Functional Fas Expression in Human Thymic Epithelial Cells

By Nathalie Moulian, Claire Renvoizé, Colette Desodt, Alain Serraf, and Sonia Berrih-Aknin

Fas, a cell surface receptor, can induce apoptosis after cross-linking with its ligand. We report that Fas antigen is constitutively expressed in medullary epithelial cells of the human thymus. Expression is decreased in cultured thymic epithelial cells (TEC), similarly to HLA-DR antigen. TEC are resistant to anti-Fas-induced apoptosis after 4 days of primary culture, and this resistance is reversed by concomitant addition of cycloheximide. Cycloheximide also downregulated the expression of Fas-associated phosphatase-1, which has been found to inhibit Fas-induced apoptosis. This phosphatase could have been involved in the resistance to Fas-induced apoptosis observed on day 4 of TEC culture. When TEC were subcultured after 10 to 13 days of primary culture, exposure to interleukin-1β, tumor necrosis factor-α, and interferon-γ, alone or together, reinduced Fas mRNA and protein expression. In coculture with activated thymocytes, TEC also upregulated Fas protein expression. Cytokine-activated TEC became sensitive to apoptosis induced by an agonistic anti-Fas antibody. This apoptosis was inhibited by Z-VAD-fmk but not by Z-DEVD-fmk and DEVDase activity was slightly increased in Fas-stimulated TEC, suggesting that DEVDase activity is not sufficient to induce TEC apoptosis. Taken together, these data show that the Fas receptor is expressed in medullary epithelial cells of the human thymus and is able to induce apoptosis.

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Thymic tissue and TEC culture. Normal thymus fragments were obtained from infants aged from 5 days to 2 years undergoing heart surgery at Marie-Lannelongue Hospital (Le Plessis-Robinson, France). For some experiments, fragments of thymic tissue were flash-frozen in liquid nitrogen and stored at −80°C. Primary TEC cultures were established as previously described.33 Briefly, small fragments of human thymic tissue were washed in RPMI medium and transferred to 75-cm² culture dishes. The culture medium, RPMI 1640 supplemented with 20% horse serum (Life Technologies, Cergy-Pontoise, France), 0.2% Utrorser (Life Technologies), 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin was replaced twice a week. After 10 to 13 days of primary culture, the confluent monolayers were washed with phosphate-buffered saline (PBS) and treated with 0.075% trypsin (Life Technologies) and 0.16% EDTA for 5 minutes at 37°C. In some experiments, TEC were collected

MATERIALS AND METHODS

Thymic tissue and TEC culture. Normal thymus fragments were obtained from infants aged from 5 days to 2 years undergoing heart surgery at Marie-Lannelongue Hospital (Le Plessis-Robinson, France). For some experiments, fragments of thymic tissue were flash-frozen in liquid nitrogen and stored at −80°C. Primary TEC cultures were established as previously described.33 Briefly, small fragments of human thymic tissue were washed in RPMI medium and transferred to 75-cm² culture dishes. The culture medium, RPMI 1640 supplemented with 20% horse serum (Life Technologies, Cergy-Pontoise, France), 0.2% Utrorser (Life Technologies), 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 µg/mL streptomycin was replaced twice a week. After 10 to 13 days of primary culture, the confluent monolayers were washed with phosphate-buffered saline (PBS) and treated with 0.075% trypsin (Life Technologies) and 0.16% EDTA for 5 minutes at 37°C. In some experiments, TEC were collected
after 2, 4, 7, or 10 days of primary culture. The epithelial nature of the cells was checked by immunocytochemical analysis of cytocentrifuged cells, using the antikeratin monoclonal antibody CK-1 (Dako, Trappes, France). Our culture conditions select medullary epithelial cells, because cells collected after 10 to 13 days of culture strongly express antikeratin CK-1, a marker of the medullary epithelial network. The proportion of keratin-positive cells was consistently greater than 95%.

After 10 to 13 days of primary culture, TEC were subcultured (5 × 10⁵ cells/well) in 24-well Primaria plates (Polylabo, Paris, France) and incubated for 24 hours to allow them to adhere. After two washes with Hank’s Balanced Salt Solution (HBSS), the medium was replaced with RPMI supplemented with 5% horse serum and the following cytokines: 1 ng/mL recombinant human IL-1β (Sigma Chemical Co, Saint Quentin Fallavier, France), 10 ng/mL recombinant TNF-α (Genzyme, Cergy Saint Christophe, France), and 500 U/mL recombinant human IFN-γ (Genzyme), separately or together. TEC were treated with trypsin-EDTA after 24, 48, or 72 hours. After three washes, the cells were used for immunofluorescence studies.

**Immunofluorescence studies.** TEC were labeled fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR (Immunotech, Marseille, France) or anti-Fas antibodies (Dako). TEC were first incubated with anti-Fas (anti-CD95) monoclonal antibody (clone UB2; Immunotech) for 30 minutes at 4°C, then washed twice in Hank’s solution (HBSS) supplemented with 5% fetal serum calf, stained with biotin-coupled goat antiserum IgG antibody (Amersham), washed twice, and incubated with Quantum Red-conjugated streptavidin (Sigma).

Cell labeling was analyzed on a FACScan flow cytometer (Becton Dickinson, Grenoble, France) using Cell Quest software. A gate was set on intact cells by using forward- and side-scatter analysis; 10⁶ cells were analyzed in the gate. The proportion of cells expressing HLA-DR among total cells or the mean fluorescence intensity (MFI) of Fas staining was measured.

**TEC/thymocytes cocultures.** After 10 to 13 days of primary culture, TEC were subcultured in 24-well plates (0.5 × 10⁶ cells/well). Heterologous thymocytes were mechanically isolated by gently scraping fresh thymic tissue, filtering the cells through sterile gauze, and washing them with HBSS. After two washes of adherent TEC, 5 × 10⁶ thymocytes (in 1 mL RPMI containing 5% horse serum) were added per well. When indicated, 5 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) and 500 ng/mL ionomycin (Boehringer Mannheim, Meylan, France) were added to the coculture. After a 3-day coculture period, wells were washed three times and TEC were collected as previously described. After staining with anti-Fas antibody, a gate was set on TEC by using forward- and side-scatter analysis during the acquisition of data on the FACScan flow cytometer.

**Immunohistochemical analysis of thymic sections.** Acetone-fixed frozen sections 6-µm thick were incubated for 30 minutes at room temperature with polyclonal rabbit antikeratin antibody (Dako) and monoclonal anti-Fas antibody (clone UB2). They were then washed three times in PBS and incubated with rhodamine-coupled goat antiserum IgG antibody (Immunotech), washed twice, and incubated with Quantum Red-conjugated streptavidin (Sigma).

Cell labeling was analyzed on a FACScan flow cytometer (Becton Dickinson, Grenoble, France) using Cell Quest software. A gate was set on intact cells by using forward- and side-scatter analysis; 10⁶ cells were analyzed in the gate. The proportion of cells expressing HLA-DR among total cells or the mean fluorescence intensity (MFI) of Fas staining was measured.

**RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR).** Total RNA was extracted using the RNeasy plus kit (Qiagen, Paris, France), and then purified with 0.5 vol of 7.5 mol/L ammonium acetate and 2.5 vol of 100% ethanol, and centrifuged at 15,000 rpm for 30 minutes at 4°C. The pellet was washed in 75% ethanol, dried under a vacuum, and stored at −80°C after dissolution in water. The total RNA concentration was determined by measuring absorbance at 260 nm with a Gene Quant spectrophotometer (Pharmacia, Cambridge, UK). The purity of the RNA preparation was checked by measuring the 260 nm/280 nm ratio.

The oligonucleotide primers used for RT-PCR were from Genset (Paris, France) and the sequences were as follows: Fas primers, forward 5'-GACA-AAGGCCCATTTTCTTCCTC-3' and reverse 5'-ATTTACGGCAGTGTTCAGG-3'; FAP-1 primers, forward 5'-GGATACCGAAGTTGCAAAATTCCG-3' and reverse 5'-GGGTTGCAAGATCTGCTGCTG-3'; and reverse 5'-GGTCTC-CAACATGACGAGTGTTTG-3'. A 50-µL reverse transcription reaction mixture containing 1 µg of total RNA, 5 µL of 10X RT buffer (Eurobio, Les Ulis, France), 1.5 mM/L each dNTP (Eurobio), 2 U/RNAse inhibitor, and 4 U of avian myeloblastosis virus (AMV) reverse transcriptase (Eurobio) was incubated at 42°C for 60 minutes. PCR was performed in a total volume of 100 µL containing 10 µL of RT reaction mixture, 10 µL of PCR buffer (Eurobio), 1.5 mM/L MgCl₂, 0.5 µM/L of each primer, 0.2 mM/L of each dNTP, and 5U Taq DNA polymerase. A 3 µL aliquot of each sample was electrophoresed through a 2% agarose gel (Bioprobe, Paris, France), then purified with 0.5 vol of 7.5 mol/L ammonium acetate.
2.5 U of EurobioTaq II polymerase (Eurobio). The mixture was overlaid with mineral oil and amplified in a PHC3 thermal cycler (Techne, Cambridge, UK) as follows: denaturation at 94°C for 1 minute; annealing at 53°C (Fas), 60°C (FAP-1), or 58°C (β-actin) for 1 minute; and extension at 72°C for 2 minutes. The final elongation step lasted 10 minutes at 72°C. PCR products were analyzed on 1.5% agarose gel containing ethidium bromide.

**Western blotting.** In some experiments, TEC were collected and solubilized in a lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris, pH 8.0, 5 mmol/L EDTA, 1% Triton X-100, 0.02% NaN₃, 1 mmol/L PMSF (Sigma), and 0.15 U/mL aprotinin (Sigma) for 20 minutes at 4°C. Insoluble material was removed by centrifugation at 4°C for 10 minutes. After boiling, the samples (20 µg total protein) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (7.5%) and proteins were transferred to polyvinylidene difluoride (PVDF) membranes. A second SDS-PAGE gel was colored with Coomassie blue to check that similar amounts of protein were loaded into the gel. Blots were saturated in PBS containing 0.1% Tween-20 and 5% dry nonfat milk and incubated for 4 hours at 4°C in PBS containing 0.1% Tween-20, 0.1% dry nonfat milk, and 0.2 µg/mL polyclonal goat anti–FAP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibody was detected by using peroxidase-conjugated antigoat Ig (Santa Cruz Biotechnology). Immunoreactivity was determined using the ECL chemiluminescence reaction (Amer sham France S.A., Les Ulis, France).

**Statistical analysis.** Differences between groups were identified by using the Mann-Whitney or Wilcoxon test (Instat software). A difference was considered statistically significant if the P value was less than .05.

**RESULTS**

*Fas antigen is expressed in situ in human thymus.* Fas expression was analyzed on cryosections of human thymuses in double-staining experiments with antikeratin to visualize the epithelial network. We used a polyclonal antikeratin antibody that stains thymic epithelial cells more strongly in the medulla than in the cortex. Fas was mainly expressed in the medulla (Fig 1). Double-staining showed that most cells expressing keratin were also Fas-positive, whereas cortical epithelial cells were clearly Fas-negative. Thus, a subset of TEC express Fas antigen in the human thymus. A subset of human thymocyte (<3% of

**Fig 1.** Double immunofluorescence distribution of Fas and keratin in the human thymus. Frozen thymus sections were fixed in acetone and then double-stained with antikeratin (left) and anti-Fas (right) antibodies. After three washes they were stained with rhodamine-coupled antirabbit and FITC-coupled antimouse antibodies. The top plates (×30) show that Fas expression is mainly observed in the medullary area of the human thymus. Control sections (×20) were incubated with the fluorescent conjugates only. The lower plates are enlargements of the framed areas in the upper plates. The white arrows indicate medullary epithelial cells expressing keratin and Fas antigen.
total thymocytes) with a strong expression of Fas was previously described using cytofluorimetry. We could rarely distinguish Fas expression in some medullary thymocytes; thus, Fas expression in the human thymus is mostly epithelial.

**Fas antigen expression falls in cultured TEC.** We examined Fas and HLA-DR expression by immunofluorescence in TEC after 2, 4, 7, or 10 days of culture. Only about 1% of cultured TEC expressed HLA-DR after 10 days in primary culture, whereas 51% of TEC were HLA-DR–positive cells after 2 days (Fig 2). Fas antigen expression followed the same pattern and decreased threefold during primary culture, between day 2 and day 10.

**Fas antigen expression by cultured TEC is upregulated by cytokines.** Fas and HLA-DR expression were monitored by means of flow cytometry after cytokine treatment (IL-1-β, TNF-α, and IFN-γ) of TEC subcultures. All three cytokines individually upregulated Fas expression (Fig 3A). The Fas MFI in the presence of one or several cytokines was expressed as a ratio relative to control values. Fas expression by cultured TEC increased more strongly when the three cytokines were added together than when they were added separately (Fig 3A and C); the Fas MFI ratio was 2.4 ± 0.4 after 48 hours in the presence of IL-1-β, TNF-α, and IFN-γ, compared with 1.4 ± 0.05 with IL-1-β, 1.4 ± 0.1 with TNF-α, and 1.7 ± 0.1 with IFN-γ. Like HLA-DR, the effect was maximal after 48 hours of incubation (Fig 3C and D). HLA-DR expression was increased by IFN-γ, as previously described, but not by IL-1-β and TNF-α (Fig 3D); the combined effect of the three cytokines was slightly less potent than that of IFN-γ alone.

Moreover, Fas mRNA levels monitored by RT-PCR were low or undetectable in control conditions and were strikingly increased after 24 hours in the presence of the three cytokines, suggesting that Fas upregulation occurred at the transcription level (Fig 3B).

**Activated thymocytes induce Fas upregulation in TEC.** Heterologous thymocytes were cocultured with TEC for a 3-day period. Fas expression was analyzed in TEC only, i.e., in cells in the R region (Fig 4A) according to forward-and side-scatter parameters. Thus, thymocytes (that have smaller size) were excluded from the analysis. In the absence of any activation, Fas expression was not modified in TEC (Fig 4). In the absence of thymocytes, the addition of PMA, a phorbol ester that activated protein kinase C, and ionomycin, a calcium ionophore, did not have any effect on Fas expression in TEC. By contrast, when thymocytes activated by these agents were cocultured with TEC, we measured an increase in Fas MFI in TEC: it was 52.0 ± 5.0 in TEC cocultured with thymocytes in medium and 82.7 ± 9.9 in TEC cocultured with activated thymocytes (n = 3). Thus, activated thymocytes are able to upregulate Fas expression in TEC.

**Susceptibility of TEC to cell death induced by an agonistic anti-Fas antibody on day 4 of culture.** Because Fas antigen expression decreased gradually during culture, we examined whether the remaining Fas antigen was functional and whether TEC in primary culture were sensitive to an agonistic anti-Fas antibody. TEC on day 4 were treated with 0.5 µg/mL anti-Fas IgM antibody (clone CH-11) or with 0.5 µg/mL mouse IgM. Susceptibility to Fas-induced apoptosis was assessed by (1) the number of viable cells recovered and (2) the proportion of annexin-V–positive cells (Fig 5). Although Fas antigen expression was still strong on day 4 of primary culture, TEC were not sensitive to Fas-induced apoptosis at this time. This resistance to Fas-induced apoptosis was reversed by concomitant addition of 10 µg/mL cycloheximide, an inhibitor of protein synthesis. In these conditions, cell density was reduced (Fig 5A), cell recovery was about 70% of that in the presence of IgM and cycloheximide or in the absence of cycloheximide (Fig 5B), and the proportion of annexin-V–positive cells was significantly increased (P < .05, n = 4; Fig 5C and D). TEC on day 4 of culture were also resistant to 0.5 µg/mL recombinant soluble Fas ligand and this resistance was similarly reversed by concomitant addition of cycloheximide (not shown).

**FAP-1 regulation in TEC.** Because expression of FAP-1, a Fas-associated phophatase, protects cells from Fas-induced apoptosis, we wondered whether FAP-1 expression was altered by cycloheximide treatment. TEC collected on day 3 of primary culture and left to adhere for 24 hours were further cultured for 24 hours in the presence or absence of 10 µg/mL cycloheximide. They were then harvested and FAP-1 mRNA was examined. Cycloheximide strongly reduced FAP-1 mRNA levels, whereas it did not affect β-actin mRNA levels (Fig 6). Fas mRNA levels were not significantly affected by the cycloheximide treatment (data not shown). In similar experiments, TEC protein extracts (20 µg protein in each condition)
**Fig 3.** IL-1β, TNF-α, and IFN-γ, alone and in combination, upregulate Fas expression in cultured TEC. TEC subcultured after 10 to 13 days of primary culture were incubated with the cytokines. At 24, 48, or 72 hours, TEC were collected by trypsin treatment and labeled with anti–HLA-DR or anti-Fas antibody. (A) A representative experiment shows that, after 48 hours of incubation, IL-1β, TNF-α, and IFN-γ, both alone and together, increased Fas MFI. Vertical bars on Fas staining histograms indicate the Fas MFI level in TEC cultured in control conditions, ie, in medium. (B) mRNA was extracted from TEC cultured without (−) or with (+) 1 ng/mL IL-1β, 10 ng/mL TNF-α, and 500 U/mL IFN-γ. Lane 1 corresponds to the molecular weight (MW) marker (pUC18 DNA Marker HaeIII digest; Sigma). mRNA was submitted to RT-PCR. In three independent experiments, cytokine-activated TEC showed a strong increase in Fas mRNA levels in comparison to TEC cultured in control conditions, whereas β-actin expression was not modified. (C) The MFI ratio is the ratio between Fas MFI measured in the presence of one or several cytokines and Fas MFI measured in control conditions. MFI ratios are expressed as a function of time. Results are means ± SEM of four independent experiments. (D) The proportion of HLA-DR–positive cells was analyzed in the same experiments.

**Fig 4.** Fas upregulation is induced in TEC cocultured with activated thymocytes. TEC subcultured after 10 to 13 days of primary culture were cocultured with heterologous thymocytes in the presence or in the absence of 5 ng/mL PMA and 500 ng/mL ionomycin (PMA/I). After 3 days, TEC were collected. (A) Fas expression was analyzed in TEC, ie, in cells gated in the R region. (B) A representative experiment shows that, without thymocytes, PMA and ionomycin did not induce any effect on Fas expression in TEC. When cocultured with thymocytes, Fas expression is upregulated in TEC when PMA and ionomycin were added. (C) Data are the mean ± SEM from three independent experiments.
were analyzed in Western blot assay. We observed a major protein band (apparent molecular weight, 200 kD) detected by anti–FAP-1 antibody; it was clearly reduced in the presence of cycloheximide. These results show that FAP-1 downregulation coincides with the acquisition of susceptibility to Fas-induced apoptosis in human TEC.

An agonistic anti-Fas antibody induces apoptosis of cytokine-activated TEC. To determine if human TEC activated by cytokines and reexpressing Fas antigen were susceptible to Fas-induced apoptosis, subcultured TEC were incubated for 48 hours with IL-1β, TNF-α, and IFN-γ, alone or in combination, and then treated with various concentrations of IgM or anti-Fas antibody (clone CH-11). TEC were collected by trypsin treatment after 24 hours and annexin-V binding was examined. TEC cultured in the absence of cytokines were not susceptible to apoptosis induced by agonistic anti-Fas antibody (Fig 7) or recombinant soluble Fas ligand (not shown); this resistance was not reversed by concomitant addition of cycloheximide (not shown). By contrast, TEC previously treated with IL-1β, TNF-α, and IFN-γ, especially in combination, underwent apoptosis. Thus, the level of anti-Fas–induced apoptosis was thus related to the level of Fas expression. Apoptosis was maximal with 0.5 µg/mL agonistic anti-Fas antibody.

Effect of Z-DEVD-fmk and Z-VAD-fmk peptides on anti-Fas–induced apoptosis in TEC and DEVDase assay in Fas-stimulated TEC. Tripeptide Z-VAD-fmk is a cystein protease inhibitor of broad specificity, whereas Z-DEVD-fmk inhibits more specifically cystein proteases from the caspase-3 family (DEVDases). In preliminary experiments, Z-VAD-fmk and Z-DEVD-fmk alone did not have any effect on TEC apoptosis. To study the effects of these caspase inhibitors on anti-Fas–induced apoptosis, TEC were first cultured for 48 hours with the combination of IL-1β, TNF-α, and IFN-γ. After two washes, TEC were incubated for 2 hours with 20 µmol/L Z-VAD-fmk or...
molecular weight, 200 kD) was decreased in the presence of cycloheximide. A major protein band (apparent molecular weight, 200 kDa) was clearly involved,36 DEVDase activity was increased 12.1 and 15.6 times, respectively, in K562 target cells killed by Z-DEVD-fmk, and 0.5 µg/mL agonistic anti-Fas antibody or mouse IgM was then added. After 24 hours, the cells were collected by trypsin treatment. As expected (Fig 8), the agonistic anti-Fas antibody depleted viable cells relative to the control IgM antibody. Z-DEVD-fmk did not modify the number of cells recovered or the proportion of annexin-V–positive cells. By contrast, Z-VAD-fmk restored the number of cells collected and inhibited anti-Fas–induced apoptosis. Using a specific colorimetric substrate (DEVD-pNA), DEVDase activity was measured in cytokine-activated TEC that were cultured during 7 hours in the presence of 0.5 µg/mL IgM or 0.5 µg/mL CH-11. We also evaluated the proportion of apoptotic cells among living cells using annexin-V staining. As shown in Table 1, after 7 hours of incubation with agonistic anti-Fas antibody, TEC already undergone Fas-specific apoptosis, because the proportion of annexin-V–positive cells among living cells was increased in the presence of CH-11. DEVDase activity was slightly increased (2.1 and 2.4 times in 2 independent experiments). In comparison, in a granule-mediated apoptosis in which caspase-3 family was clearly involved,39 DEVDase activity was increased 12.1 and 15.6 times, respectively, in K562 target cells killed by lymphokine-activated killer cells compared with K562 alone. Thus, the slight activation of DEVDase in Fas-stimulated TEC is well correlated to the noninhibition of apoptosis induced by z-DEVD-fmk and is probably not sufficient to induce TEC apoptosis.

DISCUSSION

We report that Fas antigen is constitutively expressed by medullary epithelial cells. Fas expression is lost by cultured TEC but can be restored by cytokines. TEC were sensitive to anti-Fas–induced apoptosis in the presence of cycloheximide on day 4 of culture (when Fas expression was still high) and when subcultured on day 10 to 13 if they had been activated by cytokines. These results point to functional expression of Fas antigen by human medullary TEC.

Fas and Fas ligand expression in the human thymus. Fas antigen expression has been examined in mouse and human thymic lymphoid cells. Most mouse thymocytes are Fas-positive,18,19 whereas only a minor subset strongly express Fas in the human thymus.28-30 Fas expression in the epithelial compartment also seems to differ between mice and humans. Indeed, Fas expression was not found in the epithelial network of mouse thymus. French et al37 characterized the expression of Fas and its ligand in the mouse thymus by using in situ hybridization and immunohistochemistry. They detected Fas expression in thymocytes and strong Fas ligand expression in thymic epithelial cells and dendritic cells. We found weak expression of Fas ligand and strong Fas ligand expression in thymic epithelial cells and dendritic cells. We found weak expression of Fas ligand mRNA in human thymus by means of RT-PCR, but no protein expression was found in situ (not shown). These results suggest that the Fas/Fas ligand system is differently expressed in the mouse and human thymus and could have different functions.

We found that Fas antigen expression decreased during primary human TEC culture, reaching minimal levels after 10 days. This effect was unlikely to be due to in vitro selection of cortical epithelial cells, which do not express the Fas receptor, because epithelial cells collected after 2, 4, 7, or 10 days of primary culture were strongly labeled by an antikeratin antibody (clone CK1) that mainly stains the medullary epithelial network (not shown). Because Fas was detected in situ, its expression could be downregulated in the absence of the thymic environment, as previously shown for HLA-DR in these cells.33 This is supported by the moderate upregulation of Fas expression in TEC cocultured for 3 days with activated human thymocytes. Fas expression in vivo could be maintained by contact with thymocytes. Fas is mostly expressed in the medulla, where most mature thymocytes are found. Mature human CD3high thymocytes (both double- and single-positive CD4+/CD8+ cells) can secrete consistent amounts of cytokines38 and play a key role in the differentiation of medullary TEC.39 Thus, medullary mature thymocytes might interact with medullary TEC, resulting in the maintenance of Fas expression.

Role of Fas in epithelial cells of the human thymus. Most nonlymphoid tissues constitutively coexpressing Fas and its ligand in adult mice40 and humans41 are characterized by apoptotic cell turnover, possibly regulated by the Fas system. Fas is directly involved in the regression of the vaginal epithelium after ovariectomy and during the estrous cycle in mice.52 Moreover, Fas ligation induces apoptosis in various
epithelia in vitro, including ovarian surface epithelial cells, thyroid epithelial cells, and colon epithelial cells. Our findings show that Fas can also induce apoptosis of thymic epithelial cells in vitro.

Regarding the physiologic role of Fas in the thymus epithelial compartment, Fas could regulate the turnover of TEC, especially when thymus involutes after childhood. Although no substantial expression of Fas ligand protein is detected in the human thymus, activated rat thymocytes and activated human thymocytes and TEC (not shown) can express Fas ligand. Both Fas and its ligand can be produced by TEC in vitro, but we did not observe cell death in those cells in the absence of agonistic anti-Fas antibody. Further studies are needed to determine if Fas ligand produced by activated TEC is also secreted into the extracellular medium, because membrane-associated and secreted Fas ligand seem to have different capacities to induce apoptosis.

Fig 7. Cytokine-activated TEC are sensitive to anti-Fas-induced apoptosis. TEC subcultured after 10 to 13 days of primary culture were incubated in the presence of 1 ng/mL IL-1β, 10 ng/mL TNF-α, and 500 U/mL IFN-γ, alone or in combination, for 48 hours. After two washes, various concentrations of agonistic anti-Fas antibody or control IgM were added. After 24 hours, cells were collected by trypsin treatment and labeled with annexin-V-FITC and propidium iodide. Dead cells, ie, cells incorporating propidium iodide, were excluded from the analysis. A representative analysis is shown. (A) The proportion of annexin-V-positive cells among total living cells is expressed as a function of the concentration of IgM or anti-Fas. Increasing concentrations of IgM were inactive, whereas Fas-mediated apoptosis was concentration-dependent. (B) The analysis of annexin-V-FITC binding in TEC previously activated by cytokines and incubated with 0.5 μg/mL anti-Fas CH-11 or IgM is presented.
apoptosis, and soluble Fas ligand can block Fas-induced cell death.  

**Intracellular partners of the Fas receptor in the induction of cell death.** On day 4 of primary culture, when they still expressed significant amounts of Fas antigen, TEC were resistant to Fas-induced apoptosis, and cycloheximide reversed this resistance. Metabolic inhibitors such as actinomycin D and cycloheximide can sensitize some cells to anti-Fas–mediated apoptosis in both mice and humans, suggesting that a labile protein might inhibit Fas-mediated cell death. We found that cycloheximide downregulated FAP-1, a phosphatase able to inhibit Fas-mediated apoptosis. Similarly, Mori et al showed that actinomycin D induced sensitivity to Fas-induced apoptosis and downregulated FAP-1 mRNA in Kaposi’s sarcoma cells. FAP-1 may thus be involved in TEC resistance to Fas-induced apoptosis on day 4 of primary culture. Other molecules could be involved. Intracellular glutathione, levels of which are reduced by cycloheximide, can mediate Fas resistance in human T lymphocytes. Fournel et al showed that human T cells required IL-2 to acquire susceptibility to Fas-mediated apoptosis, and the investigators suggested that IL-2 may decrease FAP-1 expression. TEC subcultured with or without cytokines contained significant levels of FAP-1 mRNA (not shown), and cytokine-activated TEC were sensitive to Fas-mediated apoptosis, showing that FAP-1 expression was not sufficient to induce resistance to Fas-mediated cell death in cytokine-activated cells. Thus, activation by cytokines can overcome the resistance to Fas-induced cell death induced by FAP-1. In immature T lymphocytes, additional signals were shown to interfere with the Fas pathway. We show here that Fas-induced apoptosis of cultured TEC requires (1) Fas expression and (2) another signal that can be provided by the removal of a labile protein able to inhibit the Fas signal (eg, the phosphatase FAP-1) or by cell activation by cytokines. In lymphoid and nonlymphoid cells, additional signals that interfere with the Fas pathway (other than the one produced by FAP-1) need to be clarified.

**Table 1. DEVDase Activity in Fas-Stimulated TEC**

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>DEVDase Activity (OD Units)</th>
<th>Activity Index</th>
<th>Annexin-V-Positive Cells</th>
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</thead>
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<tr>
<td></td>
<td>IgM</td>
<td>CH-11</td>
<td>IgM</td>
</tr>
<tr>
<td>1</td>
<td>0.046</td>
<td>0.097</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>0.048</td>
<td>0.114</td>
<td>2.4</td>
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Human TEC were previously activated for 48 hours with 1 ng/mL IL-1-β, 10 ng/mL TNF-α, and 500 U/mL IFN-γ. After 7 hours of incubation with 0.5 µg/mL anti-Fas CH-11 antibody or mouse IgM, TEC were harvested and annexin-V-FITC binding (among living cells) was measured. Remaining cells were lysed and DEVDase activity was evaluated using a colorimetric substrate. The activity index is the ratio between DEVDase activity in the presence of agonistic anti-Fas antibody and DEVDase activity in control conditions. Results are from two independent experiments. In comparison, in a granule-mediated apoptosis in which caspase-3 family was clearly involved, DEVDase activity quantified in the same experiment was increased 12.1 and 15.6 times, respectively, in K562 target cells killed by lymphokine-activated killer cells compared with K562 alone.

Caspase-8 plays a pivotal role in Fas-induced apoptosis and links the Fas signaling complex and other ICE-like caspases. Caspase-3 is a major cysteine protease activated after Fas triggering. However, in caspase-3 knock-out mice, Fas-induced apoptosis and poly(ADP-ribose) polymerase (PARP) cleavage were not impaired. We found that Fas-induced apoptosis of cytokine-activated TEC was inhibited by Z-VAD-fmk, a cysteine protease inhibitor, but not by Z-DEVD-fmk, a specific inhibitor of caspases from the caspase-3 family or DEVDases. Moreover, DEVDase activity is slightly increased in Fas-stimulated TEC, compared with a granule-mediated apoptosis in which DEVDase is implicated and compared with
Fas-stimulated cells whose apoptosis is clearly related to DEVDase activity as Jurkat cells (in which DEVDase activity is 26 times increased in the presence of agonistic anti-Fas antibody\textsuperscript{55}). This suggests that Fas-induced apoptosis of TEC is mediated by cysteine proteases but DEVDase activity is probably not sufficient to induce Fas-mediated apoptosis in TEC.

In conclusion, human medullary TEC express Fas in situ and are able to undergo Fas-induced apoptosis. This original system of Fas-mediated cell death in nonlymphoid cells could serve as a model to examine the intracellular signals conferring susceptibility to Fas-mediated apoptosis or to its execution.

ACKNOWLEDGMENT

The authors are grateful to Dr E. Dulmet and S. Plante (Service d’Anatomo-Pathologie, Hôpital Marie-Lannelongue, Le Plessis-Robinson, France) for technical advice in the histological experiments, to Dr J. Bréard (INSERM U461) for the generous gift of Z-DEVD-fmk and Z-VAD-fmk and for helpful discussions, and to N. Riché (INSERM U461) for technical assistance.

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