluoride, 1mM β-glycerophosphate, 5 mM sodium pyrophosphate, 1µM okadaic acid) and centrifuged at 15,000g for 10 min at 4 °C. Supernatants were incubated for 2 hr at 4 °C with 10-20 µg of goat anti-PKB antibody conjugated protein G-agarose. Immunoprecipitates were washed in lysis buffer and three times in assay buffer (20 mM MOPS pH 7, 25 mM β-glycerophosphate, 5 mM EDTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol). The final agarose pellets were resuspended in assay buffer and
incubated with Akt substrate in assay buffer. The reaction is started by adding 10 µ Ci $[^{32}\text{P}]$-ATP diluted with 0.5 mM cold ATP and the samples were incubated for 15 min at 37°C. Equal amount of supernatant was transferred to Pierce Phosphocellulose Unit and washed 3 times by Pierce binding buffer. The membrane portion of the Unit was counted.

**MTT assay**

Cell survival was measured using the MTT (3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide) assay as described by Hansen et al. (113). Briefly, the cells were seeded onto 96-well culture plates at a density of 20,000 cells/100µl medium per well. After treatment of the cells, 25 µl of a 5 mg/ml stock solution of MTT was added to each well, and incubated at 37 °C for 2 hrs to allow cells to take up the dye and convert it to the formazan derivative. The formazan was solubilized by the addition of 100 µl of lysis buffer (20% SDS in 50 % DMF, pH 4.7), and optical density was measured after a 24 hrs incubation at 37 °C using a Dyna Tec-700 plate reader.

**Measurement of GSH level**

Cells are seeded at $2 \times 10^5$ in 6-cm Petri dishes in 4 ml of complete medium. Two days later, cells are treated with the desired agents as described above. Treated cells are detached by trypsinization, washed (three times) with ice-cold PBS, and solubilized in 150 µl of water. 5-Sulfosalicylic acid is added to a final concentration of 2%, and the supernatant is separated from the acid-precipitated proteins by centrifugation. GSH content in the supernatant was determined by the Griffith modification of the Tietze's enzymatic procedure as described (Liu, 1997). Protein content is determined by the dye binding assay using bovine serum albumin as standard.

**Alamar Blue Assay**

Alamar Blue is a cell growth and cytotoxicity indicator dye and considered to be good replacement for MTT. Plate cells on 96 well tissue plate in final volume of 1 ml.
When treatment time is done. Add 100µl Alamar Blue and incubate for 2h. Read it on any microplate spectrophotometer or fluoromater (Fluorescence with excitation at 560nm and emission at 590nm; Absorbance with excitation at 570nm and absorbance at 600nm)

**LDH Release Assay**

Lactate Dehydrogenase catalyzes the interconversion of lactate and pyruvate. After treatment, collect culture media. Add 0.05ml sample and 1ml LD-L reagent. Mix well and read the absorbance at 340nm using a spectrophotometer every minute up to 5 times.
Results

**Inhibition of PI3-kinase/Akt-kinase pathway induces cell death in CD4^+ T cell line**

PI3–kinase/Akt have been shown to be an important anti-apoptotic pathway. Overexpression of constitutively activated forms of PI3–kinase or Akt-kinase result in decreased apoptosis in response to serum/growth factor deprivation, UV-B irradiation, or loss of matrix attachment (Kauffmann-Zeh, 1997; Kennedy, 1997; Khwaja, 1997). First, we investigated whether inhibition of PI3–kinase/Akt pathway without any further stimuli is sufficient for CD4^+ T cells to undergo apoptosis. We treated Jurkat cells with two structurally unrelated specific PI3–kinase inhibitors, LY294002 (LY) and wortmannin (WT) respectively. After three hours, the activity of Akt kinase was evaluated. Cell lysate was immunoprecipitated with Akt antibody. Then substrate of Akt kinase was applied to the immunoprecipitates to evaluate the activity of Akt kinase. In Fig. 21, we can see that both LY and WT inhibit Akt kinase activity in a dose-dependent fashion. Cell survival was measured by MTT assay after twelve hours of treatment. We found that LY and WT dose-dependently induce cell death in Jurkat T cells (Fig. 22).

**Inhibition of PI3-kinase/Akt-kinase pathway sensitizes cells to undergo apoptosis.**

After examining the effect of PI3-kinase inhibitor on cell viability, we further studied its effect on apoptotic signaling. Jurkat cells were treated with increasing concentrations of LY294002 for 5h. Cytoplasmic extracts were isolated and assayed for (I) activity of caspase-3, which is a hallmark of apoptosis, using caspase-3 specific tetrapeptide substrate Ac-DEVD-AMC (7-amino-4-trifluoromethyl coumarin) as a fluorogenic substrate (II) protein level of caspase3 by western blot and (III) DNA fragmentation using an antihistone DNA ELISA kit. Data are normalized to untreated values. We found that inhibition of PI3-kinase/Akt-kinase pathway by LY294002 induces caspase-3 activity as documented by a dose-dependent increase in DEVD-ase activity (Fig. 23) and a decrease in the protein level of 32 kd procaspase 3 (Fig. 24). Also LY294002 elicited DNA fragmentation in a dose-dependent fashion (Fig. 25). These data
Figure 22. LY294002 and Wortmannin, two specific and structurally distinct PI3-kinase inhibitors dose-dependently induced cell death in CD4+ T cells. Jurkat cells were seeded on 96-well plate and treated with increasing concentrations of LY294002 (5, 10, 15, 25, 50, 100µM in A) and Wortmannin (0.1, 0.5, 1, 1.5, 2, 3 µM, in B) for 5 hrs. Cell viability was measured by MTT assay.
Figure 23. LY294002 dose-dependently induces caspase 3 activity. Cells were treated with increasing concentrations of LY 294002 (5, 10, 15, 25, 50 μM). Cells were harvested and cytoplasmic extracts were prepared for caspase3 activity assay.
Figure 24. LY294002 dose-dependently induces proteolysis of procaspase 3. Cells were treated with increasing concentrations of LY 294002 (5, 10, 15, 25, 50, 75, 100µM). Cells were harvested and total cell extracts were prepared for Western Blot analysis with antibody specific for procaspase 3.
Figure 25. LY294002 dose-dependently induced DNA fragmentation in Jurkat cells. Cells were treated with increasing concentrations of LY 294002 (5, 10, 15, 25, 50µM), and harvested. Cytoplasmic extracts were prepared and analyzed by Cell Death ELISA.
showed that inhibition of PI3-kinase/Akt-kinase pathway induces apoptotic signaling and eventually leads to apoptotic cell death.

**Oxidative stress induces apoptosis in CD4+ T cells.**

Many agents, which induce apoptosis, are either oxidants or stimulators of cellular oxidative metabolism. Conversely, many inhibitors of apoptosis have antioxidant activities or enhance cellular antioxidant defenses. Mammalian cells exist in a state of oxidative siege in which survival requires an appropriate balance of oxidants and antioxidants (Buttke, 1994). Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) is a well-known oxidant and has been shown to induce apoptosis (Lennon, 1991) and NF\kappa B activation (Schreck, 1991) in a variety of cell types. Therefore we chose H\textsubscript{2}O\textsubscript{2} as the stimulator to investigate the interaction of oxidative stress induced apoptosis and PI3-kinase/Akt-kinase pathway. Jurkat T lymphocytes were exposed to increasing concentrations of H\textsubscript{2}O\textsubscript{2} for 12h. Then cell viability was determined by trypan blue exclusion (Fig. 26) and alamar blue analysis (Fig. 27). We found that in the dose range from 100\mu M to 500\mu M, H\textsubscript{2}O\textsubscript{2} caused significant loss of cell viability in a dose-dependent pattern. Caspases are a novel class of cysteine proteases, which are activated during apoptosis by proteolytic processing at specific aspartate cleavage sites (Kim, 2000). Among more than 10 caspases identified so far, caspase-3, a member of DEVD-specific caspases (DEVDase), has received particular attention because it is commonly activated by a broad range of apoptotic stimuli. So in the next step, we evaluated the activation of caspase-3 in H\textsubscript{2}O\textsubscript{2} induced apoptosis in Jurkat T cells. Cells were treated with H\textsubscript{2}O\textsubscript{2} at increasing concentrations from 25 to 500\mu M for 6h. Cytoplasmic extracts were prepared and DEVDase activity was measured by its ability to hydrolyze a specific peptide substrate. We showed that H\textsubscript{2}O\textsubscript{2} at 100\mu M significantly induced caspase-3 activity and this effect was further augmented by higher concentrations (Fig. 28).
Figure 26. Oxidative stress induced cell death in CD4$^+$ T cells. Jurkat cells were treated with increasing concentrations of H$_2$O$_2$ for 12h. Cell viability was evaluated by Trypan Blue exclusion.
Figure 27. Oxidative stress induced cell death in CD4+ T cells. Jurkat cells were treated with increasing concentrations of H$_2$O$_2$ for 12h. Cell viability was evaluated by Alamar Blue Assay.
Figure 28. H$_2$O$_2$ dose-dependently induces caspase 3 activity. Cells were treated with increasing concentrations of H$_2$O$_2$. Cells were harvested and cytoplasmic extracts were prepared for caspase 3 activity assay.
**Hydrogen Peroxide induced apoptosis involves loss of Akt-kinase**

Akt-kinase is a key antiapoptotic regulator. Earlier we have shown that inhibition of PI3-kinase/Akt-kinase pathway without any other stimuli could lead to apoptosis in Jurkat T lymphocytes. Next we were curious to see what happened to Akt-kinase in H$_2$O$_2$ induced cell death. Jurkat cells were either untreated or treated with H$_2$O$_2$ at 100, 250 and 500µM respectively. Cells were collected at four different time points, namely 3h, 6h, 12h and 24h. Total cell lysates were prepared and evaluated by western blot (Fig. 29, 30). Two antibodies were used. One can specifically recognize phospho-Akt, the active and functional form of Akt-kinase. The other can react with both wild type Akt (unphosphorylated) and phospho-Akt. At 3h, we found that H$_2$O$_2$ at 100µM did not affect the level of phospho-Akt. However, H$_2$O$_2$ at 250 and 500µM cause significant loss of phospho-Akt in a dose-dependent pattern (Fig. 29). If comparing the levels of phospho-Akt with the same concentration of H$_2$O$_2$ treatment at different time points, we can clearly see that phospho-Akt further went away along with the prolonged treatment time (i.e. in Fig. 29, compare between Lane 2,6,10 and 14). Its level was almost undetectable after 6h with the concentration of H$_2$O$_2$ at 250 and 500µM respectively. As shown in Fig. 30, the level of total Akt (phospho-and wildtype) is roughly unchanged over the first 6 hours. The 12h and 24h treatments show a downregulation of total Akt level. Pretreatment of cells with DEVD-cho, a peptide inhibitor for caspase 3, was unable to rescue Akt inactivation (Fig. 31). We will further discuss these observations in the discussion section.

**N-acetyl-cysteine rescues CD4$^+$ T lymphocytes from hydrogen peroxide induced apoptosis while GSH depletion enhances it.**

GSH is the predominant thiol present in many cells, and its presence seems to be a prerequisite for protection against oxidative damage. We proposed that depletion of GSH may sensitize cells to oxidative stress induced apoptosis. We pretreated cells with 2.5mM BSO, which is known to lower the intracellular GSH level by inhibiting its biosynthesis.
Figure 29. Time course of oxidative stress induced phospho-Akt (P-Akt) downregulation. Cells were treated with increasing concentrations of H$_2$O$_2$. Total cell lysates were evaluated using antibody specific for P-Akt.
Lane Number   1                2               3                4                5                 6                7               8  
Time (hour)    3                3               3                3                 6                6                6               6  
$\text{H}_2\text{O}_2$ (µM) -               100           250             500             -               100            250          500

Lane Number    9                 10               11               12              13              14              15             16  
Time (hour)    12                12               12               12              24              24              24             24  
$\text{H}_2\text{O}_2$ (µM) -               100           250             500             -               100            250           500

Figure 30. Time course of oxidative stress induced Akt downregulation. Cells were treated with increasing concentrations of $\text{H}_2\text{O}_2$. Total cell lysates were evaluated using antibody recognizing both P-Akt and Akt.
Figure 31. H$_2$O$_2$ induced P-Akt downregulation may be caspase 3 independent. Cell were treated with H$_2$O$_2$ in the presence or absence of DEVD-cho. Total cell lysates were analyzed by western blot.
We showed that the GSH levels were significantly depleted after treating with BSO (Fig. 32). With or without BSO pretreatment for 12h, Jurkat cells were exposed to increasing concentrations of H$_2$O$_2$ from 25 to 500µM for another 12h. LDH release assay was performed to evaluate cell viability (Fig. 33). The LDH activity in the culture medium correlates with the extent of cell death. Without BSO pretreatment H$_2$O$_2$ induced moderate to substantial cell death at 250 and 500µM. There is no significant cell death observed at low dose range from 25 to 100µM. However, with BSO pretreatment, the effect of H$_2$O$_2$ induced cell death is greatly and synergistically enhanced. Even low dose H$_2$O$_2$ can induce substantial amount of cell death. NAC is a well-known antioxidant and is a precursor for GSH biosynthesis. To study its potential protective effect on H$_2$O$_2$ induced apoptosis, we exposed cells to increasing concentrations of H$_2$O$_2$ with or without the pretreatment of 2.5mM NAC for 6h (Fig. 34). Cell death was examined by LDH release assay. As expected, NAC pretreatment completely blocked H$_2$O$_2$ induced cell death. At the same time, cytoplasmic extracts were prepared for measurement of caspase-3 activity after 6h exposure to H$_2$O$_2$ at 100 and 250µM. In Fig. 35, NAC pretreatment significantly down-regulated H$_2$O$_2$ induced caspase-3 activation.

In summary, the above results demonstrate that oxidative stress can strongly induce caspase 3 activation and apoptosis in CD4$^+$ T lymphocytes. Depletion of intracellular GSH by BSO enhanced oxidative stress induced apoptosis, while antioxidant NAC suppress it. Akt kinase is an important antiapoptotic factor and indispensable for the survival of CD4$^+$ T lymphocytes. Inactivation and further degradation of Akt kinase is involved in oxidative stress induced cell death.
Figure 32. Depletion of GSH level by BSO. Cells were treated with BSO at 2.5 mM and harvested at several time points (O/N overnight). Intracellular GSH levels were measured by HPLC.
Figure 33. BSO sensitizes Jurkat cells to $\text{H}_2\text{O}_2$ induced cell death. Cells were treated with increasing concentrations of $\text{H}_2\text{O}_2$ for 24h with/without BSO pretreatment at 2.5mM for 12h. Cell death is evaluated by LDH release assay.
Figure 34. NAC rescues Jurkat cells from H$_2$O$_2$ induced cell death. Cells were treated with increasing concentrations of H$_2$O$_2$ for 24h with/without NAC pretreatment at 2.5 and 5.0mM for 12h. Cell death is evaluated by LDH release assay.
Figure 35. NAC inhibits H₂O₂ induced caspase 3 activity. Cells were treated with increasing concentrations of H₂O₂ for 6h with/without NAC pretreatment. Cells were harvested and cytoplasmic extracts were prepared for caspase3 activity assay.
Discussion

Human immunodeficiency virus (HIV) infection is considered to lead to full-blown acquired immunodeficiency syndrome (AIDS) through a progressive and complete loss immune competence in the infected host. Recent research has highlighted that HIV may trigger an active cell suicide process, termed programmed cell death or apoptosis, that contributes to the decline in lymphocyte count throughout the course of the infection. The most important difference between apoptosis and necrosis is of a functional nature. The active cell suicide process requires activation signals, signal transduction, and sometimes gene expression as well as RNA and protein synthesis in the dying cell. In general, the apoptotic process is associated with a breakdown of intracellular calcium homeostasis, cytoplasm condensation, disruption of cytoskeleton, DNA fragmentation, activation of endonucleases and proteases and phagocytosis (Bursch, 1990; Kroemer, 1995). Apoptosis presents morphological characteristics differing from cell necrosis, which is capable of inducing inflammatory responses. It has been suggested that the disruption of the balance between cell proliferation and cell death could be implicated in several pathological disorders. For example, cancer could be considered as a result of excess of cell proliferation or inhibition of cell death, while degenerative diseases may result from excess of cell death or activation of apoptosis. Taking this assumption into account, AIDS can be considered as a disease associated with excessive death of CD4+ T cells (Weiss, 1993). Various agents appear to be able to trigger apoptosis in CD4+ T lymphocytes, including viral proteins (i.e. gp120, Tat), inappropriate secretion of inflammatory cytokines (i.e. TNFα) and toxins produced by opportunistic microorganisms. Since oxidative stress can also induce apoptosis, it has been suggested that such a mechanism could participate in CD4+ T cell apoptosis observed in AIDS (Harakeh, 1990). This correlates strongly with the observation that AIDS patients present low levels of antioxidants most likely due to inappropriate nutrition, alcohol and drug consumption, and digestive problems associated with the disease. Furthermore, coadministration of the antiviral drug with antioxidants increases its therapeutic potential (Roederer, 1990; Roederer, 1992; Reisinger, 1990; Hersh, 1991). The above facts suggested that oxidative stress induced apoptosis in CD4+ T cells could play a critical
role in HIV pathogenesis, however, the underlying mechanisms still remain to be elucidated. Protein kinase B (PKB)/Akt has been increasingly linked to enhanced cell survival. PKB is activated downstream of phosphoinositide 3-kinase (PI3K) in a wide range of signaling cascades including those for growth and survival factors (Marte, 1997). In the present study, we evaluated the possible involvement of PI3K/Akt pathway in oxidative stress induced apoptosis. First, we showed that PI3K/Akt pathway is indispensable for the survival of CD4\(^+\) T cell line. Jurkat cells were treated with two structurally distinct PI3-kinase inhibitors, LY294002 and Wortmannin, both of which can effectively and specifically inhibit PI3-kinase activity and consequently block the downstream Akt kinase activity (Fig. 21). MTT assay (Fig. 22) and DNA fragmentation ELISA (Fig. 25) showed decreased cell viability and increased DNA fragmentation in response to the two inhibitors in a dose-dependent fashion. Caspase 3 activation, one of the hallmarks of programmed cell death, was enhanced in Jurkat cells treated with either PI3-kinase inhibitors (Fig. 23, 24). In summary, this study suggests that PI3-kinase/Akt pathway is critical for the survival of CD4\(^+\) T cells. Then, we moved on to study the effect of oxidative stress on the survival of CD4\(^+\) T cells. Jurkat cells were treated with a known oxidant, hydrogen peroxide. As expected, H\(_2\)O\(_2\) induced cell death dose-dependently (Fig. 26, 27). Further, the characteristic apoptotic signal, caspase-3 activation, was evaluated and found greatly elevated in H\(_2\)O\(_2\) induced cell death (Fig. 28). This confirmed that apoptotic mechanism is actively involved in oxidative stress related cell death. One novel finding of this study was that multi-level anti-apoptotic effector PKB/Akt was down regulated in H\(_2\)O\(_2\) induced apoptosis. The Akt kinase was known to phosphorylate a proapoptotic bcl-2 family member bad and maintain the integrity of mitochondria. It has been shown to phosphorylate caspase-9 leading to a reduction in the protease activity of this caspase and blocking of the further relay of apoptotic signaling (Cardone, 1998). A recent study has also revealed that PKB can inhibit apoptosis by phosphorylating and inhibiting the Forkhead transcription factor FKHRL1 (Brunet, 1999), which is then sequestered in the cytoplasm in association with 14-3-3 proteins. This results in blocking FKHRL1 mediated transcription of death-inducing genes such as Fas ligand, with obvious implications for the apoptotic program. Clearly PKB is able to act at several points along the apoptotic cascade. This is unlikely to represent functional redundancy.
Since apoptosis is a multistep process, the multiple actions of PKB make it more efficient and effective on blocking the apoptotic machinery. Therefore, it is very natural to take out this anti-apoptotic force in order to commit the cell to death. Our result showed that in the early phase (0-6h after H₂O₂ treatment) of apoptosis only the phospho-Akt level (Fig. 29) was reduced while the total Akt level (Fig. 30) was roughly unchanged. However, in the late stage (12h after H₂O₂ treatment and later) the total amount of Akt kinase is also significantly dropped. Similar to the result from chapter 3, the inactivation of Akt seems caspase 3 independent (Fig. 31), again implicating that PI3K/Akt pathway primarily functions in the early stage of apoptosis. We can try to speculate that in the early stage of apoptosis in H₂O₂ treatment in Jurkat cells the PKB proteins were mostly inactivated or unphosphorylated to their inactive form. If this is true, it implicates that at this phase the apoptotic process is probably reversible. If appropriate intervention takes place, the apoptotic process can be stopped and cell can be rescued. It may be a good potential therapeutic window to let clinical interventions come in and turn things around. However, in the late phase of apoptosis, the cells are determined to die, the endonucleases and certain protease started to chop off DNA and other protein targets possibly including Akt. Secondary necrosis could also be involved in this process. Last but not least, we found that depletion of GSH greatly sensitized cells to H₂O₂ induced cell death (Fig. 33). Antioxidant NAC pretreatment protected Jurkat cells from H₂O₂ associated caspase 3 activation (Fig. 35) and cell death (Fig. 34). This supports the fact that antioxidant coadministration is usually beneficial to oxidative stress involved disease processes (i.e. AIDS).

Taken together, in this study, we investigated oxidative stress induced apoptosis and importantly the involvement of PI3K/Akt, a common anti-apoptotic pathway, in this process in CD4⁺ T cells. We demonstrate that in Jurkat T lymphocytes hydrogen peroxide is able to activate caspase signaling cascade leading to DNA fragmentation and apoptosis. Importantly, Akt kinase is significantly and dose-dependently down-regulated in H₂O₂ induced cell death. Pretreatment of antioxidant NAC can make cells resistant to H₂O₂ treatment and depletion of GSH by BSO sensitize cells to it.
Chapter Five

Summary

The human immunodeficiency virus (HIV-1) was first identified in 1983 (Barr-Sinoussi, 1983) and is now considered as the cause for the progressive immune system degeneration leading to the development of AIDS. HIV-1 infection is transmitted sexually, through intravenous contact with contaminated blood, or across broken mucosal or epidermal epithelia. HIV-1 can also be transmitted vertically from infected mothers in utero or through lactation after birth (McCune, 1991). The typical course of HIV-1 disease begins with a primary infection followed by an acute viral syndrome characterized by a mononucleosis-like illness, generalized lymphadenopathy, intense plasma viremia, and a significant decrease in circulating CD4\(^+\) T cells approximately 3 to 6 weeks after the initial infection. This is followed by extensive viral dissemination to surrounding tissues such as lung, bone marrow and liver but particularly to the lymph nodes (Pantaleo, 1993). Then as a result of an immune response specifically against HIV-1, the acute syndrome resolves, the number of circulating CD4\(^+\) T cells recovers, and the infection may enter a period of clinically latency that can last for 10 years or even longer. During this period, HIV-1 persists in the lymphoid tissue, where it continues to replicate and infect circulating T cells. During this phase, virus is replicated and destroyed at a very high rate, and so do CD4\(^+\) T cells. Such a dynamic replication rate yields an extremely high mutation rate (Ho, 1995). The result, over a time course of years, is the net decline in the number of CD4\(^+\) T cells and the gradual deterioration of multiple aspects of immune responses. In later stage of HIV-1 disease or AIDS, virus is usually detectable in the peripheral blood because of the breakdown in the ultrastructure of lymph nodes that permits virus to escape into the circulation. Because of the extremely low level of circulating CD4\(^+\) T cells, patients may become increasingly susceptible to life-threatening opportunistic infections and malignancies (Fauci, 1993).

The course of HIV/AIDS disease is a course of progressive degeneration of the
immune system. At the primary infection stage, the HIV-1 specific immune response is pretty effective and is able to clear most of the virus in the body and drastically restore the number of CD4+ T cells. Unfortunately, the HIV seems smart enough to find a way to escape from a total elimination and keep replicating and infecting CD4+ cells. The immune system tries very hard to hold itself by replenishing CD4+ T cells and clearing virus at very high rate until running out of ‘fuel’. This rather long latency period presents a great opportunity for therapeutic interventions. Intense amount of investigation and research have been and are being undertaken to unveil the underlying mechanisms and design potential therapeutic strategies.

During the latent phase of human immunodeficiency virus type 1 (HIV-1) infection, CD4+ T cells carrying replication competent proviral HIV-1 DNA play an important role in persistence of the virus. Several cofactors can induce and or amplify HIV-1 replication and negatively impact disease progression and pathogenesis. Ethanol consumption is an important risk factor for HIV-1 infection and has been implicated it may adversely affect HIV-1 replication and disease progression. Since TNFα is an important modulator of HIV-1 replication, we investigate the possible effects of ethanol on TNFα inducible signaling associated with HIV-1 replication in human CD4+ T cells (Jurkat E6-1). We demonstrate that clinically relevant ethanol concentrations significantly potentiate TNFα inducible NFκB. Although ethanol effectively collaborated with TNFα, by itself it does not have a direct effect on NFκB activation. The ethanol dependent potentiation of TNFα inducible NFκB nuclear translocation is observed to involve the enhanced degradation of IκBα. Additionally, the ethanol mediated potentiation of TNFα inducible NFκB activation, is abrogated by the known antioxidant pyrrolidinedithiocarbamate (PDTC), suggesting an important mechanistic role for reactive oxygen species (ROS) in this process. In correspondence with its effect on NFκB, ethanol is also able to significantly enhance HIV-1 long terminal repeat (HIV-1-LTR) dependent transcription induced by TNFα. Overall, the data provides a molecular basis for the possible role of ethanol as a cofactor that can adversely affect HIV-1 infection and pathogenesis.
Apoptosis, also known as programmed cell death (PCD), is an active cell suicide process and has long been recognized as an important feature in embryonic development, in particular in shaping the immune and nervous systems. In the early 1990’s, it was proposed that inappropriate induction of apoptosis may play a central role in HIV/AIDS pathogenesis. Most immunological and nonimmunological defects leading to AIDS, including severe conditional infection, brain atrophy and dementia, could be related to an activation induced suicide process in CD4⁺ T cells and neurons, caused by indirect interference of HIV with inter- or intracellular signaling. Sphingomyelin lipid is structural component of the plasma membrane of eukaryotic organisms. Its breakdown product, ceramide, has been shown to exert potent apoptotic effect in a variety of cell types. In HIV-infected patient, intracellular ceramide levels has been significantly elevated in CD4⁺ and CD8⁺ T cells, which displayed markedly increased apoptosis compared to a normal population (Dimarzio, 1997; Cifone, 1997). Hence, it is very relevant to examine the mechanism of ceramide induced apoptosis in CD4⁺ T cells. In the current study, we treat human CD4⁺ T cell line Jurkat E6-1 with cell-permeable short-chain ceramide and evaluate two characteristic apoptotic markers, caspase 3 activity and DNA fragmentation. As expected, ceramide potently induces caspase 3 activity and DNA fragmentation in a dose-dependent pattern. Pretreatment with Zn²⁺ is able to almost completely rescue ceramide induced DNA fragmentation. Interestingly, caspase 3 is identified as a noval target of zinc. Zinc significantly suppress the activity of caspase 3 while the processing from procaspase 3 to active caspase 3 is unaffected by zinc. We have also found that the Fas ligand protein level is significantly increased in ceramide induced apoptosis in CD4⁺ T cell line Jurkat. We speculate this could serve as a positive feedback to further exacerbate the apoptotic effect of ceramide. This effect seems to be caspase 3 dependent because it is blocked by a DEVDase specific inhibitor, DEVD-cho. This implies that caspase 3 may play a role in the regulatory phase of apoptosis rather than traditionally being considered just as an executor.

A coin has two sides. Similarly, the survival of cell is under tightly regulation by both apoptotic pathway and its counterpart, antiapoptotic pathway. Recently, PI3K/Akt pathway has emerged as a common survival pathway. That how this pathway is involved
in the process of CD4⁺ T cells apoptosis is our motivation to conduct the following research. First, in ceramide induced apoptosis, we have found that the PKB/Akt activity is greatly down-regulated. Pretreatment of DEVD-cho can not restore the Akt kinase activity, indicating that this process is caspase 3 independent. This result is in concordance to the fact that Akt/PKB has been suggested to function upstream of caspase 3 activation.

Oxidative stress has long been proposed as a mediator of apoptosis in CD4⁺ T cells in HIV pathogenesis. The current study has evaluated the role of PI3K/Akt pathway in the context of oxidative stress induced cell death in CD4⁺ T cells. Without further stimuli, Jurkat cells treated with PI3K specific inhibitors underwent significant caspase 3 activation and massive cell death. This result confirms that the PI3K/Akt pathway is essential for the survival of CD4⁺ T lymphocytes. A known oxidant hydrogen peroxide from 100 to 500μM is also able to activate caspase cascade and further lead to DNA fragmentation and apoptosis. More importantly, Akt kinase activity is significantly and dose-dependently down-regulated in H₂O₂ induced cell death. Pretreatment of antioxidant NAC can rescue cells from H₂O₂ induced caspase 3 Activation and apoptosis, while depletion of GSH by BSO exacerbate H₂O₂ induced cell death. Recent report shows that the extracellular HIV-1 Tat protein can activate PI3K/Akt pathway in Jurkat cells (Paola, 1997), implicating that this pathway is truly ubiquitous and may be a future target for potential therapeutic strategies for disorders associated with excessive cell death (i.e. AIDS).

Taken together, HIV/AIDS is a major worldwide epidemic disease and has killed millions of people since it was first reported in the early 1980’s. Two major aspects in HIV pathogenesis are virus replication and depletion of CD4⁺ T cells. The current study provides molecular basis for the adverse effect of ethanol on latency HIV replication and in-depth insight into the possible mechanisms underlying CD4⁺ T cell apoptosis. This work along with many others will help to generate future therapeutic strategies to battle diseases such as HIV/AIDS that are devastating to the individuals afflicted and an enormous economic burden for this world.
Reference


Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O'Rourke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M.E., Dixit, V. M. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J Biol Chem. 1996 Mar 1; 271(9):4961-5.


Cifone MG, Roncaiolli P, De Maria R, Camarda G, Santoni A, Ruberti G, Testi R. Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement
of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. EMBO J. 1995 Dec 1;14(23):5859-68.


Cook RT. Alcohol and human immunodeficiency virus infection. Alcohol Clin Exp 1996;20:210A-215A


Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S. Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. J Immunol. 1993 Jan 15;150(2):353-60.


Harhaj EW, Maggirwar SB, Good L, Sun SC. CD28 mediates a potent costimulatory signal for rapid degradation of IkappaBbeta which is associated with accelerated activation of various NF-kappaB/Rel heterodimers. Mol Cell Biol 1996;16:6736-43.


Hohmann HP, Remy R, Poschl B, van Loon AP. Tumor necrosis factors-alpha and -beta bind to the same two types of tumor necrosis factor receptors and maximally activate the transcription factor NF-kappa B at low receptor occupancy and within minutes after receptor binding. J Biol Chem. 1990 Sep 5;265(25):15183-8.


Oyaizu N, McCloskey TW, Coronesi M, Chirmule N, Kalyanaraman VS, Pahwa S. Accelerated apoptosis in peripheral blood mononuclear cells (PBMCs) from human immunodeficiency virus type-1 infected patients and in CD4 cross-linked PBMCs from normal individuals. Blood. 1993 Dec 1;82(11):3392-400.


Tyor WR, Middaugh LD. Do alcohol and cocaine abuse alter the course of HIV associated dementia complex?. J Leukoc Biol 1999; 65:475-81.


Vita

Contact Info
700 Woodland Ave., C-101
Lexington, KY 40508
859-323-7604
qdong0@pop.uky.edu

Date of birth
March 23rd, 1971

Place of birth
Beijing, China

Education
Graduate Center for Nutritional Sciences, University of Kentucky, Lexington, KY. Aug. 1996 - Present. GPA: 4.0

Bachelor of Medicine, Peking University Medical School, Beijing, China, Aug. 1990 – July 1995. GPA: 3.9

Experience
Research Assistant, Department of Internal Medicine, GI Division, University of Kentucky Medical Center, Lexington, KY, Aug. 1996 - present.

Publication

Talks and Presentations
Dong Q, Joshi-Barve S, McClain CJ and Barve S. PI3-kinase/Akt pathway is indispensible for the survival of CD4+ T cells. Annual Meeting of Experimental Biology (FASEB), April 2000.

S. Kelkar, Dong Q, Joshi-Barve S, McClain CJ and Barve S. Ethanol sensitizes CD4+ T cells to activation induced cell death. Annual Meeting Experimental Biology (FASEB), April 2000.


Awards

Kentucky Opportunity Fellowship
Graduate Fellowship (University of Kentucky)