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Fas Ligand–Induced Apoptosis as a Mechanism of Immune Privilege

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The eye is a privileged site that cannot tolerate destructive inflammatory responses. Inflammatory cells entering the anterior chamber of the eye in response to viral infection underwent apoptosis that was dependent on Fas (CD95)–Fas ligand (FasL) and produced no tissue damage. In contrast, viral infection in *gld* mice, which lack functional FasL, resulted in an inflammation and invasion of ocular tissue without apoptosis. Fas-positive but not Fas-negative tumor cells were killed by apoptosis when placed within isolated anterior segments of the eyes of normal but not FasL-negative mice. FasL messenger RNA and protein were detectable in the eye. Thus, Fas-FasL interactions appear to be an important mechanism for the maintenance of immune privilege.

That some sites in the body are immunologically "privileged" has been recognized for more than 120 years (*1*). Classically, an immune-privileged site is where allogeneic or xenogeneic (*2*, *3*) tissue grafts enjoy prolonged survival relative to other areas. Thus, these sites have been defined as regions in the body where the immune system appears not to function. One example of such a site is the eye, where even minor episodes of inflammation can result in impaired vision or even blindness if the inflammation proceeds unchecked. The concept of immune privilege in the eye has been extended to include infectious organisms and tumor cells (*4*, *5*), because placing these into immune-privileged sites does not elicit destructive or protective immunity. Although several explanations

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have been offered for the phenomenon of immune privilege in the eye (*2–5*), we examined the possibility that infiltrating cells might be promptly induced to undergo apoptosis, inasmuch as this has been demonstrated for both central (*6*, *7*) and peripheral (*8*) tolerance in lymphocytes. Furthermore, because Fas-FasL interactions have been implicated in at least one form of peripheral tolerance (*9*), we investigated whether the apoptotic signal generated by this interaction may function in controlling immune traffic into the privileged site of the eye.

We examined the fate of cells that infiltrate the eye in response to infectious agents by injecting herpes simplex virus–type 1 [HSV-1 (KOS)] into the anterior chamber of the eyes of C57BL/6 mice (Fig. 1A). This induced an infiltration of neutrophils and lymphocytes (*5*). Extensive apoptosis was observed in infiltrating cells 24 hours after infection, as detected by in situ terminal deoxytransferase (TdT)–catalyzed DNA nick end labeling (TUNEL) (*10*). To test the possible role of Fas-FasL interactions in this apoptosis, we infected animals lacking Fas [*lpr* mice (*11*)] or FasL [*gld* mice (*12*)] with HSV-1. Extensive infiltration without associated apoptosis occurred in both cases (Fig. 1, B and C). Thus, apoptosis in the infiltrating cells appears to depend on functional Fas and FasL expression.

Under normal circumstances, the inflammatory response resulting from HSV-1 infection in the anterior chamber of the eye is contained within the anterior segment, with only minimal spread of inflammatory cells into the posterior cavity and no invasion of the retina by inflammatory cells or virus. Therefore we examined the consequences of defective FasL by comparing the course of the infection in B6 mice with that

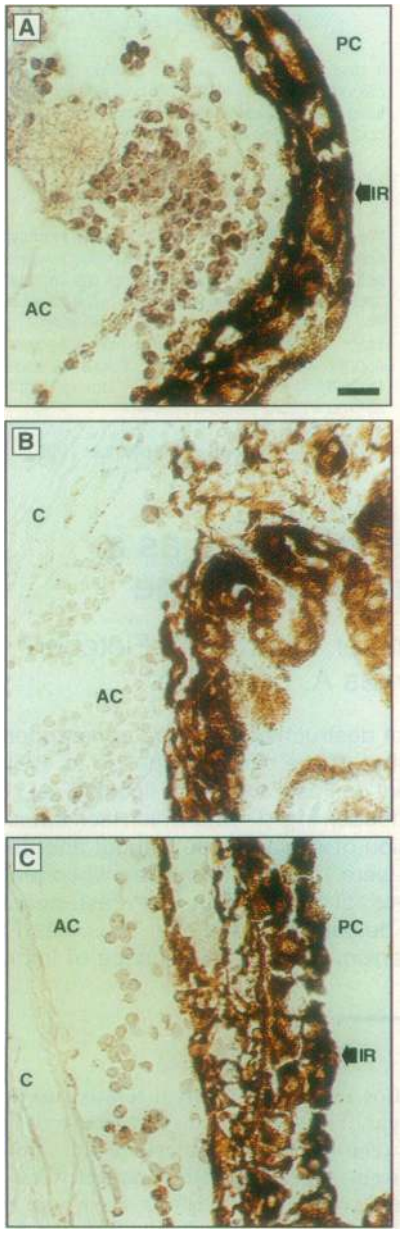

Fig. 1. In situ detection of apoptosis in HSV-1–infected eyes by TdT-mediated deoxyuridine 5′-triphosphate nick end labeling (TUNEL) staining. Paraffin sections of (**A**) B6, (**B**) B6^{lpr}, and (**C**) B6^{gld} eyes 24 hours after injection of HSV-1 were stained for apoptotic cells through the labeling of nuclear DNA fragments. AC, anterior chamber; C, cornea; IR, iris; PC, posterior chamber (*26*). Scale bar, 0.0058 μm.

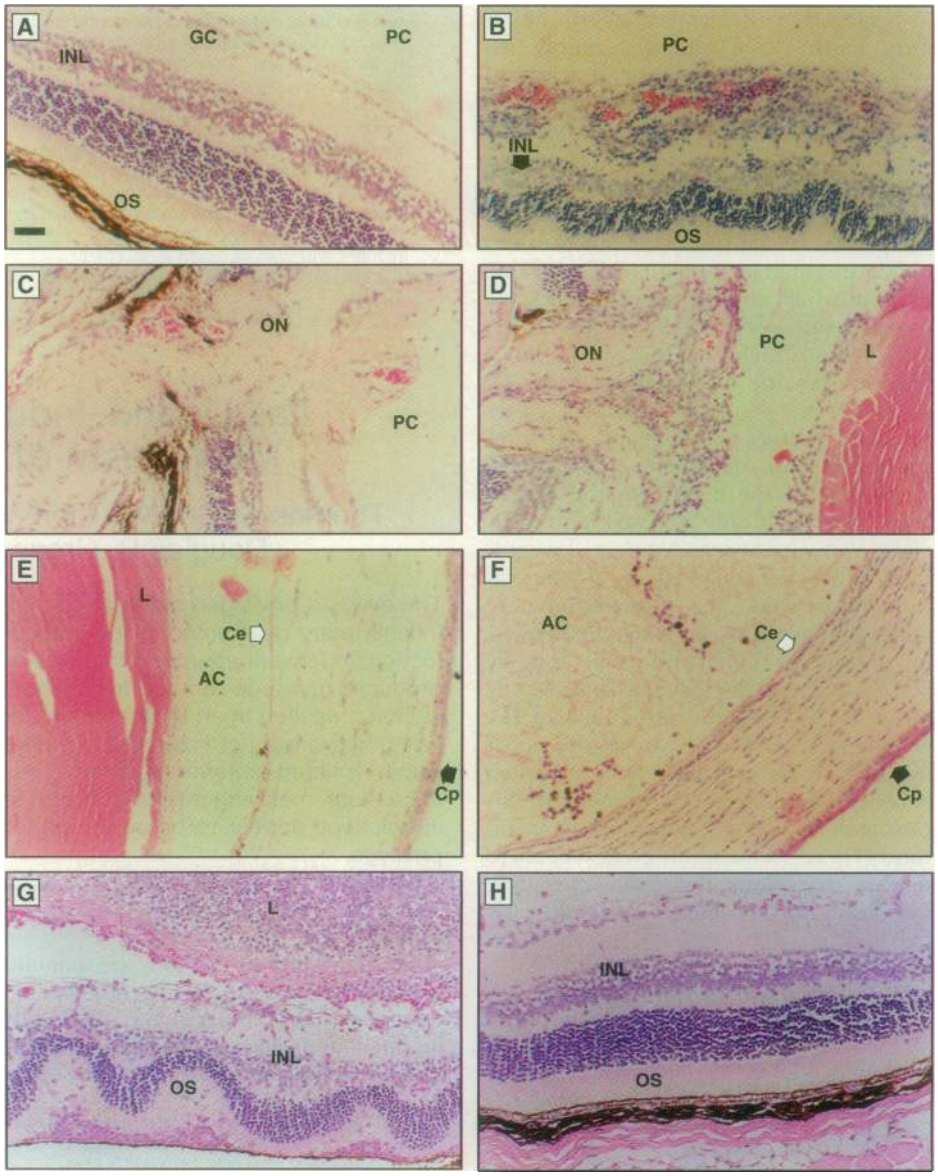

Fig. 2. Consequences of not expressing functional FasL in the eye. (**A**, **C**, and **E**) B6, (**B**, **D**, and **F**) B6^{gld}, (**G**) chimeric B6 → *gld*, or (**H**) chimeric B6 → B6 mice (*13*) were injected in the AC of the eye as described (Fig. 1). Eyes were removed 10 days after infection, processed for paraffin sections, and stained with hematoxylin and eosin. AC, anterior chamber; Ce, corneal endothelium; Cp, corneal epithelium; GC, ganglion cell layer; INL, inner nuclear layer; L, lens; ON, optic nerve; OS, outer segments; PC, posterior chamber. Scale bar, 0.0116 μm.

in B6^{gld} mice (Fig. 2). A minimal number of inflammatory cells associated with the retina, optic nerve, and cornea were observed 10 days after HSV-1 injection in B6 mice. In contrast, comparable sections from B6^{gld} mice showed numerous inflammatory cells in the posterior cavity, with cells attached to, and invading, the retina and the optic nerve. Numerous inflammatory cells attaching to and invading the cornea induced keratitis. Although this suggested that FasL expression in the eye was important, it was still possible that the inflammatory cells in *gld* mice were abnormal such that they either produced a toxic effect in the eye or simply could not be killed because of their lack of functional FasL. To test this, we prepared radiation bone marrow chimeras (*13*) in which irradiated B6 or *gld* mice were reconstituted with bone marrow from normal B6 mice. After infection, the *gld* mice reconstituted with B6 bone marrow (Fig. 2G) had an intense inflammatory response, with retinal invasion similar to that of *gld* mice (Fig. 2B). In contrast, B6 mice reconstituted with B6 bone marrow contained the inflammatory response (Fig. 2H). Thus, defective FasL function results in a loss of immune privilege in the eye with severe consequences after infection. Furthermore, the function of FasL in maintaining this privilege is associated with expression in compartments not derived from the bone marrow.

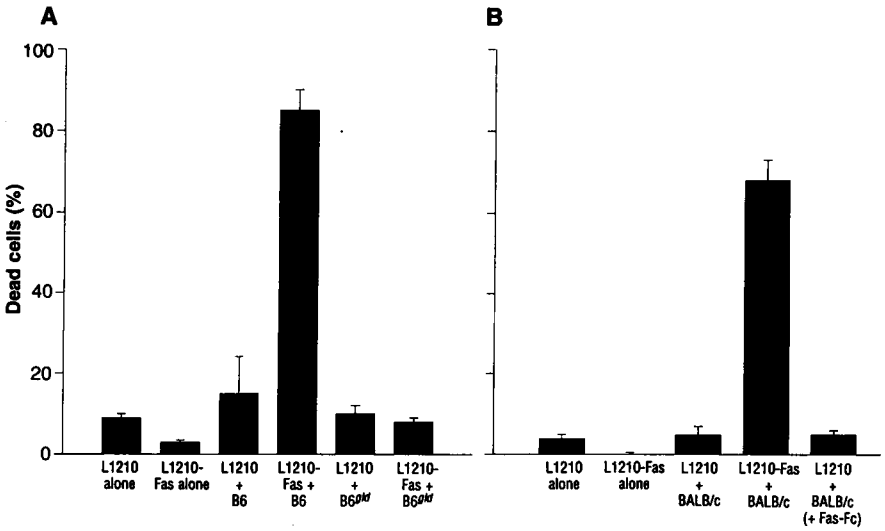

Fig. 3. In vitro killing of L1210-Fas T cell lymphoma by the anterior segment of the eye. (**A**) Co-incubation of L1210 or L1210-Fas cells with B6 or B6^{gld} eye segments led to killing of L1210-Fas by B6 eyes only. (**B**) Inclusion of the fusion protein Fas-Fc inhibited killing of L1210-Fas cells (*27*).

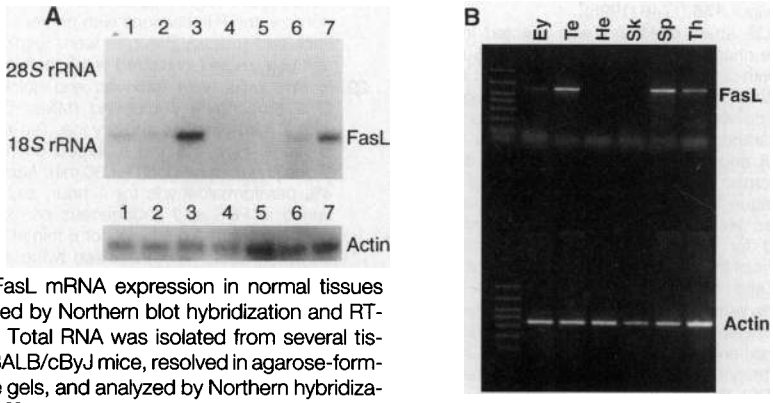

Fig. 4. FasL mRNA expression in normal tissues determined by Northern blot hybridization and RT-PCR. (**A**) Total RNA was isolated from several tissues of BALB/cByJ mice, resolved in agarose-formaldehyde gels, and analyzed by Northern hybridization with ^{32}P-labeled DNA fragments derived from PCR for FasL or β-actin. Tissue samples are as follows: 1, eye (sample 1); 2, eye (sample 2); 3, testis; 4, heart; 5, tail skin; 6, spleen; 7, thymus; rRNA, ribosomal RNA. (**B**) RT-PCR was performed on mRNA isolated from the eye (Ey), testis (Te), heart (He), tail skin (Sk), spleen (Sp), and thymus (Th) (*28*).

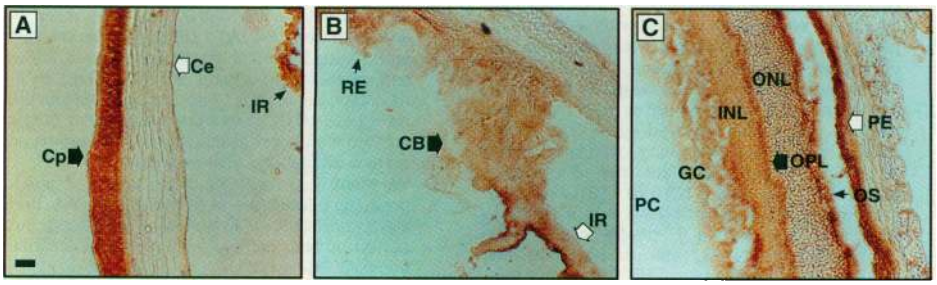

Fig. 5. (**A** to **C**) Localization of FasL protein in the eye by immunohistochemistry with anti-FasL. CB, ciliary body; Ce, corneal endothelium; Cp, corneal epithelium; GC, ganglion cells; INL, inner nuclear layer; IR, iris; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segments of photoreceptors; PC, posterior chamber; RE, retina (*29*). Scale bar, 0.0116 μm.

In mice, Fas is expressed on a variety of cell types, including lymphocytes (*14*) and neutrophils (*15*), whereas FasL is expressed predominantly on activated T cells (*16*, *17*). Our results suggest that FasL is also expressed on nonlymphoid tissue in such immunologically privileged sites as the eye. To examine this possibility in the absence of infiltrating T cells, we placed isolated anterior segments from the eyes of uninfected mice into culture and inoculated them with L1210 or L1210-Fas cells [murine T cell lymphoma cells that are negative (low) or strongly positive for Fas expression, respectively, which do not express FasL (*18*)]. Despite the absence of infiltrating T cells, the Fas-positive cells underwent cell death in the eyes from wild-type mice but not in those from *gld* mice (Fig. 3A). Fas-negative cells did not die in either case. Furthermore, the death of the Fas-positive cells in eyes from normal animals was blocked by the addition of a chimeric protein, Fas-Fc (Fig. 3B), composed of extracellular Fas on human immunoglobulin (Ig) heavy chain (*16*). Thus, the environment of the eye induces Fas-dependent cell death in the absence of infiltrating lymphocytes.

These results suggested that FasL was present in the eye before the appearance of infiltrating lymphocytes. We therefore examined the expression of FasL by Northern (RNA) blot and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of total RNA isolated from the eye and from several other organs. We confirmed that FasL was expressed in the testis, spleen, and thymus, but not in the heart or skin (Fig. 4, A and B) (*14*, *19*). FasL mRNA, however, was also expressed in the eye. The location of FasL expression in the eye was then determined immuno-

histochemically with an antibody to FasL (Fig. 5). Intense staining was found on the corneal epithelium and endothelium, iris and ciliary body, and throughout the retina. Addition of a competitive peptide inhibited staining in these locations, verifying the specificity of the antibody reaction with the ocular tissue. Staining was also observed in the spleen and testis, which were positive for FasL mRNA expression. The heart, which does not express FasL mRNA, was negative (*20*).

Cellular immune reactions and their associated inflammatory responses can cause nonspecific injury to nearby tissue. Although most organ systems can tolerate such inflammation without permanent consequences, this is not true for the eye. The immune privilege of the eye is thought to be one mechanism by which the visual axis is protected from dangerous immune reactions. It has long been thought that immune privilege was maintained by the sequestration of antigens, the lack of lymphatic drainage, and the blood-ocular barrier (*2*, *3*, *21*). These mechanisms would prevent cells from entering and antigen from leaving the eye, and thus the immune system would simply ignore the area. It is now known that not only do antigens placed in the eye gain access to the immune system, but activated cells can enter the eye as well (*22*). It has been shown that immune privilege may be maintained partially by the local production and release of immunosuppressive cytokines (*23*, *24*) and neuropeptides (*25*). Our results here show that the induction of apoptosis by Fas-FasL interactions is a potent mechanism of immune privilege; the consequences of defective FasL for the eye can be the spread of dangerous inflammatory responses. Through the expression of FasL, the eye directly kills activated cells that might invade the globe and destroy vision by reacting with (or near) important structures such as the retina. FasL expression in the testis may perform a similar function for this immune-privileged site (*14*, *19*). Thus, immune privilege is not simply a passive process involving physical barriers; rather, it is an active process that uses an important natural mechanism to induce cell death in potentially dangerous infiltrating lymphoid and myeloid cells.

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13. Radiation bone marrow chimeras were prepared as described [G. J. Spangrude, in *Current Protocols in Immunology*, J. Colligan, A. Krusbeek, D. Marguiles, E. Shevach, W. Strober, Eds. (Wiley, New York, 1994), vol. 1, section 4.6]. Mice received 11 Gy (1 Gy = 100 rads) of irradiation before receiving 1×10^7 bone marrow cells and were injected with HSV-12 weeks later. The dose of 11 Gy was used because we had previously determined that this dose established fully allogeneic bone marrow chimeras in C57BL/6 mice (E.-K. Gao and T. A. Ferguson, unpublished observations).
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26. The KOS strain of HSV-1 was injected into the anterior chamber (AC) of the eye in 0.005-ml volume with a 0.25-ml Hamilton microliter syringe (Hamilton, Reno, NV) fitted with a 33-gauge needle. Mice were anesthetized with Metofane methoxyflurane (Pitman-Moore, Washington Crossing, NJ), and injections were done under a dissecting microscope. Each injection contained 2.5×10^4 plaque-forming units of virus. After 24 hours, the eyes were removed, fixed in Formalin, and processed for paraffin sectioning. Ten-micrometer sections of the eye were mounted onto microscope slides and incubated overnight at 55°C. Tissue sections were deparaffinized by washing twice for 5 min in xylene, twice for 5 min in absolute ethanol, and once each for 3 min in 95% ethanol, 3 min in 70% ethanol, and 5 min in phosphate-buffered saline (PBS). Protein present in the sections was digested with proteinase K (20 μg/ml) for 15 min at room temperature. After four washes in distilled water, endogenous peroxidase was quenched with 2.0% H_2O_2 for 5 min at room temperature and sections were washed twice in PBS. Labeling of 3′-OH–fragmented DNA ends was performed with an in situ apoptosis detection kit (ApopTag; Oncor, Gaithersburg, MD) following the manufacturer's instructions. We detected labeled ends with kit-supplied antibody to digoxigenin peroxidase and development of diaminobenzidene (DAB) substrate (DAB substrate kit; Vector, Burlingham, CA).
27. Anterior eye segments from B6 or B6^{gld} mice were isolated from freshly removed eyes under a dissecting microscope by cutting directly posterior to the limbus. Tissue was then placed in round-bottom 96-well plates. L1210 or L1210-Fas cells (10^5) were placed within the AC with a tuberculin syringe fitted with a 27-gauge needle. After 24-hour culture, the AC was removed from the well, placed in Trypan blue–PBS, and gently vortexed to remove the cells. Cell viability was determined on a hemocytometer by Trypan blue exclusion. Isolated BALB/c anterior segments were incubated with L1210-Fas cells containing Fas-Fc (10 μg/ml). Viable and dead cells were counted at 24 hours by Trypan blue exclusion.
28. Total RNA was isolated from frozen and ground tissues with TRIzol reagent (Life Technologies, Gaithersburg, MD) as per the manufacturer's instructions, and 5 μg was loaded onto an agarose (1%, w/v)-formaldehyde (4%, v/v) gel and resolved by electrophoresis. RNA was transferred to nylon membrane (maximum strength Nytran Plus; Schleicher & Schuell, Keene, NH). The immobilized RNA was probed with ^{32}P-labeled PCR products made from FasL and β-actin complementary DNAs. Labeling of PCR fragments was done by random priming with the Prime-a-Gene labeling system (Promega, Madison, WI). Primers used to generate the probes were FasL (forward, 5′-AAGCTTCAGCTCTTCCACCTG-3′; reverse, 5′-ATGAATTCCTGGTGCCCATG-3′) and β-actin (forward, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′; reverse, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′), which gave a 590-base pair and a 349-base pair product, respectively. Conditions for PCR were optimized to give only the desired products. FasL conditions were 95°C for 1 min, 65°C for 1.5 min, and 72°C for 2 min for 40 cycles; β-actin cycle conditions were 95°C for 1 min, 65°C for 1.5 min, and 72°C for 2 min for 22 cycles. Hybridization was performed under high-stringency conditions, with prehybridization and hybridization performed at 55°C in Rapid-hyb buffer (Amersham) and the ^{32}P-labeled probe added at 750,000 cpm/ml of buffer. Stringency washes of probed membrane consisted of one wash for 20 min in 2× standard saline citrate (SSC) and 0.1% (w/v) SDS at room temperature, followed by two washes for 15 min in 0.2× SSC and 0.1% (w/v) SDS at 60°C. RNA samples (1 μg each) were tested for DNA contamination by 40 cycles of PCR with β-actin primers. Complementary DNA was synthesized with an RNA PCR kit (Perkin-Elmer, Norwalk, CT) with the supplied oligo$(dT)_{16}$ primer. Reverse transcription was done by using a thermal program of 25°C for 10 min, 42°C for 30 min, and 95°C for 5 min, and PCR was done on the RT reactions with primers specific for FasL and β-actin. Samples were resolved on 2% agarose gel and visualized with ethidium bromide.
29. Normal eyes were removed and quick-frozen in OCT embedding compound (Miles, Elkhart, IN) with 2-methylbutane and dry ice. Ten-micrometer sections were cut and mounted on microscope slides and then air-dried for 30 min. After fixation in 4% paraformaldehyde for 1 hour, sections were rinsed in PBS and endogenous peroxidase was quenched with 1.0% H_2O_2 for 5 min at room temperature. Sections were rinsed twice in PBS and then blocked with 5% normal goat serum and 0.3% Triton X-100 in PBS for 30 min at room temperature. A polyclonal antibody to FasL (anti-FasL; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the sections at 0.1 μg/ml in 1% normal goat serum and 0.3% Triton X-100 in PBS for 1 hour at room temperature. Sections were washed three times for 5 min in PBS, followed by the addition of peroxidase-conjugated goat antibody to rabbit Ig (1 μg/ml; Jackson ImmunoResearch) for 1 hour at room temperature. After washing three times for 5 min in PBS, antibody location was determined with the addition of DAB substrate (ImmunoPure metal-enhanced DAB substrate kit; Pierce) for 5 min. Color development was stopped by washing in water. The antiserum was raised to a peptide corresponding to amino acids 2 to 19 at the NH_2-terminus of rat FasL and cross-reacted with rat and mouse FasL by immunoblot (Santa Cruz Biotechnology) and immunohistochemistry (*20*). Staining in the presence of inhibitor peptide (amino acids 2 to 19 at 1 μg/ml) and staining with rabbit IgG were negative (*20*).

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