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Fas Is Expressed Early in Human Thymocyte Development But Does Not Transmit an Apoptotic Signal

Morgan Jenkins,* Mary Keir,* and Joseph M. McCune²*†

We investigated the expression and function of Fas on human thymocytes prepared from fetal and pediatric tissue specimens and from SCID-hu Thy/Liv grafts. Unlike mouse thymocytes, human thymocytes exhibited a pattern of Fas expression skewed to immature cells, in that the highest expression was seen on double negative thymocytes and on intrathymic T progenitor cells. Fas expression was intermediate on double positive human thymocytes, and low or negative on mature single positive CD4 and CD8 medullary thymocytes. In spite of this relatively abundant surface expression, cross-linking of Fas with agonist mAb was incapable of triggering an apoptotic signal in human thymocytes. Apoptotic signaling was not enhanced by treatment with cycloheximide, nor by restoring a cosignaling milieu by addition of thymic stromal cells. Mouse thymocytes were induced to apoptosis by cross-linked recombinant soluble human Fas ligand both in vitro and in vivo, though human thymocytes were also resistant to this mode of receptor ligation. Membrane-bound Fas ligand also induced apoptotic death in murine thymocytes but not in human thymocytes. Human thymocytes were as sensitive as Jurkat cells, however, to apoptosis induced by TNF-α, suggesting that these cells have a signaling defect before activation of the earliest caspases. These data demonstrate a durable and specific resistance of human thymocytes to apoptosis induced through Fas receptor engagement, and reveal significant species-specific differences in the biology of thymocyte-programmed cell death. The Journal of Immunology, 1999, 163: 1195–1204.

F as (CD95, APO-1) is a member of the TNF-receptor superfamily that plays a unique role in programmed cell death (PCD) of lymphoid cells (1–3). Fas is required for the PCD of mature T cells by activation-induced cell death (4–7), and loss-of-function defects in Fas in humans (8–10) and mice (11–13) result in lymphoproliferative disorders. Recent thymic emigrants appear to lack expression of Fas, and naive T cells with the CD45RO phenotype and neonatal T cells do not express Fas (14). Up-regulation of Fas occurs during activation of T cells, whether by activation through the TCR (15), mitogens (14), or superantigen (16). Fas up-regulation on activated T cells is often followed by sensitization of the cell to apoptosis mediated by ligation of Fas (17, 18), but exceptions to this sequence of events have been increasingly recognized (19–22).

A role for Fas in early lymphocyte development is less well characterized, though it is clear that Fas signaling in the periphery frequently differs from that found in the central hematolymphoid organs. Bone marrow hematopoietic progenitor cells are largely negative for Fas surface expression (23, 24), but Fas may be transiently expressed during the development of hematopoietic precursors (25, 26). Exposure of CD34⁺ hematopoietic progenitor cells to TNF-α or IFN-γ induces Fas expression followed by sensitivity to Fas-induced apoptosis (23, 25, 27). The Fas-dependent apoptotic death of progenitor cells has been cited as a possible pathogenetic mechanism for aplastic anemia (27). In contrast to bone marrow cells, thymocytes are reported to constitutively express abundant Fas both in mouse (24, 28, 29) and human tissues (30, 31). Conflicting data have been reported, however, regarding the pattern of Fas expression on different thymocyte subpopulations. Fas-positive thymocytes in the mouse appear to be heterogeneous in their response to Fas ligation: all subpopulations of mouse thymocytes beyond the double positive (CD4⁺CD8⁺) stage express equivalent amounts of Fas on the cell surface, but single positive (CD4⁺CD8⁻ and CD4⁻CD8⁺) cells appear to be relatively resistant to apoptosis induced by mAbs to Fas, while double positive CD4⁺CD8⁺ cells are readily induced to apoptosis (29, 32).

It has been postulated that Fas may participate in the normal apoptotic death of thymocytes, but a clear role for Fas in negative selection is not well established. lpr mice with a loss-of-function mutation in Fas appear to have normal negative selection (33), as do mice in which the Fas gene has been knocked out (13), suggesting that Fas is not required for the apoptotic elimination of self-reactive thymocytes. Mouse transgenic models of negative selection also support the notion that Fas is responsible for peripheral deletion of at least some classes of T cells but does not participate in thymic deletion (34). Mice made genetically deficient in Fas-associated death domain protein (FADD)³ (35), an essential signal protein in the Fas pathway, are defective in thymocyte development, suggesting that Fas or related receptors that signal through FADD participate in thymocyte development. When a dominant negative FADD is expressed in mouse thymocytes, a somewhat different phenotype is seen, with enhanced apoptosis and negative selection of thymocytes (36).

In this study, we have evaluated the expression and function of Fas on different subpopulations of human thymocytes. In contrast

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³ Abbreviations used in this paper: FADD, Fas-associated death domain protein; TC, Tri-Color; GFP, green fluorescent protein; rs, recombinant soluble; DN, double negative; DP, double positive; SP8, single positive CD8; SP4, single positive CD4; ITTP, intrathymic T progenitor cells.
to the situation described for the murine thymus, we found high levels of Fas expressed on immature classes of human thymocytes. However, human thymocytes displayed marked resistance to Fas-mediated apoptosis, whether triggered by mAb to Fas receptor, soluble Fas ligand, or membrane-bound Fas ligand. Human thymocytes are nonetheless sensitive to apoptosis induced by TNF-α, suggesting that resistance to apoptosis induction is Fas-specific and operates at an early step in Fas signaling.

Materials and Methods

Animals

SCID-hu Thy/Liv, C57BL/6, and BALB/c mice were housed in specific pathogen-free conditions. SCID-hu Thy/Liv mice were constructed as described (37, 38) from fetal human thymus and liver tissue implanted under the kidney capsule, and used within 8 mo after transplantation. Protocols for the care and use of animals were approved by the University of California, San Francisco (UCSF) Committee on Animal Research.

Human tissue

Human fetal thymic tissue was obtained from tissues obtained at second trimester elective abortions after informed consent. Surgical specimens from children aged 1 wk to 9 years of age undergoing cardiac repair procedures were the source of pediatric thymocytes. Protocols governing the collection and use of human fetal and pediatric tissues were approved by the UCSF Committee on Human Research.

Cell culture

Jurkat E6 clone 1 cells were obtained from Arthur Weiss (UCSF) and 293-T cells were obtained from David Baltimore (Massachusetts Institute of Technology, Cambridge, MA). Jurkat cells were maintained in RPMI medium with 10% FCS, and 293-T cells in DMEM with 10% FCS. Ficol-Sepharose purification was performed with mononuclear cells of PBMC separated by density gradient centrifugation. Cells were cultured in RPMI supplemented with 1.35 mM 2-deoxyguanosine (Sigma) to deplete thymocytes. Tissue fragments were digested with collagenase B (Boehringer-Mannheim, Indianapolis, IN) and IL-2 (10 U/ml) (Boehringer Mannheim, Indianapolis, IN) for 2 days before addition to apoptosis assays.

Fetal thymic organ culture

The method for human fetal thymic organ culture has been described in detail (39). In brief, human fetal thymus was minced into fragments of ~1 mm³ and cultured on prewetted raft constructs of Gelfoam (Upjohn, Kalamazoo, WI) overlaid with 0.8-μm Nucleopore filters (Corning, Acton, MA). Culture medium was RPMI with 10% FCS and 293-T cells in DMEM with 10% FCS. Ficol-Sepharose purification was performed with mononuclear cells of PBMC separated by density gradient centrifugation. Cells were cultured in RPMI supplemented with 1.35 mM 2-deoxyguanosine (Sigma) to deplete thymocytes. Tissue fragments were digested with collagenase B (Boehringer Mannheim) and Dnase (Calbiochem, La Jolla, CA) and washed in PBS before depletion of CD3 + cells with biotinylated anti-human CD3 (Becton-Dickinson, San Jose, CA) and streptavidin-coated magnetic beads (Dynal, Lake Success, NY). Purified thymic stromal cells were added in a ratio of 1:6 to 1:10 freshly disaggregated fetal human thymocytes from another donor and suspended in 40 μl RPMI with 10% FCS and supplements. Replicate suspension cultures were pipetted into wells of a sterile support (GENuc modules; Nunc, Naperville, IL). The support was inverted in a humidified dish and incubated at 37°C in 5% CO₂. Medium was supplemented daily to replenish evaporative losses.

Flow cytometric analysis of Fas expression on cells

Jurkat cells, mouse thymocytes, cells from human fetal thymus, pediatric thymocytes, or SCID-hu Thy/Liv grafts were stained and analyzed within 6 h of harvest. Longer storage of tissue or cells was found to result in altered Fas expression levels. Magnetic bead depletion of CD8-positive human fetal thymocytes was accomplished using biotinylated anti-human CD8 Ab (Becton Dickinson) and streptavidin-coated magnetic beads. Surface staining for Fas employed one of three methods: 1) using affinity-purified N-18 rabbit anti-human Fas Ab or an affinity purified Ab control (rabbit anti-mouse IgG) (Santa Cruz Biotechnology, Santa Cruz, CA) as a secondary stain; 2) using mouse monoclonal IgM anti-Fas (CH-11; Immunotech, Westbrook, ME) or mouse IgM as a primary stain, and FITC-conjugated anti-mouse IgM (Caltag) as a secondary stain; 3) using recombinant soluble Fas ligand (see below) as a primary stain (at 25 μg/ml) followed by biotinylated anti-Flag Ab (M2; Sigma) and then by FITC-conjugated avidin (Becton Dickinson) or Tri-color (TC)-conjugated streptavidin (Caltag). For three-color analysis, either FITC- or PE-conjugated anti-human CD4 (Becton Dickinson) and TC-conjugated anti-human CD8 (Caltag) were included with the secondary Ab. For four-color analysis, the staining regimens included FITC-conjugated anti-human CD4, PE-Cy5-conjugated streptavidin (Coulter, Palo Alto, CA) or TC-conjugated (Caltag) anti-human CD8, and APC-conjugated anti-human CD3 (Caltag) with the secondary stain. Three-color analysis was performed on a FACS (Becton Dickinson) and four-color analysis on a FACSVantage (Becton Dickinson). Cytometric analysis utilized CellQuest software (Becton Dickinson). Forward and side gates defining live thymocytes were used for analysis, and isotype controls were used to set thresholds defining 99th percentile for negative stains. Four-color analysis with the combination of APC and TC excluded cells positive in the FL-3 channel to eliminate fluorescence channel spillover.

Apoptosis assays

In vitro apoptosis assays used suspensions of dissociated human fetal thymus, mouse thymus, or Jurkat cells (Jurkat E6 clone 1) cultured in round-bottomed 96-well trays at a density of 1–2 × 10⁶ cells/ml for thymocytes or 2–5 × 10⁵ cells/ml for Jurkat cells in RPMI medium with 10% FCS and incubated 18–20 h at 37°C in 5% CO₂. Culture additives, including monoclonal mouse IgM anti-human Fas Ab (CH-11), hydrocortisone (5 μM), and dexamethasone (1 μM; Sigma) were added just before incubation. In other experiments, cycloheximide (Sigma) was added (to 30 μM final concentration) 30 min before addition of other agents. Secondary cross-linking agents, e.g., goat anti-human IgM (Caltag) or biotinylated anti-Flag Ab (M2; Sigma), were added 10 min after other culture additives. Apoptotic cells were quantitated by flow cytometric analysis after staining with green fluorescent protein (GFP)- annexin V (generous gift of Joel Ernst (UCSF)) (41). Primary cells (i.e., those from SCID-hu Thy/Liv grafts, fetal thymic organ cultures, mouse liver, or mouse thymus) were permeabilized by fixing in PBS with 2% paraformaldehyde and calcium chloride (staining and wash solution), followed by pipette trituration and filtering through a 70-μm nylon mesh. Cells from reaggregate cultures were dispersed by pipette trituration and filtering. Cell suspensions (1 × 10⁶ cells/ml) were incubated with 1.5 μg/ml GFP- annexin V for 20 min at 4°C and washed once before analysis. For analysis of apoptosis in thymocyte subpopulations, cells were costained with PE-conjugated anti-human CD4 and TC-conjugated anti-human CD8 or PE-conjugated anti-mouse CD4 and TC-conjugated anti-mouse CD8A (Caltag). In many experiments, propidium iodide (Molecular Probes, Eugene, OR) at 5 μg/ml was added to an aliquot of cells before flow cytometric analysis to confirm specificity of GFP-annexin V staining of apoptotic cells. Forward and side scatter gates were set to exclude debris, and percent GFP-annexin V-positive cells was enumerated for each sample in duplicate or triplicate wells. Specific apoptosis induced was calculated as follows: % apoptosis = (% GFP-annexin V positive cells (experimental)) − (% GFP-annexin V positive cells (control))/[100 − (% GFP-annexin V positive cells (control))]. SDs for replicate samples were consistently within 10% of mean values.

Cloning and expression of Fas ligand and Flag-tagged recombinant soluble Fas-ligand

The full-length transcript for human Fas ligand was amplified from reverse transcribed RNA isolated from 2-activated primary human fetal thymocytes porating restriction sites EcoRI and XbaI (sense, CCGCGATATTCTCGACCGCTTCTCAATTAC; antisense, CGCTTCTAGATAGGCTTATAT AACGCC). The PCR product was digested to create overhangs and cloned into the monolayer cultures expressing Fas ligand or control monolayers transduced with the parent vector.

The strategy for preparing a recombinant soluble Fas ligand protein tagged with the Flag epitope (rFas Flag-ligand) was based on the method of Schneider et al. (42, 43). A Flag-tagged vector incorporating the multiple cloning restriction sites EcoRI and XbaI (sense, CCGGAATTCTATGAGCAGCCCTTCAATTAC; antisense, GGCTTGAGATAGGCTTATATT AAGCC). The PCR product was digested to create overhangs and cloned into the monolayer cultures expressing Fas ligand or control monolayers transfected with the parent vector.

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TGTTAAGCTTCGCATTGAAGCTTATAAATG). The restriction enzyme-cleaved product was ligated in frame to a HindIII/KpnI fragment encoding the first 90 nucleotides of the prolactin signal sequence, followed by a modified Flag epitope (44) and ligated into pcDNA3. Expression of rs Fas ligand-Flag in 293T cells was accomplished through calcium phosphate transfection, and cells expressing recombinant protein were selected with G418. Supernatants collected from selected cell cultures were clarified by centrifugation and passed through a 0.2-μm filter. Supernatants were either used unpurified for tissue culture experiments or purified on an anti-Flag affinity column (M2, Sigma) according to the manufacturer’s instructions, and the neutralized eluate further concentrated with Microcon10 centrifugal concentrators (Amicon, Beverly, MA).

In vivo treatment with rs Fas ligand-Flag. Two regimens for in vivo administration were used. SCID-hu Thy/Liv mice were injected i.v. with 12.5 μg affinity-purified rs Fas ligand-Flag protein in 0.1 ml volume, followed immediately by i.v. injection of 50 μg of anti-Flag Ab in the same volume. Control animals received saline in place of rs Fas ligand-Flag. Alternatively, mice received 5 mg biotinylated M2 Ab in 0.2 ml PBS. Twenty-four hours later, or given signs of obvious illness, animals were euthanized, and cells from mouse liver, spleen, and human cells from the Thy/Liv grafts were prepared for annexin staining.

Results

Fas expression is highest on the least mature human thymocytes

Expression of Fas on various populations of differentiating human thymocytes was assessed with three- and four-color multiparameter flow cytometry. Human thymocytes were obtained from second trimester fetal tissue, thymus specimens of infants and children obtained at cardiac surgery, and from human fetal thymus grafted together with human fetal liver cells under the kidney capsule of SCID mice (SCID-hu Thy/Liv). Fas was highly expressed on CD4+CD8− (double negative, DN) cells and to a moderate degree on CD4+CD8+ (double positive, DP) thymocytes (Fig. 1A). CD4+CD8− (single positive CD8, SP8) thymocytes were low or negative for Fas expression, while CD4+CD8− (single positive CD4, SP4) thymocytes were heterogeneous in Fas staining intensity; a minority of SP4 cells expressed Fas at equal or higher levels than CD4+CD8− thymocytes, while the majority of CD4+CD8− cells were low or negative for Fas expression (Fig. 1A). As described previously (45, 46), CD4+CD8− human thymocytes were found to comprise two subpopulations differing in CD3 surface expression: CD3+CD4+CD8− thymocytes (mature, medullary thymocytes) and CD3−CD4+CD8− cells (also termed intrathymic T progenitor cells (ITTP cells)). The latter subpopulation of immature thymocytes represented ~10–25% of CD4+CD8− human thymocytes (or 1–4% of total human thymocytes). Consistent with earlier reports (45), CD3−CD4+CD8− thymocytes were found to have higher forward light scatter and a higher fraction of cells in cycle than double positive or single positive thymocytes (data not shown). In addition to these characteristics of a dividing cell population, many CD3−CD4+CD8− fetal thymocytes were found to express CD34 and have the capacity to mature into CD4+CD8− thymocytes on adoptive transfer into thymic reaggregate cultures (data not shown). This population thus contains true progenitor cells and is not merely comprised of more mature CD4+CD8− cells that have down-regulated CD3 expression.

Four-color flow cytometric analysis was used to directly determine whether the ITTP subpopulation had the highest expression of Fas. Among CD4+CD8− cells in most thymic tissues sampled, the expression of surface CD3 was inversely related to that of Fas (Fig. 1B), confirming that ITTPs are the Fas-high cells within the single positive CD4 gate. Identical results were obtained when SCID-hu Thy/Liv grafts were used as the source of thymocytes and depleted of CD8+ cells with mAbs and magnetic beads (Fig. 1C).

To determine whether Fas expression in the human fetus is different from that in the postnatal organ, thymic tissue was obtained from four human fetuses (17–22 wk of gestation), from two children (both 9 yr of age) undergoing cardiac repair procedures, and from two Thy/Liv grafts established in SCID-hu mice 4–6 mo before analysis. Bars show mean ± SE of measurement (SEM) for geometric mean fluorescence for Fas staining intensity in each subset.

FIGURE 1. Fas expression on the surface of differentiating human thymocytes. A. Three-color flow cytometric analysis of human fetal thymocytes using N-18, a rabbit polyclonal Ab to the N-terminal portion of Fas. Histograms show Fas surface staining intensity on gated subsets: CD4+CD8− (DN), CD4+CD8+ (DP), CD4+CD8+ (SP8), and CD4+CD8− (SP4). Data are shown from one experiment representative of seven. B. Four-color cytometric analysis of human fetal thymocytes employing FITC-CD4, TC-CD8, and APC-CD3, gated on SP4 cells. Indirect staining for Fas using purified polyclonal N-18 rabbit Ab and PE-conjugated goat anti-rabbit Ab was performed as described in Materials and Methods. C, Fas expression on human fetal thymocytes from a SCID-hu Thy/Liv graft depleted of CD8+ cells with biotinylated Ab to CD8 and streptavidin beads. The residual CD4+CD8− cells were stained with TC-conjugated anti-CD3 and N-18 rabbit polyclonal anti-Fas Ab. D, Fas surface expression on subsets of human thymocytes from different sources assessed by four-color flow cytometric analysis. Thymus tissue was obtained from four human fetuses (17–22 wk of gestation), from two children (both 9 yr of age) undergoing cardiac repair procedures, and from two Thy/Liv grafts established in SCID-hu mice 4–6 mo before analysis. Bars show mean ± SE of measurement (SEM) for geometric mean fluorescence for Fas staining intensity in each subset.
from two children undergoing cardiac repair procedures and from SCID-hu Thy/Liv grafts 4–6 mo after transplantation, a time frame when these cells may be expected to have matured developmentally from the fetal thymus source. As shown in Fig. 1D, these tissues displayed a pattern of Fas expression similar to that of human fetal thymocytes, with the highest Fas expression on immature thymocyte subpopulations, intermediate levels of expression on CD4+CD8+ thymocytes, and lowest expression on mature single positive cells.

Cross-linking of Fas with agonist mAbs does not induce apoptosis in human thymocytes

Since cross-linking of Fas receptor by Abs is sufficient to generate a death signal in susceptible cells (47–49), we tested whether Fas expressed on human thymocytes could transmit a death signal. Cells were incubated with various death-inducing stimuli, including a mouse IgM mAb to human Fas (CH-11), which has well-characterized apoptosis-induction properties, and apoptosis was quantitated using GFP-annexin V binding. Jurkat T cells, and to a lesser degree PBMC blasts, were induced to apoptosis after 18 h of incubation with CH-11 Ab (Fig. 2A). In contrast, human fetal and pediatric thymocytes did not undergo apoptosis after cross-linking of surface Fas receptors with CH-11 Ab, even though they were sensitive to apoptosis induced by hydrocortisone treatment. Doses of CH-11 Ab from 50 ng/ml through 1 μg/ml, well above the 50% effective dose for Jurkat cells, were all ineffective in inducing thymocyte apoptosis (Fig. 2B). Indirect immunofluorescent staining of thymocytes with the CH-11 anti-Fas monoclonal confirmed that the Ab bound to human thymocytes, although to a lesser degree than the polyclonal N-18 rabbit anti-Fas antiserum (see below). CH-11 Ab also failed to induce apoptosis of human thymocytes after it was cross-linked with anti-IgM Ab or after it was immobilized on tissue culture plates (data not shown).

FIGURE 2. Human fetal thymocytes are resistant to apoptosis following cross-linking of Fas with IgM mAb to human Fas. A, Indicated cells were cultured in the presence of 5 μM hydrocortisone (HC), 1 μg/ml mouse monoclonal IgM anti-Fas (CH-11), or without additives for 18 h, and apoptosis assessed by GFP-annexin V staining. Bars show mean ± SD of percent apoptosis observed in triplicate cultures. B, Anti-Fas monoclonal CH-11 was added at indicated concentrations to replicate cultures of human fetal thymocytes or Jurkat cells. Bars show mean ± SD of percent apoptosis. C, Kinetic analysis of apoptotic response of human fetal thymocytes and Jurkat cells.

FIGURE 3. Fas-high subsets of human thymocytes are not induced to apoptosis by cross-linking CH-11 anti-Fas mAb. A, Human fetal thymocytes were incubated with CH-11 anti-Fas IgM mAb or hydrocortisone (HC) before apoptosis assessment with multiparameter flow cytometry. Mean percent apoptosis and SD of triplicate cultures are shown. B, Human fetal thymocytes expressing high levels of Fas (above 97th percentile for fluorescence intensity) or with the phenotype of intrathymic T progenitor cells were sort-purified and cultured in parallel with total, unselected fetal thymocytes, and apoptosis induced with hydrocortisone or anti-Fas mAb. C, Cycloheximide does not augment the apoptotic response of human thymocytes to cross-linking anti-Fas Ab. Human fetal thymocytes were incubated in the presence or absence of 30 μM cycloheximide for 30 min before addition of anti-Fas monoclonal CH-11, hydrocortisone, or no further addition. Bars show mean ± SD for percent annexin-positive cells (rather than for percent apoptosis) to demonstrate inhibition of spontaneous apoptosis by cycloheximide.
membrane-bound Fas ligand-Flag or control Flag-tagged protein, followed 24 h later by i.p. injection of cross-linking anti-Flag Ab, resulted in survival of injected animals for an additional 24 h; at this time, they were sacrificed, and mouse liver and human Thy/Liv grafts were analyzed for apoptosis. Mouse hepatocytes from SCID-hu mice injected with the rs Fas ligand-Flag were significantly injured, with over half of cells staining positive with GFP-annexin V, compared with 9% of control hepatocytes. Apoptosis in mouse spleen was comparable in rs Fas ligand-Flag-injected and control animals (7.0 vs 5.9% GFP-annexin V positive). Likewise, apoptosis of human thymocytes in the Thy/Liv grafts was not significantly higher in rs Fas ligand-Flag treated animals than in controls (5.0 vs 4.6% GFP-annexin V positive.)

**Membrane-bound Fas ligand fails to induce apoptosis in human fetal thymocytes**

Experiments in mice indicate that the soluble form of Fas ligand may not be as efficient as membrane-bound forms in the induction of apoptosis (52, 55, 56). To examine whether human thymocytes are resistant to apoptosis induced by membrane-bound Fas ligand,
cell monolayers expressing high levels of Fas ligand (Fig. 5, A and B) were cocultured with Jurkat cells, thymocytes from BALB/c or C57BL/6 mice, or fetal human thymocytes. As with soluble Fas ligand, the membrane-bound human form efficiently induced apoptosis. We therefore compared the sensitivity of Jurkat cells to human Fas ligand-induced apoptosis (Fig. 1C). Analysis of thymocytes from BALB/c mice and from C57BL/6 mice gave equivalent results. In contrast, no subpopulation of human thymocytes appeared to be sensitive to apoptosis induction by membrane-bound Fas ligand.

Presentation of Fas ligand on the surface of transfected cells may not recapitulate the signaling milieu of the human thymus. Human thymic stromal cells express Fas ligand (57), and apoptosis signaled through Fas has also been reported to either require, or to be facilitated by, cosignaling through CD3 or other surface molecules (51). We therefore investigated whether thymic stromal cells contribute to Fas-induced apoptosis of human thymocytes with in vitro culture conditions that preserve the microenvironment of the thymus, including thymic epithelial cells, macrophages, and dendritic cells. We first treated established organ cultures of human fetal thymus fragments either with hydrocortisone or anti-Fas mAb and monitored apoptosis induction. As shown in Fig. 6A, while hydrocortisone efficiently penetrated the organ culture and induced apoptosis of thymocytes above spontaneous levels, anti-Fas Ab had no effect over a time period of 48 h. We next examined reaggregated human thymus tissue in hanging drops, as these have been shown to preserve the normal stromal architecture of thymus and to allow for positive and negative selection. As shown in Fig. 6B, addition of hydrocortisone to reggregate thymic cultures in hanging drops resulted in the apoptotic death of thymocytes over 1–2 days, while anti-Fas Ab had no effect.

An early step in the Fas apoptosis signal pathway is impaired in human thymocytes

By analogy to other TNF/nerve growth factor receptor systems, inhibition of apoptotic signaling pathways through Fas expressed on human thymocytes may occur at a prereceptor level (e.g., ligand binding by soluble receptor), at the receptor level, or at a postreceptor level (3). Apoptotic signaling pathways for TNF-α and Fas converge at FADD, and because dominant negative FADD can inhibit apoptosis induced through Fas as well as TNF-α (58, 59) it is possible to assess the integrity of the distal Fas signaling pathway in human thymocytes by testing the sensitivity to TNF-α-induced apoptosis. We therefore compared the sensitivity of Jurkat cells and freshly isolated human thymocytes to apoptosis induced

FIGURE 5. Induction of apoptosis by monolayer cells expressing membrane-bound Fas ligand. A and B, FACS analysis of expression of Fas ligand in 293-T cells 2 days after transfection. Histograms show isotype staining (dotted line) and staining by biotinylated anti-human Fas ligand Ab (solid line) costained with TC-conjugated streptavidin for 293-T cells transfected by pDNA3 (A) or human Fas ligand expression vector (B). C, Induction of apoptosis in cells cocultivated with Fas ligand-expressing monolayers. After 18 h coculture, supernatant cells were washed free of monolayer cells and apoptosis induction was quantitated using percent GFP-annexin V staining in cells exposed to vector-transfected monolayers as the spontaneous apoptosis control. Data are mean and SD from four cultures in two separate experiments. D, Subset analysis of apoptosis induced by Fas ligand monolayers. Fetal human and mouse (BALB/c and C57BL/6) thymocytes were analyzed by three-color flow cytometric analysis, and percent apoptosis was calculated for gated subsets. Data are mean and SD for duplicate cultures.

FIGURE 6. Human thymocytes cocultured with thymic stromal cells are resistant to Fas-induced apoptosis. A, Human fetal thymus fragments were maintained in fetal thymic organ culture atop Gelfoam rafts as described in Materials and Methods. Hydrocortisone (■) or CH-11 anti-Fas Ab (□) was added to culture medium at day 0 and replicate cultures harvested after 24 or 48 h and stained with GFP-annexin V. B, Thymic stromal cells from organ cultures treated with 2-deoxyguanosine were added to freshly-isolated human thymocytes and cultured in hanging drops with no addition, or with hydrocortisone or anti-Fas IgM mAb. Percent apoptosis ± SD of replicate cultures was assessed after 24 and 48 h incubation.
by TNF-α or by anti-Fas mAb. As expected, the degree of apoptosis induced after 18 h exposure to TNF-α was low in Jurkat cells, likely due to concomitant activation of NF-κB (60). Nevertheless, TNF-α induced low but highly reproducible levels of apoptosis that were equivalent in Jurkat cells and human fetal thymocytes (Fig. 7A). Murine thymocytes were also sensitive to TNF-α-induced apoptosis to a similar degree in 18-h assays (data not shown). While anti-Fas Ab was ineffective against human thymocytes at any dose (Fig. 7B), Jurkat cells and thymocytes were both sensitive, in a dose-dependent manner, to TNF-induced apoptosis (Fig. 7C). Taken together, these data show that the resistance of human thymocytes to apoptosis is Fas-specific and that the signal step(s) likely to be involved are those before FADD activation of caspase 8 (FLICE).

Discussion

Concomitant with the discovery that Fas signaling was associated with the death of lymphocytes in the periphery (5, 6, 15), it became apparent that cells bearing the Fas receptor differed in their response to Fas ligation (14, 18, 23). The apparent disconnect between surface expression of Fas and its capacity to direct apoptosis upon engagement was also apparent among subsets of mouse thymocytes, where mature single positive CD4 and CD8 cells express Fas at levels equivalent to double positive cells but are not induced to apoptosis upon exposure to cross-linking anti-Fas mAbs (29, 61). Appreciation of the complexity of Fas signaling has been expanded by the recent discovery of a multiplicity of Fas signaling pathways that may operate in a tissue-specific fashion. These pathways differ in their response kinetics and in their dependence on mitochondrial transmembrane potential (62). Cell-specific Fas signal pathways have also been described with different requirements for particular caspases in the initiation of the effector cascade (63).

In this study, we have addressed the expression and functionality of Fas in the human thymus. Our data highlight not only the tissue specificity but also the species specificity of apoptotic responses initiated through the Fas receptor.

We found, first, that the expression of Fas on developing thymocytes differed importantly from that of the mouse. As reported previously (30, 31), mature human CD4 and CD8 thymocytes were found to express low levels of Fas, while double positive CD4+CD8+ cells were intermediate in Fas expression. In contrast with these reports, and similar to the findings of others (22), we consistently found heterogeneity of Fas expression among single positive CD4 thymocytes. CD4+ cells with the highest expression of Fas were confirmed to have the phenotype CD3+CD4+CD8−, which identifies intracellular T progenitor cells (45). In addition, Fas was highly expressed on human CD4+CD8− cells. These two classes of thymocytes are the most immature thymocytes and account for ∼5% of total thymocytes. Fas expression in the human thymus thus appears to vary across a developmental spectrum: highest on the earliest intrathymic progenitor cells, dropping to intermediate levels among CD4+CD8− cells, and falling to low or negative levels on single positive CD4 and CD8 cells. This pattern of high Fas expression differs markedly from that described for the mouse thymus, in which Fas is first expressed on double positive CD4+CD8+ cells and is retained at equal levels on single positive thymocytes (24, 29).

Secondly, and also in contrast with mouse thymocytes, we observed that human thymocytes were resistant to Fas-induced apoptosis. This was the case whether the thymocytes were from fetal tissue (15–23 wk gestational age), from pediatric thymus (through 9 years of age), or from SCID-hu Thy/Liv grafts (harvested from 4 to 6 mo following transplantation). Given the consistency of this finding across these age groups, it is unlikely that resistance of human thymocytes to Fas-induced apoptosis is characteristic of a single stage of development (64). Few experimental data have been reported concerning the sensitivity of human thymocytes to Fas-induced apoptosis. Yonehara et al. (31) reported that an IgM anti-Fas Ab (AX6) lacked the ability to induce apoptosis of human thymocytes, though limited DNA fragmentation was reported. Here, we also show that human thymocytes resisted the induction of apoptosis by several other agonists, including mAbs to Fas, cross-linked rs Fas ligand, and membrane-bound Fas ligand. This last test was important because of the observation that soluble ligands of Fas may have diminished apoptotic activity (43) or may actually antagonize Fas signaling in mouse (56) and human cells (55). It has been argued that “agonist” mAbs to Fas may, under
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certain conditions of receptor density, mimic this antagonistic function (52).

Our data demonstrate that human thymocytes, regardless of source and of method of receptor ligation, resist apoptosis induction through Fas both in vitro and in vivo. The durable resistance of human thymocytes to Fas-signalized apoptosis adds to the list of important differences in Fas biology reported between mice and humans. These observations include: 1) Soluble Fas ligand released from human cells is cytotoxic to Fas-positive cells (15, 65), while soluble Fas ligand from mouse cells generally is not (55); 2) Agents capable of triggering apoptosis in mouse cells may not effectively trigger apoptosis in human cells (42); 3) Superantigen-mediated deletion of human thymocytes cells requires Fas (31), while Fas appears to be dispensable for this purpose in the mouse (13, 51); 4) Ligation of Fas may synergize with anti-CD3 in stimulating proliferation of human T lymphocytes (66), while the same combination synergizes to kill mouse thymocytes (51). These observations point to a fundamental difference between these species in the regulation and utilization of Fas signaling in maintenance of lymphocyte homeostasis.

These data provide some insight into the biology of apoptotic signaling in the human thymus. First, although mouse models have suggested that some thymocytes may depend upon Fas for negative selection, our data suggest that Fas is unlikely to participate in negative selection in the human thymus. Second, unlike mouse thymocytes, in which the apoptotic response to Fas engagement is enhanced by protein synthesis inhibition (29, 51), apoptosis of human thymocytes is not augmented by exposure to cycloheximide. In fact, we found that cycloheximide reduced levels of spontaneous apoptosis in cultures of dissociated human thymocytes by a small but significant degree. These data suggest that the balance of proapoptotic and antiapoptotic forces in human thymocytes is fundamentally different from that of PBMC or murine thymocytes. The signaling and effector proteins responsible for apoptosis of cells through Fas are already synthesized in the cell (67, 68), which accounts for the speed with which an apoptotic signal may result in cell death. Negative regulators of apoptosis that act at early steps of the Fas signal cascade, such as Toso (69), FLIP (70), and cIAP (71), may mediate the observed enhancement of Fas-induced apoptosis by cycloheximide in mouse cells, but are unlikely to account for resistance of human thymocytes. In addition, these proteins all operate at proximal signaling steps and would be expected to impact apoptosis signaled through TNF-α as well as through Fas, and so are unlikely to account for the Fas-specific effect we have described.

Data presented in this report do not point conclusively to the mechanism(s) of thymocyte resistance to Fas-induced apoptosis, but do permit some inferences. Our data do not seem to be consistent with the hypothesis that soluble Fas receptors, such as those encoded by alternatively-spliced transcripts found in both SLE patients and normal subjects (72), could account for such resistance. Elaboration of soluble receptors would be unlikely to block the binding of Fas ligand to freshly isolated and washed cells. In addition, alternatively-spliced Fas transcripts encoding soluble receptors have been found in activated PBMC (48) and in mouse thymocytes (73), although these cells do not display marked resistance to Fas-induced apoptosis. Moreover, we found that the binding of other anti-Fas Abs to human thymocytes was not blocked, suggesting that the mechanism was not a soluble decoy receptor.

Our data suggest a more complex picture regarding the likely mechanism of resistance of human thymocytes to Fas-induced apoptosis. The failure to induce thymocyte apoptosis with cross-linking IgM mAb to Fas implies that a step distal to receptor binding may be responsible for human thymocyte Fas resistance. Impaired multimerization of Fas receptor, or the inability of the Fas signal complex to recruit and activate FADD, are candidate mechanisms for this mode of resistance, as the pathways distal to these steps appear to be intact.

The apparent inability of Fas expressed on human thymocytes to signal apoptosis begs the question of what purpose is served by its expression in these cells at all. Hematopoietic progenitor cells in the liver have been reported to express Fas, which is likewise incapable of directing an apoptotic signal (74), leading to the suggestion that Fas may mediate different nonapoptotic functions in developing cells. Fas may participate in nonapoptotic signals important to development of thymocytes, either by promoting entry into the cell cycle (35, 36, 75) or by activation of Jun kinase though Daxx (76). Fas would thus join the list of receptors (including nerve growth factor receptor p75 (77), e-kit (78), and the receptors for TGF-β (79), and platelet-derived growth factor (80)), which are capable of delivering a death signal at one stage of development and a proliferative or developmental signal on another. Whether such nonapoptotic signaling pathways from Fas are triggered by specialized conditions for ligand binding, or by a unique ligand, remains to be investigated.

Regulation of Fas-mediated apoptosis may be therapeutically exploited for treatment of autoimmune diseases, tissue rejection, cancer, and immunosuppression resulting from HIV-1 infection. Control of apoptotic pathways in a tissue-specific manner represents a major challenge confronting such therapies. More detailed understanding of the regulation of Fas signaling in the thymus may provide additional insight into this problem.

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References


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