SUMMARY AND CONCLUSION

Programmed cell death (apoptosis) is a fundamental biological phenomenon of organisms. It is a physiologic process of selective cell deletion that occurs during organogenesis in embryos, metamorphogenesis, tissue atrophy, and tumor regression as a pathologic process in response to various injuries. In the immune system, apoptosis contributes to the deletion of autoreactive lymphocytes, development of mature B and T lymphocytes and to the maintenance of homeostasis and control of immune responses. The process of apoptosis is characterized by morphological and biochemical changes such as shrinkage of cell plasma, blebbing of membrane, condensation and disintegration of chromatin into fragments and formation of apoptotic bodies. The latter are rapidly removed by neighboring phagocytes without any evidence of inflammation.

In the T cell development, the process of apoptosis is tightly controlled by various genes and their protein products that belong to a large family of tumor necrosis factor (TNF) and TNF receptors (TNFRs). These include Fas receptor and TNF receptors (TNFRI and TNFRII). The trimerization of these receptors by their respective ligands leads to recruitment of adaptor molecules and proteases (known as caspases). The activation of caspase cascade results in DNA fragmentation and apoptosis.

Human aging is characterized by various physiological and immunological compromised states. The deterioration of the immune system appears to contribute to increased morbidity and mortality from infections, cancer and possibly from autoimmune diseases in elderly. The T cell deficiency in aging is characterized by altered production of cytokines (including increased production of TNF-α) and a low
proliferative response to antigens, mitogens, allo- and autoantigens, signaling via T cell receptor (TCR).

In this study, we have proposed that in aging, there is an increased susceptibility of T cell subsets to undergo apoptosis. We have studied the signaling and intracellular pathways of Fas and TNF-mediated cell death in aging as compared to young controls. Additionally, we have extended our investigation to study the role of Fas and TNF in apoptosis of cord blood lymphocytes and to study the role of apoptosis in primary immunodeficiency including DiGeorge’s anomaly.

Methods

The expression of mediators of apoptosis at the protein level was determined using dual or triple color flow cytometry and Western blotting and at the mRNA level using quantitative RT-PCR and Northern blotting. The activity of proteases (caspases) was determined using Western blotting and colorometric assays. Apoptosis was induced in anti-CD3 activated cells by cross-linking with anti-Fas monoclonal antibody or by TNF-α. The percent cells undergoing apoptosis were determined using propidium iodide staining, TUNEL assay, DNA ladder assay using gel electrophoresis and morphological changes by staining with Hoechst 33342 dye. The activities of proteases (caspases) were determined using Western blotting and colorometric assays.

Fas-induced T cell apoptosis in aging

Fas/FasL Expression

The expression of fas gene and Fas protein in freshly isolated lymphocytes were analyzed in aging and young. At the basal level, there was a significant increase in the proportions of CD4+ and CD8+ T cell subsets as well as their memory (CD45RO+) and naïve (CD45RA+) T cell subpopulations that expressed Fas as compared to young
controls. Fas mRNA was also increased in lymphocytes from aging as compared to young controls. Fas ligand (FasL) is expressed only on activated T cells. Therefore, we compared the Fas ligand expression on activated T cells from aging and young. Following activation, a higher number CD4$^+$ and CD8$^+$ T cell subsets from aging expressed FasL as compared to young controls.

**Fas-induced apoptosis**

The susceptibility of T cell subsets from aging and young to undergo apoptosis was compared. Following anti-Fas monoclonal antibody treatment, we observed a significant increase in the proportions of both CD4$^+$ and CD8$^+$ T cell subsets from aging to undergo apoptosis as compared to young controls. There was an increase in number of cells in sub-Go peak as determined by propidium iodide staining and an increased number of TUNEL positive cells. Increased DNA ladders of the extracted DNA were also observed on agarose gel electrophoresis. Furthermore, upon staining with Hoechst 33342 dye revealed evidence of a higher number of lymphocytes with chromatic condensation and formation of apoptotic bodies in aging as compared to young controls.

The increased susceptibility of T cell subsets from aging to undergo Fas-mediated apoptosis as compared to controls correlated with increased Fas and FasL expression in T cells from aging, thereby suggesting its role in increased Fas-induced T cell death.

**Downstream signaling**

Next, we analyzed the execution pathway in T cell apoptosis in aging and young. We compared the activity and expression of caspases (caspase-8 and caspase-3) and other mediators (FADD) involved in Fas-mediated apoptosis. The caspase activity was compared in T cells from aging and young at 0, 4, 8 or 16 hours following anti-Fas treatment. The cleaved forms of caspase-8 appeared within four hours of anti-
Fas treatment in aging as compared to 16 hours in young subjects. Similarly, in aging, cleavage of caspase-3 and its substrates (including PARP and DEVD-pNA) were observed within four hours of anti-Fas treatment as compared to 16 hours in young.

We also compared the constitutive expression of caspase-8 and caspase-3 at the protein and at the mRNA levels. A higher expression of caspase-8 and caspase-3 both at the protein and at the mRNA level was observed in aging as compared to young.

FADD (Fas-associated death domain) protein is an adaptor molecule which is recruited to the cytoplasmic death domain of the Fas receptor upon its trimerization. We observed an increased expression of FADD at the protein level in aging as compared to young controls; however, no significant differences was observed at the FADD mRNA level, suggesting a post-transcriptional modification of FADD in aging.

**Bcl-2 family expression**

The Bcl-2 family of homologs comprises of both pro-apoptotic and anti-apoptotic members. They play an important role in regulating apoptosis and therefore, we compared the expression of Bcl-2 and its anti-apoptotic homolog, Bcl-xL, and pro-apoptotic member Bax, in T cells from aging and young both at the protein and at the mRNA level. In aging, a decreased expression of Bcl-2 and an increased expression of Bax was observed both the protein and at the mRNA levels as compared to young controls. The decreased Bcl-2 expression in aging was also present in both the memory and naïve T cell subpopulations of CD4⁺ and CD8⁺ T cell subsets. Furthermore, the ratio of Bax:Bcl-xL both at the protein and at the mRNA level was increased in aging as compared to young subjects suggesting that differential expression of Bcl-2 family of proteins may play a role in increased susceptibility of T cells from aging to apoptosis.
TNF-induced apoptosis

In order to determine the role of TNF-α in T cell apoptosis in aging, we analyzed the expression of TNF receptors (TNFRI and TNFRII) both at the protein and the mRNA level and the susceptibility of T cells to undergo TNF-induced apoptosis. In aging, there was an increased expression of TNFRI and a decreased expression of TNFRII as compared to young controls. An increased susceptibility to undergo TNF-induced apoptosis was observed in T cells from aging as compared to young controls, as determined by TUNEL assay and DNA fragmentation assay. As TNFRI is primarily involved in mediating apoptotic signal and TNFRII mediates anti-apoptotic signals, we also studied the expression and activity of adaptor molecules (TRADD, TRAF-2 and RIP) and caspases (caspase-8 and caspase-3) involved in TNFR/TNF signaling pathway. In aging, there was an increased expression of TRADD, an adaptor molecule which interacts with TNFRI, and a decreased expression of TRAF-2 (an adaptor molecule which interacts with TNFRII) both at the protein and at the mRNA level as compared to young. No differences in RIP expression, which interacts with both TNFRI and Fas, was observed between aging and young both at the protein and at the mRNA level. Furthermore, upon TNF-α treatment, there was an early and increased activity of caspase-8 and caspase-3 as compared to young controls. These data suggest that T cells from aging have increased susceptibility to TNF-induced apoptosis as compared to young controls.

Fas- and TNF- induced apoptosis in cord blood T cells

Cord blood cellular immunity is developmentally immature as compared to adult peripheral blood. Therefore, we analyzed the role of Fas and TNF-induced apoptosis in T cells from cord blood and compared to young control.
In cord blood T cells, there was a significant decrease in susceptibility to anti-Fas-induced and TNF-induced apoptosis as compared to young controls. The decreased T cell apoptosis was associated with a decreased expression of Fas, FasL and TNFR (TNFRI and TNFRII) as compared to young. Furthermore, lymphocytes from cord blood have decreased expression of FADD, TRADD, TRAF-2 and RIP as compared to young controls. No increase in caspase activity was seen following anti-Fas treatment, whereas, upon TNF-α treatment, the increase in caspase-8 and caspase-3 activities was significantly lower as compared to young controls.

Furthermore, we observed that there was a decreased expression of Bcl-2 and Bax and an increased expression of Bcl-x\textsubscript{L} in cord blood lymphocytes as compared to young controls, suggesting thereby a role of Bcl-2 family of protein in decreased susceptibility to Fas-induced and TNF-induced apoptosis in T cells from cord blood.

**Spontaneous apoptosis in T cells from patient with DiGeorge's anomaly**

DiGeorge's anomaly is an immune deficiency which is characterized by excessive infections and T cell deficiency. Therefore, we studied the role of apoptosis in T cells from patient with DiGeorge's syndrome. There was a significant increased expression of Fas and FasL and a decreased expression of Bcl-2 on T cells from the patient as compared to an age-matched control both at the protein and at the mRNA level. Furthermore, T cells from patient underwent an excessive T cell death as compared to control as measured by propidium iodide staining and DNA fragmentation ladder assay. These data suggest that increased T cell apoptosis in DiGeorge's anomaly plays a role in decreased T cell function associated with the disease.
CONCLUSION

We have shown that in aging, there is an increased susceptibility of T cells to undergo Fas-induced and TNF-induced apoptosis. This increased apoptosis is associated with differential expression of genes and their products regulating Fas/FasL and TNFR/TNF apoptotic pathways both at the signaling phase and the execution phases. Furthermore, increased T cell apoptosis and differential gene expression is characteristic of primary immune deficiencies including DiGeorge’s syndrome. On the other hand, there is a decreased Fas-mediated and TNF-mediated T cell apoptosis in cord blood lymphocytes as compared to young.