Glucocorticoid-induced, caspase-dependent organ apoptosis early after burn injury

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Fukuzuka, Kunitaro, Carl K. Edwards III, Michael Clare-Salzler, Edward M. Copeland III, Lyle L. Moldawer, and David W. Mozingo. Glucocorticoid-induced, caspase-dependent organ apoptosis early after burn injury. Am J Physiol Regulatory Integrative Comp Physiol 278: R1005-R1018, 2000.—Immune suppression and increased apoptotic loss of circulating lymphocytes have been reported after burn injury. However, little is known about the underlying mechanisms responsible for the increased apoptosis of lymphoid and parenchymal cells in solid organs and the role played by inflammatory mediators, such as tumor necrosis factor-α (TNF-α) and Fas ligand (FasL), as well as by glucocorticoids. To evaluate the role of endogenously produced glucocorticoids and FasL, mice subjected to a 20% steam burn were pretreated with a glucocorticoid receptor antagonist (mifepristone) or a neutralizing murine Fas fusion protein. Three and twenty-four hours after burn injury, histological analysis, caspase-3 activity, and in situ terminal deoxynucleotidyl transferase dUTP nick-end labeling staining and phenotyping of lymphocyte populations for apoptosis were evaluated. Burn injury increased the number of apoptotic cells and caspase-3 activity in thymus and spleen, but not in other solid organs. Increased apoptosis was seen in several T and B cell populations from both thymus and spleen. Mifepristone pretreatment significantly reduced the apoptosis and caspase-3 activity after burn injury, whereas blocking FasL activity had only minimal effects. We conclude that corticosteroids, and not FasL, are primarily responsible for the increased caspase-3 activity and apoptosis in thymus and spleen cell populations early after burn injury.

mouse; cytokines; spleen; thymus; tumor necrosis factor

SEVERE BURN INJURY is often accompanied by immune suppression. These complications contribute to a greater incidence of infection and multisystem organ failure leading to increased mortality (23). Clinically, increased apoptosis in lymphoid organs and decreased numbers of peripheral lymphoid cells have been reported after burn injury (38). These losses of immune cell populations are presumed to be secondary to activation-induced cell death, provoked by the release of stress mediators such as tumor necrosis factor-α (TNF-α), Fas ligand (FasL; CD95L, Apo1L), and granzymes, as well as by glucocorticoids (15). Circulating TNF-α has been occasionally detected in the serum of burned patients, and increased glucocorticoid concentrations have been well described early after a burn injury in both patients and rodents (10, 21, 30). It is less clear whether FasL expression is increased after a burn, although recent studies suggest that surgical injury will induce FasL expression in peripheral blood leukocyte populations (35).

In a previous report, we noted that increased apoptosis in spleen and thymus after a steam burn injury was not dependent on either TNF-α expression or lipopolysaccharide (LPS), but was secondary to increased caspase-3 activation (11). During the course of those studies, we noted that FasL expression was increased in spleen and thymus from burned mice concordant with increased caspase-3 activity and apoptosis. Because glucocorticoid and Fas/CD95 signaling involve activation of caspase-8 and caspase-3 (13, 36), we proposed that the increased expression of FasL and/or glucocorticoid release may play a crucial role in regulating this caspase-3-dependent apoptosis early after burn injury. To test this hypothesis, a Fas agonist or dexamethasone was administered to healthy mice in an effort to replicate the apoptotic tissue changes that occur after a burn injury. In addition, mice were pretreated with a mouse soluble Fas IgG1 fusion protein (mFasFc) to neutralize FasL (7) or a glucocorticoid receptor inhibitor (mifepristone) (5) before the burn injury. Finally, mice lacking a functional FasL ([B6Smn.C3H-Faslglδ (B6.Faslgδ)]) were also subjected to the burn injury.

We report here that glucocorticoid administration in the healthy mouse increases apoptosis and caspase-3 activity in the spleen and thymus, but not in other organs. In contrast, Fas agonist administration produces profound apoptosis and caspase-3 activation in the liver and more modest effects in the spleen and thymus. The apoptotic changes and induction of caspase-3 activity seen 3 and 24 h after a steam burn were limited primarily to the spleen and thymus, although some increases in apoptosis were also seen in the lung after 24 h. The apoptotic changes in lymphoid organs after a burn injury were primarily dependent on endogenous glucocorticoid release, because pretreatment of mice with mifepristone significantly reduced apoptosis and caspase-3 activity in both spleen and thymus. Blocking FasL activity with a soluble Fas fusion protein did not reduce caspase-3 activity or...
apoptosis in the thymus and had only variable effects in the spleen. Apoptosis and caspase-3 activity were actually increased in FasL-deficient mice (gld) receiving a steam burn compared with burned mice on a similar genetic background. Although several mediators such as FasL, TNF-α, and glucocorticoids all can induce apoptosis in lymphoid cell populations, the present findings suggest that after a burn injury, apoptosis of lymphoid cell populations is principally glucocorticoid dependent.

MATERIALS AND METHODS

Animals. Specific pathogen-free, female C57BL/6j mice and B6.Fasgld at 8–10 wk of age were obtained from Charles River Breeding Laboratory (Wilmington, MA) and Jackson Laboratories (Bar Harbor, MA). All mice were housed in a barrier facility with viral pathogen surveillance, sterile cages, food and water, and high-efficiency particle-free air. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Florida College of Medicine before initiation of these studies. Anesthesia and euthanasia protocols were consistent with those recommendations of the American Veterinary Medical Association.

Animal burn model. Mice were randomized into burn and sham-burn groups. Each animal was anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium, and all dorsal hair was clipped. A burn injury was produced by using a modified method described by Manktelow and Meyer (20). The clipped skin on the dorsum of the burn group was exposed to steam through an insulated template for 7 s, and a 20% total body surface area full-thickness burn was obtained. Animals were immediately resuscitated with 0.08 ml/g body wt ip of saline after burn injury. Sham-burned animals underwent the same anesthesia and resuscitation procedures as those in the burn group, but did not receive a burn injury.

The burn injury produces a full-thickness anesthetizing burn. Pain and discomfort associated with the burn wound are minimal due to the destruction of innervating sensory neurons in the skin. Animals routinely begin taking food and water within 4–8 h, although food intake is markedly reduced in the first 24 h. Handling of the animals and the burn wound does not evoke any behavioral response consistent with pain or discomfort, such as guarding, avoidance, or vocalization. Postburn analgesics were not employed due to their undefined effects on immune function.

All of the mice were anesthetized and killed by cervical neck dislocation at 3 or 24 h after burn injury. Tissue samples were taken from liver, lung, kidney, thymus, and spleen. Sections of the organs were immediately homogenized for caspase-3 activity measurements; other parts of each organ were snap frozen in liquid nitrogen and stored at −80°C for analysis of mRNA or fixed in 4% phosphate-buffered Formalin for histological analysis.

Injection protocol. To neutralize FasL activity, 25 mg/kg of mFasFc (provided by Amgen, Thousand Oaks, CA) dissolved in 100 µl of saline was injected 30 min before the burn (16). The mFasFc is a chimeric immunoadhesin (or fusion protein) comprised of dimeric murine Fas covalently linked to the hinge and Fc region of human IgG (34). Vehicle-treated animals received human IgG as a control for mFasFc. Steroid receptor antagonist mifepristone (Sigma Chemical, St. Louis, MO) was dissolved in ethanol-polyethylene glycol-distilled water (1:5.4, vol/vol/vol) at a final concentration of 5 mg/ml, and 20 mg/kg of mifepristone was administered subcutaneously 30 min before thermal injury. The vehicle-treated animals received the same amount of solvent.

Healthy C57BL/6j mice were injected intraperitoneally with saline, 25 mg/kg of dexamethasone (Sigma Chemical), or 500 µg/kg body wt of the Fas agonist monoclonal antibody (J02, PharMingen, San Diego, CA) to determine the direct effect of glucocorticoids and Fas activation on organ apoptosis and caspase activation in the healthy mouse. This dose of J02 is uniformly lethal to the mouse within 6–8 h, and death has been associated with massive apoptotic liver injury (27). Mice were killed either 3 or 24 h after dexamethasone administration or 3 h after J02 administration.

Histopathological examination. The organs were fixed in 10% buffered Formalin and embedded in paraffin. Five-micrometer-thick sections were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to assess morphological changes. In situ terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using an in situ apoptosis detection kit (Apoptag, Oncor, Gaithersburg, MD). All steps were performed according to the supplier’s instructions. Briefly, paraffin-embedded sections were dewaxed and rehydrated and then incubated with proteinase K (20 µg/ml in 100 mM Tris and 50 mM EDTA) for 15 min at 25°C. After the slides were washed four times with distilled water, the sections were incubated in equilibration buffer for 5 min. The sections were then incubated with the labeling solution containing terminal deoxynucleotidyl transferase in a humidified chamber for 1 h at 37°C. The reactions were terminated by rinsing the sections in a stopwash buffer. The sections were incubated with anti-digoxigenin fluorescein for 30 min at room temperature and then rinsed three times in PBS. The TUNEL-labeled slides were photographed using a fluorescence microscope. Tissues stained with hematoxylin and eosin were also examined.

Flow cytometric detection of apoptosis using annexin V. To detect apoptotic cells, fluorescein isothiocyanate (FITC)-annexin V and the nonvital dye 7-amino-actinomycin D (7AAD) double staining was performed as previously described with minimum modification (22, 31, 39). Thymus and spleen single-cell suspensions were generated by gentle dispersion, and cells were washed twice with cold Hanks balanced salt solution. After washing twice with PBS, 1 × 106 cells were resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). FITC-annexin V (PharMingen) and 7AAD (PharMingen) were added, resulting in a final concentration of 1 µg/ml. The mixture was incubated for 15 min in the dark at 4°C and analyzed by FACSCalibur and CELLQuest software (version 3.0) (Becton Dickinson Systems, San Jose, CA).

In an attempt to identify the phenotype of cells undergoing apoptosis, we performed a four-color flow cytometric analysis. After annexin V and 7AAD treatment, cells were stained with antibodies against either CD4 (donc RN4–5, rat IgG2b), CD8a (donc 53–6.7, rat IgG2a), and CD45R/B220 (donc RA3–6B2). Rat IgG2a) (all of these antibodies were purchased from Pharmingen) conjugated to either FITC, phycoerythrin (PE), or allophycocyanin (APC). In a typical staining protocol (18), 1 × 106 cells were incubated with 1 µg of monoclonal antibodies with PBS containing 1% BSA for 10 min. After one washing in PBS-1% BSA, cell samples were submitted to flow cytometric analysis.

FITC-, PE-, and 7AAD-stained cells were excited with a 488-nm argon ion laser. Detection of FITC fluorescent emission was with a 530 ± 15-nm band-pass filter, PE emission was detected with a 585 ± 21-nm band-pass filter, and 7AAD
was detected with a 670-nm long-pass filter. APC-stained cells were excited with a diode laser to 635 nm, and detection of the emission was with a 661±8-nm band-pass filter. FITC, PE, 7AAD, and APC emission overlap was corrected by electronic compensation. The analysis for apoptosis using FITC-annexin V and 7AAD was determined after gating of cell debris and doublets for no less than 20,000 cells/sample. With the use of CELLQuest software, four typically quad-rated regions (bottom right, FITC-annexin V; top left, 7AAD single positive; top right, double-positive cells; bottom left, double-negative cells) were established. The bottom right quadrant (FITC-annexin V positive and 7AAD negative) represents apoptotic cells (31, 39). To determine the phenotype of all cells or apoptotic cells, four typically quad-rated regions (bottom right, PE-CD4; top left, APC-CD8 single-positive cells; top right, double-positive cells; bottom left, double-negative cells) were established. This phenotyping was performed for total and apoptotic cells falling into the bottom right quadrant of annexin V-7AAD analysis.

Caspase-3 activity assay. Protein extracts were prepared by homogenization of 20 mg of tissue, and caspase-3 activity was measured as previously described (9, 29). Briefly, excised organs were homogenized in 25 mM HEPES buffer (pH 7.5) containing 5 mM MgCl₂, 1 mM EGTA, 1 mM phenylmethylsul-
fonyl fluoride, and 1 µg/ml leupeptin and aprotinin. After centrifugation at 15,000 rpm for 10 min, the supernatants were collected. Forty micrograms of the extracted proteins were incubated with the synthetic fluorescent substrates benzoyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Z-DEVD-AFC; Enzyme Systems Products, Livermore, CA) for caspase-3 activity assay at concentration of 20 mM in 0.1 M HEPES buffer (pH 7.4) containing 2 mM dithiothreitol, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate, and 10% sucrose. The kinetics of the proteolytic cleavage of the substrates were monitored in a fluorescence microreader using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. The fluorescence intensity was calibrated with a standard concentration of AFC, and the caspase-3 activity was calculated from the slope of the recorded fluorescence and expressed in picomoles per minute per microgram of protein. Protein concentrations in the supernatant were assayed using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). To confirm the specificity of the assay, N-acetyl-Asp-Glu-Val-Asp-CHO

Fig. 2. Caspase-3 activity in organs after a burn injury. Caspase-3 activity was determined fluorimetrically using a synthetic substrate as described in MATERIALS AND METHODS. Increases in caspase-3 activity were seen only in thymus and spleen 3 h after burn injury, and caspase-3 activity had returned to baseline by 24 h. There was no increase in caspase-3 activity in other organs evaluated. Values represent mean and SE of at least 5 observations. *P < 0.05 by Student's t-test vs. sham-treated rats at same time point.

Fig. 3. Flow cytometric analysis of isolated thymocytes and splenocytes from sham-treated and burned mice after 3 h. Isolated splenocytes and thymocytes were subjected to flow cytometric analysis using fluorescein isothiocyanate (FITC)-labeled annexin V and 7-aminoactinomycin D (7AAD), as described in MATERIALS AND METHODS. Illustrated are results of a typical flow cytometric analysis of a single-cell suspension obtained from thymus and spleen 3 h after a sham (A) or burn injury (B). Bottom left of each panel shows viable cells; bottom right represents apoptotic cells, FITC-annexin V positive, and 7AAD negative; and top right contains nonviable, necrotic cells. Number of FITC-annexin V-positive and 7AAD-negative cells increased after burn injury. One representative experiment out of five is shown. C: statistical evaluation of data. Burn injury increased number of apoptotic cells significantly in both spleen and thymus (P < 0.05 by Mann-Whitney U test; mean ± SE; n = 5 mice sampled/group).
Pharmingen, San Diego, CA) was added to the supernatant 20 min before adding the substrate Z-DEVD-AFC.

Detection of TNF-α and FasL mRNA by RT-PCR. Total cellular RNA was isolated, and estimated quantities of TNF-α and FasL mRNA were calculated as previously described (17). Total organ RNA was isolated by guanidinium isothiocyanate and acid-phenol extraction. One microgram of total organ RNA was reverse transcribed and then amplified using

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**Fig. 4. Phenotypic analysis of total and apoptotic thymocytes and splenocytes 3 h after a burn injury.** Representative flow cytograms are presented for isolated thymocytes and splenocytes of mice 3 h after a steam or sham burn. Details of flow cytometry are presented in MATERIALS AND METHODS. Phenotyping of total cells in thymus from a sham-burned or a burned mouse (A) and in the spleen from a sham-burned or burned mouse (C). B and D: phenotyping of apoptotic cells falling into bottom right of annexin V-7AAD cytometric analyses (see Fig. 3). Phenotyping of apoptotic cells in thymus from a sham-burned and burned mouse (B) and in spleen from a sham-burned and burned mouse (D) is shown. APC, allophycocyanin; PE, phycoerythrin.
primers for murine TNF-α, FasL, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The sequences of the oligonucleotide primers were 5'-TNF-α, ATG AGC ACA GAA AGC ATG ATC; 3'-TNF-α, TAC AGG CTT GTC ACT CGA ATT; 5'-FasL, ATC AGC TCT TCC ACC TGC AGA AGC AAC; 3'-FasL, AGT TCA ACC TCT TCT CCT CCA TTA GCA CC; 5'-GAPDH, TGA AGG TCG GTG TGA ACG GAT TTG GC; 3'-GAPDH, CAT GTA GGC CAT GAG GTC CAC CAC. The PCR was performed using 2.5 U AmpliTaq (Perkin Elmer, Norwalk, CT) for TNF-α; 27 cycles, FasL; 28 cycles, GAPDH; 25 cycles as follows: 94°C for 1 min (dissociation), 60°C for 1 min (annealing), and 72°C for 2 min (primer extension). The expected fragment lengths were 276 bp for TNF-α, 390 bp for FasL, and 983 bp for GAPDH. Amplicons were visualized using 2% agarose gel electrophoresis. The gels were scanned, and the integrated area under the absorbance curves was calculated using a commercial program (SigmaGel, Jandel Scientific, San Rafael, CA). The relative quantities of TNF-α and FasL mRNA are presented as the ratio between the intensity of these bands relative to the intensity of the housekeeping gene GAPDH.

Corticosterone determination. Corticosterone levels in the serum were determined by radioimmunoassay using commercial reagents (ICN-BioPharma, Costa Mesa, CA).

Statistical analysis. All data are given as means ± SE. To determine statistical significance, a one-way analysis of variance with Bonferroni’s t-test post hoc comparison was performed. Statistical significance was determined at the 95% confidence interval.

RESULTS

Burn injury induces a marked increase in the number of apoptotic cells in thymus and spleen. Solid organs were harvested 3 and 24 h after a steam burn injury. A marked increase in the number of apoptotic cells was seen in thymus and spleen within 3 h after burn injury by both hematoxylin and eosin and in situ TUNEL staining. The apoptotic cells showed characteristic shrinking, chromatin clumping, and nuclear fragmentation. In situ TUNEL staining indicated that some modest apoptosis was present even in the thymus and spleen from the sham-burned mice, but the burn injury was associated with an increased number of apoptotic cells (Fig. 1). In both the spleen and thymus, these apoptotic cells aggregated to form clusters, whereas this clustering of apoptotic cells was not observed in organs from sham-treated animals. In the spleen, the aggregated apoptotic cells appeared to be primarily restricted to the white pulp regions.

Twenty-four hours after the steam burn, the number of apoptotic cells was reduced in the thymus; in the spleen, however, the number of cells undergoing apoptosis was further increased. Caspase-3 activity in the thymus and spleen was also significantly (P < 0.05) increased at 3 h after the burn injury, whereas levels returned to sham values by 24 h (Fig. 2).

Fig. 5. Changes in percentage of total and apoptotic T cell subsets 3 h after a burn injury. A and B: changes in percentage of total T and B cell subsets in thymus and spleen from 3 h sham-burned and burned mice. C and D: changes in percentage of cells undergoing apoptosis in thymus and spleen from 3 h sham-burned and burned mice. A burn injury significantly increased number of apoptotic CD4+/CD8+ and CD4+/CD8+ T cells in thymus and all T and B220+ cells in spleen (*P < 0.05 vs. sham-burned mice). Values represent mean and SE with an n of 5/group.
In contrast, increased apoptosis and caspase-3 activity were minimal in the liver, lung, or kidney at 3 or 24 h after a burn injury and were not different from sham-treated animals.

Figure 3 shows the results of annexin V-7AAD staining of thymocytes and splenocytes after burn injury, as determined by flow cytometry. Representative flow cytograms of mechanically dispersed cells from thymus and spleens of burned and sham-treated mice are presented in Fig. 3, A (sham) and B (burned), whereas statistical evaluations are presented in Fig. 3C. Cells falling into the bottom right quadrant of the cytograms are FITC-annexin V positive and 7AAD negative, presumably undergoing apoptosis, whereas cells in the top right quadrant are necrotic and nonviable, being positive for both FITC-annexin V and 7AAD uptake, thus indicating a damaged cell membrane (22).

The number of apoptotic cells in the bottom right quadrant increased significantly (P < 0.05) after burn injury in thymus and spleen, consistent with an increased number of cells early in the apoptotic process (Fig. 3).

To determine which lymphocyte subsets in the spleen and thymus were undergoing apoptosis after burn injury, cell-surface phenotypes were analyzed. Figure 4 shows a representative phenotypic analysis of total and apoptotic thymocytes and splenocytes, whereas Fig. 5 presents a statistical analysis of the percentage of T cells comprising the four subsets evaluated (CD4+/CD8−, CD4+/CD8+, CD4−/CD8−, CD4−/CD8+) and B-cells (B220+) in thymus and spleen. Burn injury did not affect the total percentage of each T cell subset in either the thymus or spleen; however, the percentage of B cells was increased in the spleen after burn injury.

Fig. 6. Expression of Fas ligand (FasL) and tumor necrosis factor-α (TNF-α) mRNA. Total RNA was isolated from organs 3 and 24 h after a steam burn or a sham burn. RT-PCR was used to detect presence of FasL, TNF-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (A; photograph of ethidium bromide-stained gel). Intensity of FasL (B) and TNF-α (C) mRNA amplicons was digitized, and intensity of bands was normalized for intensity of housekeeping gene (GAPDH) amplicon. Expression of FasL mRNA was increased in thymus and spleen through 24 h; however, TNF-α mRNA was increased only in thymus after 24 h (*P < 0.05 vs. sham-burned mice). Values represent mean and SE of 3–5 samples at each time point.
In thymus, the percentage of CD4<sup>+</sup>/CD8<sup>+</sup> and CD4<sup>+</sup>/CD8<sup>-</sup> cells undergoing apoptosis was increased (P < 0.05). In spleen, the percentage of all T cell subsets, especially CD4<sup>+</sup>/CD8<sup>-</sup> as well as B220<sup>+</sup>, undergoing apoptosis was also increased (all P < 0.05).

FasL mRNA expression was increased after burn injury. FasL mRNA expression was increased in thymus and spleen in burned animals early after burn injury (P < 0.05; Fig. 6). In addition, expression of FasL mRNA remained increased in both organs at 24 h (P < 0.05). In contrast, TNF-α mRNA expression was not increased at 3 h, but was increased at 24 h in thymus (P < 0.05). No increased expression of FasL or TNF-α was observed after burn injury in lung, liver, and kidney.

Serum corticosterone levels were increased after burn injury. Serum corticosterone concentrations increased significantly (P < 0.05) in burned mice 3 h after injury compared with sham-treated animals. By 24 h, corticosterone levels had returned to levels seen in the sham-treated mice (Fig. 7).

Dexamethasone and Fas agonist treatment induced apoptosis in thymus and spleen. To compare the effect of corticosteroid administration in the healthy animal with the responses seen in burned animals, histological examination and caspase-3 activity assessment in the solid organs were performed. Healthy mice received the intraperitoneal administration of 25 mg/kg body wt.
dexamethasone, and animals were killed at 3 and 24 h later. Histologically, the changes in the thymus and spleen of dexamethasone-treated mice were similar to organs in burned animals (Fig. 8). The characteristic clumping of apoptotic cells seen in the spleens of burned mice was also seen in the spleens of mice treated with dexamethasone. Caspase-3 activity was also increased transiently at 3 h in the thymus and spleen and declined thereafter at 24 h (Fig. 9). In contrast, dexamethasone treatment had no effect on apoptosis and caspase-3 activity in either lung, liver, or kidney (data not shown).

Treatment of mice with the intravenous administration of the Fas agonist Jo2 produced massive liver failure and lethality within 6–8 h. Histologically, increased apoptosis and necrosis were observed, and marked increases in caspase-3 activity were demonstrated in the liver at 3 h ($P < 0.05$; Figs. 8 and 9). In the liver, the increased apoptosis was limited primarily to hepatocytes. Increased apoptosis was not observed in thymus and spleen from mice treated with Jo2; however, increased caspase-3 activity was observed in spleen and lung at 3 h ($P < 0.05$), although the increases were modest compared with the liver.

Corticosteroid antagonist reduced apoptosis and caspase-3 activity after burn injury. Because both plasma corticosterone levels and FasL expression were increased after a burn injury and both agonists could induce apoptosis and increased caspase-3 activity in selected organs from healthy mice, inhibition studies were undertaken to determine whether these two mediators contributed to the endogenous apoptotic processes that were evident after a burn injury. To determine if glucocorticoids can modulate organ apoptosis and caspase-3 activity after burn injury, the glucocorticoid antagonist mifepristone was given to animals 30 min before injury. In situ TUNEL staining demonstrated that mifepristone prevented the increased apoptosis seen in thymus after burn injury (Fig. 10). In spleen, the magnitude of apoptosis was decreased by glucocorticoid antagonist; however, increased apoptosis was still observed compared with sham-treated animals. Mifepristone pretreatment prevented the increased caspase-3 activity in the thymus and spleen at 3 h (Fig. 11), reducing activity to levels seen in sham-treated mice ($P < 0.05$). Annexin V–7AAD flow cytometric analysis of total dispersed cells from the thymus and spleen also showed decreased apoptosis and caspase-3 activity in cells from mifepristone-treated burned animals ($P < 0.05$; Fig. 11).

In the thymus, mFasFc treatment had no effect on either caspase-3 activity or the numbers of apoptotic cells 3 h after burn injury (Fig. 11), and levels were similar to those seen in vehicle-treatment burn animals. This was confirmed by in situ TUNEL staining of thymus from burned mice treated with mFasFc (Fig. 12). To rule out the possibility that the failure to see any effect of mFasFc was secondary to inadequate drug administration, a limited number of B6.Fasl−/− mice were also burned, and apoptosis and caspase-3 activity were determined. Three hours after a burn injury, caspase-3 activity was significantly increased (Fig. 11; $P < 0.05$), and the numbers of apoptotic cells determined by flow cytometry were also increased by $>100\%$ ($P < 0.05$). In fact, the increases in caspase-3 activity and the numbers of cells undergoing apoptosis were higher than that seen in wild-type (C57BL/6) mice after the burn injury, although statistical significance was not achieved. In situ TUNEL staining also revealed increased numbers of cells undergoing apoptosis (Fig. 13).

In the spleen, however, the effects of Fas blockade were less clear. The increase in total caspase-3 activity was generally unaffected by administration of mFasFc, although there was an insignificant trend toward reduced apoptosis (Fig. 11). In situ TUNEL staining also revealed that the increased numbers of apoptotic cells in the spleen were not decreased by mFasFc treatment, and there was a trend toward increased numbers of positive cells (Fig. 12). However, the total number of cells determined to be annexin V positive and 7AAD negative (apoptotic) declined at 3 h in burned mice treated with mFasFc ($P < 0.05$). In B6.Fasl−/− mice, spleen caspase-3 activity and the number of apoptotic cells (measured by cytometric analysis) were significantly increased ($P < 0.05$) and were not different from the increases seen in wild-type C57BL/6j mice. In situ
TUNEL staining revealed a large number of apoptotic cells in the spleen of B6.Fasl<sup>gd</sup> mice (Fig. 13).

**DISCUSSION**

Increased apoptosis of lymphoid cell populations is frequently seen after burn injury and is proposed to contribute to the immune suppression that often results. The studies reported here demonstrate that a burn injury increases the numbers of lymphoid cells undergoing apoptosis in the spleen and thymus, but does not increase apoptosis or caspase-3 activity in other organs such as liver and kidney. This increased apoptosis in thymus and spleen is seen in both mature and immature T and B cells and appears to be depen-
dent primarily on glucocorticoid-mediated activation of caspase-3.

The present studies suggest that the primary cells undergoing accelerated apoptosis after a burn injury are lymphoid cells. Parenchymal cells in the liver, kidney, and lung were generally unaffected. Flow cytometric analysis of thymus and spleen cells binding annexin V and excluding the vital dye 7AAD confirmed in situ TUNEL data that an increased number of cells were undergoing apoptosis. By flow cytometry, however, the increased number of apoptotic cells was relatively small compared with the number identified by TUNEL staining. These quantitative differences can be explained by the contrasting stages of apoptosis the two independent measures are detecting. Expression of phosphatidylserine on the surface of cells undergoing apoptosis (annexin V staining) is an early event before the loss of cell membrane integrity (6, 22, 26). In contrast, in situ TUNEL staining of fragmented 3' nuclear DNA is a later event generally associated with the dissolution of the nuclear membrane. Although the two techniques are complementary and both detect cells undergoing apoptosis, the apoptotic process is dynamic and the expression of phosphatidylserine in an otherwise intact cell is more transient. Second, because apoptotic cells were observed to be clumping in the in situ TUNEL staining, the numbers of single apoptotic cells recovered for the flow cytometry are likely to be underestimates of the total numbers because of reduced recovery of individual cells.

The proportion of immature CD4+/CD8+ and mature CD4-/CD8+ cells undergoing apoptosis was increased in the thymus in the 3-h period after the burn. In the spleen, the percentage of several T cell and B cell populations undergoing apoptosis was similarly increased. Because approximately 1% of thymocytes leave the thymus per day and the bulk of thymocytes turn over every 5–7 days in young adult mice (32), this increased apoptotic death of thymocytes will likely lead to declines in the peripheral circulating lymphocyte numbers. In contrast, the percentage of viable B cells was increased in the spleen of burned animals, consistent with an earlier report that peripheral blood T cells decline on postburn day 2, whereas B cell numbers increase (28).

The increased apoptosis we observed in the spleen and thymus of burned mice is in agreement with the findings of Hotchkiss and colleagues (12) and Ayala et al. (2, 3) who observed increased thymus and spleen cell apoptosis in mice undergoing cecal ligation and puncture. This study can now confirm that increased corticosterone release can explain this increase in apoptosis, presumably through a caspase-3-dependent process. Increases in plasma corticosterone have been reported.

Fig. 12. In situ TUNEL staining of thymus and spleen of burned mice treated with mFasFc. Increased numbers of apoptotic cells from thymus and spleen of burned mice at 3 h were unaffected by mFasFc treatment.

Fig. 13. In situ TUNEL staining of thymus and spleen of B6.Fas+/+ mice. Increased numbers of apoptotic cells were observed in thymus and spleen from B6.Fas+/+ 3 h after burn injury. Increase was comparable to that seen in wild-type mice. However, in B6.Fas−/− mice, aggregates of apoptotic cells were not seen in white pulp of spleen, as was seen in C57Bl/6j mice, and many apoptotic cells were present in red pulp from B6.Fas−/− mice.
after burn injury (10), and we observed transient increases in the serum corticosterone concentrations 3 h after the burn injury.

There are, in fact, two lines of evidence to suggest a primary role for glucocorticoids, and not FasL or TNF-α, in mediating this response. When dexamethasone was administered to healthy mice, similar transient increases in apoptosis and caspase-3 activity were seen in spleen and thymus. We observed a very consistent clumping of apoptotic cells in mice after the burn injury and in response to the dexamethasone. Nakamura et al. (25) similarly observed that during glucocorticoid-induced thymocyte death, the increased apoptotic cells aggregated to form clusters being phagocytosed by macrophages. Caspase-3 activity also increases during thymocyte apoptosis induced by dexamethasone, and pretreatment with a caspase-3 inhibitor prevents apoptosis due to corticosteroid administration (1, 8). In a previous report, we noted that caspase-3 induction and the increased apoptosis in thymus and spleen after a burn injury were unchanged in either TNF-α knockout or LPS-resistant mice (11).

Second, blocking endogenous glucocorticoids with mifepristone not only reduced apoptosis in both organs, but also reduced caspase-3 activity. We previously showed that treatment of mice with a caspase-3, but not a caspase-1, inhibitor prevented apoptosis after a burn injury in both spleen and thymus (11). These findings are therefore consistent with previous work demonstrating that the increased apoptosis observed in thymus after a cecal ligation and puncture appeared to be due to glucocorticoids alone (2, 3).

However, increased glucocorticoid production could not explain entirely the increases in apoptosis and caspase-3 activity seen in the spleens of burned mice. We also noted in these studies that FasL expression was increased in the spleen and thymus of mice after a burn injury. Increased FasL expression has been previously seen in solid organs after concanavalin A hepatitis (17), endotoxemic shock, and cecal ligation and puncture (37). T lymphocytes, macrophages, and neutrophils all express FasL, and their expression is often increased after activation (14, 19). FasL binds to its receptor Fas/CD95 and can induce apoptosis of its target cell population through activation of Fas-associated death domain protein, caspase-8, and caspase-3 (24). Neutralization of FasL using mFasFc has rescued mice from endotoxin-induced liver injury (16) and concanavalin A-induced hepatitis exacerbated by pretreatment with a matrix metalloproteinase inhibitor (33). Recently, Alya et al. (4) postulated that increased FasL expression is responsible for the increased apoptosis seen in mucosal B cells after polymicrobial sepsis.

We could not confirm a major role for FasL in mediating either the increased caspase-3 activity or apoptosis in thymus or spleen after a burn injury. Treatment of mice with mFasFc had no effect on either total organ caspase-3 activity or apoptosis in the thymus, as measured by flow cytometry or in situ TUNEL. To rule out the possibility that inadequate quantities of the FasL antagonist were administrated, the studies were also conducted in FasL-deficient mice (B6.Fas-lgld), and these mice responded to the burn injury with comparable increases in caspase-3 activity and the numbers of TUNEL-positive cells.

In the spleen, the results were similar, although perhaps less conclusive. Caspase-3 activity was unaffected by treatment of burned mice with mFasFc, and in situ TUNEL labeling revealed no reduction in the numbers of apoptotic cells from the spleens of burned mice. Similarly, caspase-3 activity and in situ TUNEL labeling of the spleen were also increased in the spleens of FasL-deficient mice (B6.Fas-lgld), as were the numbers of apoptotic cells determined by flow cytometry. Surprisingly, the number of annexin V-positive, 7AAD-negative splenocytes (apoptotic cells), however, declined in wild-type mice burned and treated with mFasFc, as determined by flow cytometry. This latter finding was unexpected, and a complete explanation is not immediately forthcoming. However, passive immunization with mFasFc may only have altered the time course for the development of apoptosis, because the numbers of apoptotic cells detected by in situ TUNEL at 3 and 24 h were unaffected. Flow cytometric analysis conducted at 6 and 24 h revealed no reductions in the numbers of apoptotic cells (data not shown).

In conclusion, the findings clearly demonstrate that glucocorticoids are the predominant endocrine signaling system responsible for the increases in lymphoid cell apoptosis early after a burn injury in the thymus and spleen. On the basis of the findings reported here and in our previous publication (11), glucocorticoid-induced activation of caspase-3 appears to be a primary pathway for increased apoptosis. Because corticosteroid blockade is not a feasible clinical approach in patients with extensive burn injuries, inhibitors of caspase-3 may prevent immunodeficiency pathways after burn injury, without affecting other glucocorticoid-induced responses. The current results suggest that the beneficial effects of caspase-3 inhibition may offer a new therapeutic approach that prevents immunosuppression.

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following thermal trauma is associated with apoptotic cell death.
