Increased Apoptosis of T Cell Subsets in Aging Humans: Altered Expression of Fas (CD95), Fas Ligand, Bcl-2, and Bax

Sudeepta Aggarwal and Sudhir Gupta

*J Immunol* 1998; 160:1627-1637; ;
http://www.jimmunol.org/content/160/4/1627

**References**
This article cites 78 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/160/4/1627.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Increased Apoptosis of T Cell Subsets in Aging Humans: Altered Expression of Fas (CD95), Fas Ligand, Bcl-2, and Bax

Sudeepta Aggarwal and Sudhir Gupta

Aging is associated with lymphopenia and progressive decline in T cell functions; however, the mechanisms underlying these defects are unclear. We analyzed the expression of genes promoting apoptosis (fas/fasl and bax) and those inhibiting apoptosis (bcl-2 and bcl-xL) in lymphocytes from aging and young subjects at the protein and mRNA level, using flow cytometry/Western blotting, and at the mRNA level, using quantitative PCR. Susceptibility of T cell subsets to undergo anti-Fas-induced apoptosis was analyzed by propidium iodide staining, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay, DNA fragmentation assay, and staining with Hoechst 33342 dye. An increased expression of Fas and Fas ligand and a decreased expression of Bcl-2 were observed in both CD4+ and CD8+ T cells from aging compared with young controls. Increased Fas and decreased Bcl-2 expression were also found in memory cells of both CD4+ and CD8+ T cell subsets from aging. Bax expression was increased in lymphocytes from aging at both the protein and mRNA level. No significant difference was observed in Bcl-xL expression between aging and young; however, the ratio of Bax:Bcl-xL was increased in aging. An increased proportion of CD4+ and CD8+ T cell subsets from aging underwent apoptosis following anti-Fas Ab treatment as compared with CD4+ and CD8+ T cell subsets from young controls. These data suggest that increased apoptosis may be one of the mechanisms responsible for lymphopenia and T cell deficiency associated with human aging.

Materials and Methods

Subjects

Peripheral blood was obtained from healthy young (20–29 yr; n = 15, males) and aged (65–95 yr; n = 15, males) volunteers. The protocol was approved by Institutional Review Board, University of California, Irvine. The aging group is defined as those 65 yr or over.

Reagents

Antibodies. Anti-Fas IgM mAb (CH-11) that induces apoptosis (19, 40, 41) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Isotype IgM control was purchased from Caltag Laboratories (South San Francisco, CA). FITC-labeled anti-CD95 (UB2) and isotype control were purchased from Kamiya Biomedical Corp. (Thousand Oaks, CA). FITC-labeled anti-Bcl-2 mAb and isotype control were obtained from Dako Corp. (Carpenteria, CA). Per-CP-labeled anti-CD4, anti-CD8, PE-labeled anti-CD45RA, and anti-CD45RO mAbs and their isotype controls were purchased from Becton Dickinson (San Jose, CA). HRP-conjugated goat anti-mouse Ab, polyclonal Ab against Bcl-xL, and HRP-conjugated anti-rabbit IgG were purchased from Transduction Laboratories (Lexington, KY). Anti-Bax Ab was raised in rabbit and kindly provided by Drs. S. Kitada and J. Reed, Burnham Institute (La Jolla, CA). mAb against Fasl, raised in hamster (42), was kindly provided by Dr. S. Nagata (Osaka, Osaka, Japan).
Japan). FITC-labeled goat anti-hamster Ab and hamster IgG control Abs were purchased from Caltag Laboratories. Anti-CD3 mAb (OKT3) was purchased from Ortho Diagnostics (Raritan, NJ).

Primers. Primers used for RT-PCR were synthesized from Life Technologies (Gaithersburg, MD). Their sequences were as follows: fas, sense, 5'-ATG CTG GGC ATC TTC ACC CTA-3'; antisense, 5'-TCT GCA ATT GGT ATT CTG CGT CGG-3' (43); bcl-2, sense, 5'-CGA CTA CTT CCG CCA TGA CCG C-3'; antisense, 5'-CCG CAT GGT GGG GCC GTA CAG TTC-3' (44); bax, sense, 5'-ATG AAC GGG TCC GGG GAC CAG CCC-3'; antisense, 5'-GTT GAC GAC TCC GGC CAC AAA GAT-3' (45); and bcl-xL, sense, 5'-TTG TAC AAT GGA CGT GTG GA-3', and antisense, 5'-GTA GAG TGG ATG AGT GGT G-3' (31).

Chemicals. Propidium iodide, PHA, PMA, ionomycin, and Hoechst 33342 dye were purchased from Sigma Chemical Co. (St. Louis, MO). [α-32P]dATP was obtained from DuPont (Wilmington, DE). RT-PCR kits were purchased from Perkin-Elmer/Cetus (Branchburg, NJ). Metalloprotease inhibitors TIMP1 and TIMP2 were obtained from Calbiochem (San Diego, CA). rIL-2 was purchased from R&D Systems (Indianapolis, IN).

Cell culture

PBMC were separated from whole blood by Ficoll-Hypaque density centrifugation. Cells were resuspended in RPMI 1640 medium containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (1 mM) at 2 × 10^6 cells/ml, and subsequently cultured in the presence of anti-CD3 mAb (25 ng/ml) for 48 h, following which cells were maintained in IL-2 (10 ng/ml)-containing medium. For anti-Fas-induced apoptosis, cells were washed on day 6 of culture and restimulated with anti-Fas (1 μg/ml) mAb or its isotype (IgM) control Ab in the presence of human rIL-2 (10 ng/ml) for an additional 16 h (46, 47).

Flow cytometry

The expression of Fas, Fasl, and Bcl-2 proteins was determined using flow cytometry.

Fas expression

MNC were washed twice with PBS and incubated for 45 min on ice with FITC-conjugated anti-Fas mAb and PE-conjugated anti-CD4 or anti-CD8 mAbs. For triple-color analysis, FITC-conjugated anti-Fas, Per-CP-conjugated anti-CD4, or anti-CD8, and PE-conjugated anti-CD45RA or anti-CD45RO mAbs were used. FITC-labeled, PE-labeled, and Per-CP-labeled mouse IgG were used as isotype-matched background controls. Cells were washed with PBS, and 5000 cells were acquired using FACSscan. Dual- or triple-color analysis was performed using Consort 30 and FACSscan Research software, respectively (Becton Dickinson).

Bcl-2 expression

Bcl-2 is an intracellular protein (48), and therefore, cells were washed and sequentially fixed with 1% paraformaldehyde for 15 min at room temperature and by 70% methanol for 45 min at 4°C (49, 50). Cells were washed and incubated with FITC-labeled anti-Bcl-2 and PE-labeled anti-CD4 or anti-CD8 mAbs. For triple-color analysis, Per-CP-labeled anti-CD4 or anti-CD8, and PE-labeled anti-CD45RA or anti-CD45RO mAbs were used. FITC-labeled, PE-labeled, and Per-CP-labeled mouse IgG were used as isotype-matched background controls. Following incubation, the cells were washed twice with PBS, and 5000 cells were acquired using FACSscan. Lymphocytes were gated, and percentage of double-positive cells (CD4+ Bcl-2+ and CD8+ Bcl-2+) expressing Bcl-2 and fluorescence intensity, as measured by mean fluorescence channel (MFC) number, was determined using Consort 30 software.

FasL expression

Because FasL is expressed on activated human T cell subsets (21–23), FasL expression was determined by Western blotting. The expression of Bcl-2, Bcl-xL, and Bax at the protein level was determined by Western blotting. Cells were lysed in a buffer containing 142.5 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.2), 1 mM EDTA, 0.2% Nonidet P-40, 0.2 mM PMSF, 0.2% tropsin inhibitory U/ml aprotinin, 0.7 μg/ml pepstatin, and 1 μg/ml leupeptin. Cells were homogenized and centrifuged at 1000 rpm for 8 min to precipitate cell debris. The supernatants were centrifuged at 30,000 × g for 45 min to precipitate membrane fractions, and 25 μg protein (or increasing amounts of proteins for limiting dilution analysis) was loaded onto 4 to 20% Tris-glycine gels and electrophoresed. The proteins were then transferred onto nitrocellulose membrane. The blots were blocked with PBS containing 3% dry milk and 0.1% Tween-20 and probed with anti-Bcl-2, anti-Bcl-xL, or anti-Bax Abs (dilution of 1/1000) for 3 h at 37°C. The blots were incubated with HRP-conjugated goat anti-mouse or HRP-conjugated goat anti-rabbit (1/2000 dilution) Ab for 1 h at room temperature and developed using enhanced chemiluminescence method (Amersham, Arlington Heights, MA). The blots were quantified using densitometric analysis using ImageQuant soft-ware (Molecular Dynamics, Sunnyvale, CA) and data represented as OD.

Quantitative PCR

Total cellular RNA was extracted from unstimulated MNC from young and aging subjects. cDNA was synthesized using 200 ng total cellular RNA and 100 ng random hexamers in 20 μl of a solution containing 50 mM Tris- HCl, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 500 μM each of dNTPs, and 10 U reverse transcriptase. PCR was conducted with graded amounts of cDNA (0.1, 1, 2, 4, and 8 μl) using 1 U of Amplitaq polymerase and PCR reaction kit (Perkin-Elmer/Cetus). Each cycle of PCR included 1 min of denaturation at 94°C, 1 min of primer annealing at 60°C, and 2 min of extension/synthesis at 72°C. Fas-, bcl-2-, bcl-xL-, and bax-specific primers yielded 180-, 780-, 318-, and 615-bp primer products, respectively. Primers for β-actin were used as internal controls. Each primer was added at 37.5 pmol per reaction. For quantitation, 2 μCi (1 Ci = 37 GBq) of [α-32P]dATP was added to each reaction mixture, and experiments were performed in triplicates. PCR was conducted with Thermal Cycler (Perkin-Elmer/Cetus). PCR products were separated on 6% TBE gels and stained with ethidium bromide or excised and quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and data represented as OD.

Propidium iodide staining

MNC (1 × 10^6) were washed with PBS and incubated in the presence of FITC-labeled anti-CD4 or anti-CD8 mAbs and corresponding isotype controls for 45 min on ice. Following incubation, cells were washed twice with PBS and resuspended in 70% ethanol overnight at ~20°C. The cells were washed twice with PBS and incubated in sodium citrate buffer (0.1%) containing 0.1% Triton X-100, 50 μg/ml RNase A, and 50 μg/ml propidium iodide for 30 min at room temperature in the dark. Ten thousand cells were acquired, and percentage of cells undergoing apoptosis was determined by dual-color analysis, using FACSscan.

TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay was performed to study DNA fragmentation (53–56). Cells (5 × 10^5) were washed in Dulbecco’s PBS (DPBS) and incubated in the presence of PE-labeled anti-CD4 or anti-CD8 Abs for 45 min on ice. Following incubation, cells were washed twice in DPBS containing 0.1% BSA and 0.1% Na3A and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were washed, labeled at 4°C and permeabilized with sodium citrate buffer containing 0.1% Triton X-100 for 2 min on ice. After washing, cells were incubated with FITC-conjugated dUTP in the presence of terminal deoxynucleotidyl transferase enzyme solution for 1 h at 37°C using cell death detection kit (Boehringer Mannhein Corp., Indianapolis, IN). Following incubation, the cells were washed with DPBS, and 5000 cells were acquired and analyzed by dual-color analysis using FACSscan.

DNA fragmentation

DNA fragmentation was assessed by gel electrophoresis. Cells were harvested and centrifuged at 500 × g for 5 min and washed twice with PBS. The cell pellet was lysed in 400 μl of lysing buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.1% SDS, and 0.2% Triton X-100 and proteinase K (0.1 mg/ml) at 30°C for 16 h, followed by incubation with 50 μg/ml RNase A for an additional hour at 50°C. DNA was extracted once with phenol-chloroform-isomyl alcohol (25:24:1) and twice with chloroform-isomyl alcohol (24:1). The aqueous phase was precipitated with 2
using Cytospin 3 (Shandon, Cheshire, UK) and visualized under fluorescence microscopy. Following incubation, cells were cytospun onto microscopic slides (mean SD cpm for 1 and 2 µl fas). Increasing amounts of cDNA (0, 1, 2, 4, and 8 µl) were used for quantitative RT-PCR. Results in mean ± SD fas:β-actin cpm ratio (1 and 2 µl for fas, and 1 µl for β-actin) show an increased expression in aging as compared with young. Linearity of quantitative RT-PCR was determined using increasing amounts of cDNA (0, 1, 2, 4, and 8 µl) and is shown as OD in inset for aging (●) and young (○). B, Increased fas mRNA expression in lymphocytes from aging. Representative gel photograph for fas mRNA expression using 2 µl cDNA is shown. PCR reactions were conducted in triplicate, and PCR products were electrophoresed on 6% TBE gel. Gel exposed to x-ray films for 2 h shows increased fas expression (top panel) in aging as compared with young. Primers specific for β-actin were used as internal controls (bottom panel).

FIGURE 2. A, Increased fas mRNA expression in lymphocytes from aging. Graded amounts of cDNA from two subjects each were used for quantitative PCR. Results in mean ± SD fas:β-actin cpm ratio (1 and 2 µl for fas, and 1 µl for β-actin) show an increased expression in aging as compared with young. Vertical lines represent mean ± SD cpm for 1 and 2 µl fas and 1 µl β-actin: 0.45 ± 0.03 and 1.05 ± 0.04), demonstrating an increase of fas expression at mRNA level in aging. A representative gel photograph is shown in Figure 2B.

Aging is associated with an increase in memory cell population (58), and as memory T cells are known to have increased expression of Fas (59), we investigated whether the expression of Fas in memory and naive cell populations was different between aging and young subjects. Table I summarizes the data obtained from five aging and five young subjects. A significantly higher (p < 0.001) percentage of memory (CD45RA + CD8 - ) as well as naive (CD45RO + CD8 - ) cells in both CD4 + and CD8 - subsets expressed Fas in aging subjects as compared with their memory and naive counterparts in young subjects. Furthermore, in both of the age groups, a higher proportion of memory cells (CD4 + CD45RO + and CD8 - CD45RO + ) expressed Fas as compared with naive cells (CD4 + CD45RA + and CD8 - CD45RA - ) (p < 0.001 and p < 0.002, respectively).

FasL is expressed only on activated T cells; therefore, to compare the proportions of T cells expressing FasL, MNC from five

FIGURE 1. Increased Fas expression in T cell subsets from aging as compared with young. Freshly isolated MNC from 10 aging and 10 young subjects were stained for Fas, CD4, or CD8 using mAbs, as described in Materials and Methods. Proportions of dual-positive CD4 + and CD8 - T cells expressing Fas using flow cytometry are shown. Increased Fas expression is seen in CD4 + and CD8 - T cell subsets from aging as compared with young controls. Vertical lines represent mean ± SD from 10 experiments.

Morphologic determination of apoptosis

Changes in nuclear structure were studied after staining cells with DNA-binding fluorescent dye Hoechst 33342 (57). MNC (1 × 10 6 ) were washed and resuspended in 5 µg/ml Hoechst dye in PBS and incubated at 37°C for 30 min. Following incubation, cells were cytospun onto microscopic slides using Cytospin 3 (Shandon, Cheshire, UK) and visualized under fluorescent microscope at 365 nm excitation and 420 nm long pass emission.

Statistical analysis

All studies were done in pairs, i.e., each experiment was done using an equal number of aging subjects and young subjects simultaneously. Statistical analysis was performed using two Student sample t tests.

Results

Fas/FasL expression in T cell subsets

MNC from 10 subjects, each from aging and young groups, were analyzed for Fas expression with mAbs using dual-color analysis by FACScan. Figure 1 shows a significantly higher (p < 0.001) proportion of CD4 + and CD8 - T cells from aging subjects expressed Fas (percentage of mean ± SD: CD4 +, 45 ± 7; CD8 -, 56 ± 7) as compared with young subjects (percentage of mean ± SD: CD4 +, 29 ± 5; CD8 -, 35 ± 5). Furthermore, there was a significantly (p < 0.002) higher proportion of CD8 - T cells expressing Fas in each age group as compared with CD4 + T cells (aging CD8 - vs CD4 +, 56 ± 7 and 45 ± 7; young CD8 - vs CD4 +, 35 ± 5 and 29 ± 5).

To determine whether the age-related differences in the Fas Ag expression also existed at the mRNA level, we analyzed fas mRNA expression in MNC from aging and young subjects, using quantitative PCR. Graded amounts of cDNA (0, 1, 2, 4, and 8 µl) were used to determine the linearity of the PCR reaction (Fig. 2A, inset). As seen in Figure 2A, there was a significant increase (p < 0.001) in cpm ratio for fas/β-actin in lymphocytes from aging (mean ± SD cpm for 1 and 2 µl fas and 1 µl β-actin: 1.06 ± 0.03 and 2.4 ± 0.05) as compared with young (mean ± SD cpm for 1
subjects each from aging and young groups were stimulated with PHA (5 µg/ml) and IL-2 (10 ng/ml) for 48 h and subsequently activated with PMA and ionomycin for 16 h. As shown in Figure 3, significantly higher ($p < 0.001$) proportions of both CD4$^+$ and CD8$^+$ T cell subsets in aging subjects expressed FasL (percentage of mean $\pm$ SD: CD4$^+$, 24 $\pm$ 4; CD8$^+$, 28 $\pm$ 5) as compared with young controls (percentage of mean $\pm$ SD: CD4$^+$, 8 $\pm$ 3; CD8$^+$, 10 $\pm$ 2).

Expression of Bcl-2 and its homologues

Fas and Bcl-2 are regulated differentially in various cell types (60–62). Therefore, we examined the levels of Bcl-2 protein in aging and young T cell subsets. Bcl-2 is a membrane-bound protein found in mitochondrial membrane (48) that is expressed in almost all CD4$^+$ and CD8$^+$ T cells. Therefore, the differences in Bcl-2 protein levels were analyzed by changes in MFC intensity/numbers assessed by flow cytometry and Western blotting. We found that Bcl-2 levels in aging (n = 10) T cell subsets were significantly ($p < 0.001$) lower (MFC, mean $\pm$ SD: CD4$^+$, 513 $\pm$ 18; CD8$^+$, 476 $\pm$ 21) as compared with young (n = 10) controls (MFC, mean $\pm$ SD: CD4$^+$, 570 $\pm$ 12; CD8$^+$, 536 $\pm$ 20). Figure 4A shows representative FACS plots for Bcl-2 staining for CD4$^+$ (middle panel) and CD8$^+$ (right panel) T cells in each young (bottom panel) and aging (top panel) subject. Markers were set for the cells positive for Bcl-2 and CD4 or CD8 following staining with isotype control Abs (left panel). Furthermore, Western blotting analysis was performed on three different subjects from each aging and young. Figure 4B shows that in each of the three aging subjects, there was a decreased Bcl-2 expression (OD mean $\pm$ SD, 0.68 $\pm$ 0.05; Fig. 6B) and at the mRNA level (Fig. 7A; mean $\pm$ SD cpm ratio, 1 µl bax:1 µl β-actin: 0.62 $\pm$ 0.03, and 2 µl bax:1 µl β-actin: 1.4 $\pm$ 0.13) as compared with that from young subjects at the protein level (OD mean $\pm$ SD, 0.37 $\pm$ 0.07; Fig. 6B) and at the mRNA level (Fig. 7A; mean $\pm$ SD cpm ratio, 1 µl bax:1 µl β-actin: 0.46 $\pm$ 0.02, and 2 µl bax:1 µl β-actin: 0.91 $\pm$ 0.04). The limiting dilution for Western blotting was conducted using increasing amounts of proteins (Fig. 6A), and for quantitative PCR was conducted using increasing amounts of cDNA (0.1, 1, 2, 4, and 8 µl) to demonstrate the linearity (Fig. 7A, inset). Representative gel photographs for Western blotting and quantitative PCR are shown in Figures 6B (inset) and 7B, respectively. On the other hand, there was no significant difference ($p > 0.05$) in Bcl-x$_L$ expression in lymphocytes between aging and young both at the protein level (Fig. 8B; aging OD mean $\pm$ SD, 0.23 $\pm$ 0.02; young OD mean $\pm$ SD, 0.22 $\pm$ 0.02) and at the mRNA level (Fig. 9A; aging mean $\pm$ SD cpm ratio, 1 µl bcl-x$_L$:1 µl β-actin: 0.35 $\pm$ 0.05, and 2 µl bcl-x$_L$:1 µl β-actin: 0.68 $\pm$ 0.02; young mean $\pm$ SD cpm ratio, 1 µl bcl-x$_L$:1 µl β-actin: 0.32 $\pm$ 0.03, and 2 µl bcl-x$_L$:1 µl β-actin: 0.71 $\pm$ 0.04). Representative graphs for limiting dilution for Western blotting and quantitative PCR are shown in Figures 8A and 9A (inset), respectively. Representative gel photographs for Western blotting and quantitative PCR are shown in Figures 8B (inset) and 9B, respectively.

### Table I. Expression of Fas in T cell subsets

<table>
<thead>
<tr>
<th>Subjects</th>
<th>CD4$^+$</th>
<th>CD8$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45RA$^+$</td>
<td>CD45RO$^+$</td>
<td>CD45RA$^+$</td>
</tr>
<tr>
<td>Young</td>
<td>6 $\pm$ 2</td>
<td>25 $\pm$ 3</td>
</tr>
<tr>
<td>Aging</td>
<td>17 $\pm$ 2</td>
<td>40 $\pm$ 4</td>
</tr>
<tr>
<td>p value</td>
<td>$&lt;0.001$</td>
<td>$&lt;0.001$</td>
</tr>
</tbody>
</table>

*MNCS (1 x 10$^6$ cells) from 10 subjects each in aging and young groups were stained with mAbs against Fas, CD4, CD8, CD45RA, or CD45RO as described in Materials and Methods. Data are represented as mean $\pm$ SD percent Fas. Which were CD4$^+$ (CD45RA$^+$/CD45RO$^+$) or CD8$^+$ (CD45RA$^+$/CD45RO$^+$) T cell types.

FIGURE 3. Increased FasL expression in T cells from aging. MNCs were activated with PHA and IL-2, followed by stimulation with PMA and ionomycin. Percentage of Fas-L-expressing cells was determined by dual-color flow-cytometric analysis of CD4$^+$ and CD8$^+$-stained cells. Bars indicate percentage of mean $\pm$ SD CD4$^+$ or CD8$^+$ T cells expressing FasL from five similar experiments.

[Image of Table I and Figure 3]
Susceptibility to anti-Fas-induced apoptosis

To determine whether differential expression of Fas, FasL, Bcl-2, and Bax correlates with differential susceptibility of T cells to undergo anti-Fas-induced apoptosis between aging and young, we cultured MNC from aging and young subjects for 6 days in IL-2-containing medium following an initial stimulation with anti-CD3 (25 ng/ml) mAb for 48 h. Following culture, cells were treated with anti-Fas (1 μg/ml) mAb or its isotype-matched control for an additional 16 h, and susceptibility of T cells to undergo apoptosis was determined using propidium iodide staining and TUNEL assay. Table III summarizes data from 10 aging and 10 young subjects. An increased proportion of CD4\(^+\) and CD8\(^+\) T cell subsets from aging underwent anti-Fas-induced apoptosis as compared with CD4\(^+\) and CD8\(^+\) T cells from young controls (\(p < 0.001\)). Figure 10, A and B, shows representative histograms obtained for percentage of cells in TUNEL assay and propidium iodide staining undergoing apoptosis following anti-Fas Ab treatment (Fig. 10, A and B, bottom panel). No significant apoptosis was observed following
treatment with isotype control Ab (Fig. 10, A and B, top panel). In addition, in each aging and young group, a significantly increased proportion of CD4^+ T cells underwent apoptosis as compared with their CD8^+ T cell subsets (p, 0.05).

Next, we compared anti-Fas-induced DNA fragmentation in MNC between aging and young using gel electrophoresis. Figure 10C shows that following anti-Fas treatment, there was an increased DNA fragmentation in lymphocytes from aging compared with young controls. Furthermore, an increase in proportions of cells showing apoptotic bodies in aging MNC was observed with Hoechst dye as compared with young controls. A representative photomicrograph of cells stained with Hoechst dye following anti-Fas treatment is shown in Figure 10D.

Discussion

In this study, we have shown that there is an altered expression of genes regulating apoptosis in lymphocytes from aging humans, and that T cell subsets from aging show increased susceptibility to undergo anti-Fas-induced apoptosis as compared with T cell subsets from young humans.

The Fas receptor cross-linking results in the death of Fas^+ T cells upon ligation with FasL (26–29). This interaction helps terminate an ongoing immune response by inducing apoptosis in activated lymphocytes. Our data show an increased expression of Fas at basal levels in CD4^+ and CD8^+ T cell subsets from aging humans as compared with young controls. Furthermore, the fas expression was also increased at mRNA level in MNC from aging.

Table II. Expression of Bcl-2 in T cell subsets

<table>
<thead>
<tr>
<th>Subjects</th>
<th>CD45RA^+</th>
<th>CD45RO^+</th>
<th>CD45RA^+</th>
<th>CD45RO^+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>CD4^+</td>
<td>CD8^+</td>
<td>CD4^+</td>
<td>CD8^+</td>
</tr>
<tr>
<td></td>
<td>596 ± 10</td>
<td>570 ± 8</td>
<td>585 ± 14</td>
<td>561 ± 12</td>
</tr>
<tr>
<td>Aging</td>
<td>525 ± 12</td>
<td>499 ± 20</td>
<td>501 ± 14</td>
<td>484 ± 7</td>
</tr>
<tr>
<td>p Value</td>
<td>&lt;0.001</td>
<td>&lt;0.003</td>
<td>&lt;0.001</td>
<td>&lt;0.003</td>
</tr>
</tbody>
</table>

^MNCs (1 x 10^6 cells) from 10 subjects each from aging and young groups were fixed and stained with mAbs as described in Materials and Methods. Data are represented as mean ± SD of MFC intensity of Bcl-2^+ cells that were CD4^+ (CD45RA^+/CD45RO^+) or CD8^+ (CD45RA^+/CD45RO^+) T cell types. One aging and one sample were stained and analyzed simultaneously.
Naive cells express little or no Fas, which can be induced upon activation, whereas memory cells have increased Fas (26, 59, 60). Interestingly, we observed a significant increase in Fas expression in both naive and memory subpopulations of CD4⁺ and CD8⁺ T cell subsets in aging as compared with young. This would suggest that an increase in Fas expression observed in CD4⁺ and CD8⁺ T cells in aging is not exclusively due to a shift from naive to memory phenotype T cells. Our observations are in agreement with the findings of Shinohara et al. (62), who also reported an increased Fas expression in memory CD4⁺ or CD8⁺ T cells in elderly human population (mean age: 51 yr). However, in this report, Fas expression was not determined on naive subpopulations of CD4⁺ and CD8⁺ T cell subsets, and therefore, an increased Fas expression observed could be due to an increase in the number of memory cells, as data were not normalized for increased memory cell numbers in the elderly population. In contrast, studies on aged mice show a decrease in Fas expression on T cells (19). The reason for this discrepancy of Fas expression levels in humans and mice is not known, but could be related to species differences. Furthermore, we observed that following in vitro activation, a significantly higher proportion of CD4⁺ and CD8⁺ T cells from aging expressed FasL as compared with young. There are no published data on FasL expression in lymphocytes from aging humans.

To examine whether the increased expression of Fas and FasL in aging lymphocytes correlated with an increased susceptibility to apoptosis, we compared anti-Fas-induced apoptosis in T cell subsets between aging and young humans. Freshly isolated T cells are resistant to anti-Fas-induced apoptosis; however, they become sensitive upon prolonged in vitro activation in culture (46). Following anti-Fas treatment of cultured lymphocytes, an increased proportion of CD4⁺ and CD8⁺ T cells from aging underwent apoptosis as compared with young subjects. We also observed that following an initial stimulation with anti-CD3, a higher proportion of CD4⁺ and CD8⁺ T cells in aging underwent apoptosis as compared with young; however, the number of dying cells was very few (data not shown). Furthermore, there was no effect of IgM treatment on T cell apoptosis in both aging and young. Recently, Herndon et al. (38) have demonstrated an increased apoptosis of naive (CD3⁺CD45RO⁻) T cells in aging humans as compared with young following in vitro culture of fresh lymphocytes. Phelouzat et al. (39) have also reported an increased susceptibility of aging lymphocytes to undergo activation-induced apoptosis. However,
one aging and one young subject were analyzed simultaneously.

C

upper panel

2, 4, and 8
tative RT-PCR was determined using increasing amounts of cDNA (0, 1,

ers specific for

were gated, and percentage of cells undergoing apoptosis was determined in sub-G o peak in FL2 channel. Representative histograms show percentage of cells undergoing apoptosis following treatment of control Ab (lower panel) using FL1 channel. Representative histograms show percentage of cells undergoing apoptosis following treatment with suprapharmacologic concentrations of PMA and ionomycin. Furthermore, determination of apoptosis was done on MNC using ELISA, which quantitates the DNA fragments released following cell lysis, a rel-

logic concentrations of PMA and ionomycin. Furthermore, deter-

apoptosis was done on MNC using ELISA, which quantitates the nicks in DNA undergoing apoptosis within 4 h of initiation of the apoptotic signal. Furthermore, we observed a preferential death of CD4+ T cell subsets upon anti-Fas treatment as compared with CD8+ T cells in both aging and young groups. Using a different cell system of CD4 and CD8 clones, Zheng et al. (63) have also shown a preferential death of CD4+ T cells as compared with CD8+ T cell subsets following anti-Fas treatment. The data on apoptosis of lymphocytes in aging mice are inconclusive. Chrest et al. (64) have shown an increased death of G0 T cells from spleen of aging C57BL/6 female mice upon stimulation with anti-CD3 mAb, using flow cytometry. In contrast, Zhou et al. (65) have demonstrated a decreased susceptibility of lymph node T cells from CD1-aged mice to undergo FasL-mediated apoptosis as compared with young mice. The differences between the reports from Chrest et al. and Zhou et al. could be due to differences in the strain of experimental animals, lymphoid compartments (spleen vs lymph nodes), or the method of stimulation (anti-CD3 vs FasL) used.

Bcl-2 plays an important role in the fate of cells committed to undergo apoptosis (66). We therefore compared the expression of Bcl-2 in lymphocytes from aging and young subjects. We observed a decreased Bcl-2 expression in aging both at the protein and at the mRNA level as compared with young subjects. Decreased Bcl-2 expression in aging was observed in both CD4+ and CD8+ T cell subsets. Furthermore, decreased Bcl-2 expression was seen in naive and memory cells of both CD4+ and CD8+ T cells in aging as compared with young controls. Shinohara et al. (62) have also observed decreased Bcl-2 expression in memory cell subpopulations of CD4+ and CD8+ T cell subsets in aging; however, Bcl-2 expression on naive T cells was not analyzed. The role of Bcl-2 in the regulation of anti-Fas-mediated apoptosis is controversial and may depend on the cell types and stimulus used. Iwai et al. (67) and Yoshino et al. (68), using activated T lymphocytes, have quantitated the proportions of CD4+ and CD8+ T cell subsets undergoing apoptosis using TUNEL assay, which is specific for apoptosis and quantitates the nicks in DNA undergoing apoptosis.

Table III. Susceptibility to anti-Fas-induced apoptosis of T cell subsets

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Percent Apoptotic Cells</th>
<th>TUNEL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4+</td>
<td>CD8+</td>
</tr>
<tr>
<td>Young</td>
<td>26 ± 5</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>Aging</td>
<td>55 ± 2</td>
<td>50 ± 3</td>
</tr>
</tbody>
</table>

p Value | <0.001 | <0.001 | <0.001 | <0.001 |

*Previously stimulated MNCs (2 × 10^6 cells/ml) were rechallenged with anti-Fas MAb as described in Materials and Methods. Cells were stained with anti-CD4 or anti-CD8 mAbs and percent cells undergoing apoptosis determined by propidium iodide staining or TUNEL assay. Data from 10 subjects each in aging and young group are represented in mean ± SD percent dead cells in the apoptotic peak. All the experiments were carried out in pairs, i.e., equal number of aging and young subjects were analyzed simultaneously.

these authors have compared susceptibility of lymphocytes between aging and young following treatment with suprapharmacologic concentrations of PMA and ionomycin. Furthermore, determination of apoptosis was done on MNC using ELISA, which quantitates the DNA fragments released following cell lysis, a relatively late phase event in apoptosis, which does not differentiate between cell types undergoing necrosis and apoptosis. We have

FIGURE 10. A, TUNEL assay for anti-Fas-induced apoptosis in CD4+ and CD8+ T cell subsets. Following culture of freshly isolated MNC, as described in Materials and Methods, cells were treated with anti-Fas Ab or its isotype IgM control, and nicked DNA was stained with FITC-labeled dUTPs using TUNEL assay kit and PE-labeled CD4 and CD8 mAbs. CD4+ and CD8+ T cells were gated, and percentage of cells undergoing apoptosis was determined using FL1 channel. Representative histograms show percentage of cells undergoing apoptosis following treatment of control Ab (upper panel) or anti-Fas Ab (lower panel) from 10 experiments conducted in pairs, i.e., one aging and one young subject analyzed simultaneously. B, Propidium iodide assay for anti-Fas-induced apoptosis in CD4+ and CD8+ T cell subsets. Following culture of freshly isolated MNC, as described in Materials and Methods, cells were treated with anti-Fas Ab or isotype IgM control and stained with propidium iodide and FITC-labeled CD4 or CD8 mAbs. CD4+ and CD8+ T cells were gated, and percentage of cells undergoing apoptosis was determined in sub-G0 peak in FL2 channel. Representative histograms show percentage of cells undergoing apoptosis following treatment with control Ab (upper panel) or anti-Fas Ab (lower panel) from 10 experiments performed in pairs, i.e., one aging and one young subject were analyzed simultaneously. C, DNA fragmentation in lymphocytes following anti-Fas treatment. Following

FIGURE 9. A, Increased bcl-xL mRNA expression in lymphocytes from aging. Graded amounts of cDNA from two subjects each were used for quantitative PCR. Results in mean ± SD cpm ratio bcl-xL/β-actin (1 and 2 μl for bcl-xL, and 1 μl for β-actin) show no significant difference in bcl-xL expression in aging as compared with young. Linearity of quantitative RT-PCR was determined using increasing amounts of cDNA (0, 1, 2, 4, and 8 μl) and is shown as OD in inset for aging ( ) and young ( ) subjects. B, bcl-xL mRNA expression in lymphocytes from aging. Representative gel photograph for bcl-xL mRNA expression using 2 μl cDNA is shown. PCR reactions were conducted in triplicate, and PCR products were electrophoresed on 6% TBE gel. Gel exposed to x-ray films for 2 h shows no difference in bcl-xL expression in aging as compared with young. Primers specific for β-actin were used as internal controls (bottom panel).
anti-Fas treatment of cultured lymphocytes from aging and young, as described in Materials and Methods, cells were lysed and DNA was extracted and electrophoresed on 2% gel. A representative gel from 10 experiments is shown. Increased number of DNA ladder pattern is seen in aging (A) as compared with young (Y) following anti-Fas treatment. D, Hoechst dye staining of lymphocytes following anti-Fas treatment. Apoptotic DNA on cytocentrifuge preparations of lymphocytes from aging and young using Hoechst staining dye following anti-Fas treatment of cultured lymphocytes. Higher proportions of apoptotic bodies were seen in the lymphocytes from aging group as compared with young following anti-Fas treatment.
shown that Bcl-2 blocks anti-Fas Ab-induced apoptosis in mitogen-activated T cells. In contrast, some reports show that Bcl-2 is not involved in anti-Fas-induced apoptosis in lymphocytes (69, 70). However, in these reports, changes in Bcl-2 levels were studied following anti-Fas treatment. In our study, we have compared the basal expression levels of Bcl-2 (both at the protein and at the mRNA level) between aging and young and have suggested that lower Bcl-2 expression in T cells from aging may play a role in higher susceptibility of T cells to undergo anti-Fas-induced apoptosis. Differences in Bcl-2 expression in aging following anti-Fas treatment remain to be seen. It has been shown that in vitro activation of T cells is associated with down-regulation of Bcl-2 and up-regulation of Fas/FasL (22, 23, 71, 72). Therefore, it is possible that prolonged in vivo antigenic activation of T cells in aging may be one of the mechanisms of low Bcl-2 and increased Fas/FasL expression in aging.

Overexpression of Bcl-xL, the long form of an alternatively spliced homologue of Bcl-2, has been shown to be involved in inhibition of apoptosis (73). On the other hand, Bax, a pro-apoptotic Bcl-2 family member, can heterodimerize with either Bcl-2 or Bcl-xL to nullify their anti-apoptotic properties. The ratios of Bcl-2:Bax and Bcl-xL:Bax appear to be important determinants for apoptosis: higher ratios favor cell survival, whereas higher Bax:Bcl-2 and Bax:Bcl-xL ratios promote cell death (66, 74). We did not find any significant differences in Bcl-xL levels between young and aging using Western blotting; however, because of increased level of Bax, the ratio of Bax:Bcl-xL was higher in aging as compared with young subjects (Fig. 11).

These data would support our observation of increased susceptibility of T cells from aging to undergo Fas-induced apoptosis as compared with young subjects. Abnormalities in the regulation of apoptosis may contribute to the pathogenesis of a variety of disorders (reviewed by C. Thompson: 75, 76). Aging is associated with increase in frequency of infection and increased incidence of cancer. Increased apoptosis of both CD4+ and CD8+ in aging may contribute to both increased frequency of infection and increased incidence of cancer. Increased apoptosis of both CD4+ and CD8+ T cells has also been observed in AIDS (77–81), which, similar to aging, is also associated with T cell deficiency, increased frequency of infections, and increased incidence of malignancies.

One of the questions remains unanswered as to whether the cells expressing high levels of pro-apoptotic molecules and low levels of anti-apoptotic molecules represent a subset of lymphocytes that are normally present in young subjects, and aging represents an expansion of such a population. If this is true, increase in this population during aging would be as a consequence of increased production of this population or decreased death of a subset that does not express high levels of Fas/FasL and/or decreased level of Bcl-2. This latter possibility is unlikely because a subset of lymphocytes that expresses high levels of Fas/FasL and decreased level of Bcl-2 would undergo cell death earlier than the other subsets. Furthermore, it is unlikely that there is preferential increased production of this population in aging, since thymus during aging is almost completely involuted. Therefore, it is likely that lymphocytes with increased pro-apoptotic and decreased anti-apoptotic molecules may represent a prolonged in vivo activation as a reflection of age.

In summary, T cell deficiency in aging appears to be, at least in part, due to an increased apoptosis associated with increased expression of pro-apoptotic molecules and decreased expression of anti-apoptotic molecules.

Acknowledgments

We thank Dr. S. Nagata for providing mAb against FasL, and Drs. S. Kitada and J. Reed for gift of Bax Ab.

References


Hunter, J. J., and T. G. Parlow. 1996. A peptide sequence from


Strasser, A., A. W. Harris, D. C. S. Huang, P. H. Krammer, and S. Cory. 1996.


Subcell. Biol. 16:58.

Science 267:1449.


Yoshino, T., E. Kondo, L. Cao, K. Takashiki, K. Hayashi, S. Nomura, and T. Akagi. 1994. Inverse expression of Bcl-2 protein and Fas antigen in lympho-

Strasser, A., A. W. Harris, D. C. S. Huang, P. H. Krammer, and S. Cory. 1996.


Owen-Schaub, L. B., S. Yonehara, W. Crump III, and E. A. Grimm. 1992. DNA fragmentation and cell death is selectively triggered in activated human lympho-


