Accelerated Rejection of FAS Ligand-Expressing Heart Grafts

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*This information is current as of November 7, 2017.

J Immunol 1999; 162:518-522; ;
http://www.jimmunol.org/content/162/1/518

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Accelerated Rejection of FAS Ligand-Expressing Heart Grafts

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The Fas/Fas ligand (FasL) system plays an important role in the induction of lymphoid apoptosis and has been implicated in the suppression of immune responses. Recently, there has been renewed interest in immune privilege, as it was shown that two privileged sites (the eye and testes) constitutively express FasL, which kills lymphoid cells that invade these areas. We have established murine FasL-transgenic mice (B6) under the control of the cardiac α-myosin heavy chain promotor, and transplanted FasL-expressing F1 (B6 × C3H/HeJ) heart grafts into syngeneic (F1) and allogeneic (C3H/HeJ) recipients. FasL-expressing F1 heart allografts placed in C3H/HeJ recipients as well as FasL-expressing F1 isografts placed in nontransgenic and FasL-transgenic F1 were more rapidly rejected, and their survival was much shorter than that of nontransgenic control F1 allografts placed in C3H/HeJ. Native control and FasL-expressing hearts looked normal in mice up to 8 wk of age on hematoxylin-eosin staining. Control heart allografts undergoing ordinarily acute rejection showed moderate focal lymphocyte infiltrates, while FasL-expressing F1 allografts and isografts showed massive hemorrhage, edema, and massive neutrophil infiltration as early as 1 day after transplantation. In conclusion, FasL expression and surgical procedure (ischemia/reperfusion) were synergistic in the induction of accelerated heart graft rejection, while allogeneicity was not necessary. It may be necessary to find ways of controlling neutrophilic reaction/apoptosis in infiltrating lymphocytes to use FasL in clinical organ transplantation. The Journal of Immunology, 1999, 162: 518–522.

Fas is a type I membrane protein, a member of the TNF receptor family, which mediates apoptosis. Fas ligand (FasL) is a 40-kDa type II membrane protein, a member of the TNF family, expressed mainly in cytotoxic T cells on activation. When FasL binds to Fas on Fas-sensitive target cells, the target cells die by apoptosis (1). A family of cysteine proteases is sequentially activated leading to Fas-induced apoptosis, and Bcl-2 inhibits this process. The Fas/FasL system plays an important role in the induction of lymphoid apoptosis and has been implicated in the suppression of immune responses. Recently, there has been renewed interest in immune privilege, as it was shown that two privileged sites (the eye and testes) constitutively express FasL, which kills lymphoid cells that invade these areas (2).

The first important report on in vivo action of FasL-expressing organs in transplantation was by Bellgrau et al. (3). When normal mouse testes expressing FasL were transplanted under the kidney capsule of allogeneic animals, transplant survival was prolonged indefinitely through their induction of apoptosis in infiltrating activated T cells, whereas testis grafts derived from mutant gld mice, which express nonfunctional ligand, were rejected (3). In contrast, Seino et al. (4) reported that FasL-transfected murine tumor cells were rejected in syngeneic mice mainly by neutrophils. Kang et al. (5) reported that when pancreatic islets infected with an adenoviral vector containing FasL cDNA were transplanted into allogeneic diabetic mice, diabetes relapsed more rapidly with accelerated neutrophilic rejection in a T or B cell-independent manner than when control islet cells were transplanted. Moreover, FasL-transgenic mice expressing FasL in pancreatic β cells under the control of the insulin promotor developed destruction of β cells and consequent diabetes due to massive neutrophil recruitment (5). In a more recent report, the gene transfer of FasL by an adenoviral vector into the Fas+ murine renal cell carcinoma (renca) tumor cells eliminated the tumor by inducing tumor cell death, while the same strategy eliminated the Fas− CT26 colon carcinoma tumor by a mechanism mediated by inflammatory cells (6).

Materials and Methods

Establishment of murine FasL transgenic mice

Murine FasL cDNA was excised with XbaI from pBLC-MFLW4 (7) provided by Dr. S. Nagata (Osaka University, Osaka, Japan), gel purified, blunted ended by Klenow fragment DNA polymerase, and ligated into the blunt-ended EcoRI site of the mammalian expression vector pCAGGS (8) (pCmFasL). The 730-base pair XbaI-BamHI fragment of pCmFasL, including the poly(A) signal of rabbit β-globin, was blunted ended by Klenow fragment, gel purified, and ligated into the blunt-ended SalI site of pMHC-poly(A), yielding pMHC-mFasL, containing murine α cardiac myosin heavy chain promoter, murine FasL cDNA and poly(A) signal.
Transgenic mouse lines were generated by microinjection of fertilized eggs as described (11). The purified inserts of the NotI-Xhol fragment of pMHCMFasL were injected into the male pronuclei of fertilized eggs from C57BL/6J (Nihon Cleo, Tokyo, Japan) mice. The manipulated eggs were cultured and transferred into the oviducts of ICR (Nihon Cleo, Tokyo, Japan) pseudopregnant recipients. F₀ mice and their descendents were screened for genomic integration of murine FasL transgene as described below. Tail genomic DNA purified as previously described (12) was amplified by 30 cycles (95°C for denaturing, 55°C for annealing, and 72°C for extension) of PCR, using a thermal cycler with 1 μM sense and antisense primers (5'-GGTGGTAGAAGGAACCTCAAGA-3'/5'-TTAAAGCTTATACGCGA-3') taken from the α-MHC promoter region upstream to the inserted cDNA and murine FasL cDNA, respectively. 100 μM deoxynucleotide triphosphates, 2.5 U of Taq polymerase (Boehringer-Mannheim, Mannheim, Germany), and the buffer supplied with the enzyme. Amplified products (10 μl) were resolved by electrophoresis in 1.5% agarose gels containing ethidium bromide. Transgene-positive F₀ mice (B6) were crossed with normal B6 and normal C3H/HeJ (Nihon Cleo) mice.

Heterotopic heart transplantation

Heterotopic heart transplantation (murine FasL-transgenic and control B6 × C3H/HeJ (H-2ᵇ) F₁ as well as naive C3H/HeJ (H-2ᵃ) for donors and recipients) was performed according to Corry’s method (13). Heart grafts from male and female donors were transplanted into male and female recipients, respectively. Heart graft survival was monitored daily by palpation, and rejection (stopping of heart beat) was confirmed by laparotomy.

RT-PCR/Southern hybridization

RT-PCR/Southern hybridization was performed basically as previously described (12) using 2.5 μg of total RNAs. Sense/antisense PCR primer for murine FasL and β-actin were 5'-GTTCGATCAGCCGGCTATGA-3' / 5'-TTAATAGTTTACACACCCAAG-3' and 5'-TGGATATCTGTG GCATCTATGCCAAAC-3'/5'-TAAAGGCACGTCTACGCGA-3', respectively. Oligoprobes for murine FasL and β-actin used in Southern hybridization were 5'-TGAGGAGCGCAGCAAGTG-5'/5'-CCATGACCAGGCATGTGACG-3', respectively.

Western blotting

Protein was extracted from FasL-transgenic and control F₁ (B6 × C3H/HeJ) hearts in RIPA buffer. Protein samples (30 μg) and serum (100 μl) of murine FasL-transgenic and control F₁ mice of line 8 were electrophoresed at 20 mA in 12.5% polyacrylamide gel (Bio-Rad, Richmond, CA) and blotted to a nitrocellulose membrane. The membrane was incubated at 4°C overnight in blocking buffer (5% skim milk, 0.05% Tween 20 in Tris-buffered saline), hybridized with 0.33 μg/ml anti-human FasL rabbit polyclonal Ab (Q-20, Santa Cruz Biotechnology), or control heart and FasL-transgenic heart, respectively.

Histology

Tissues were fixed with 4% paraformaldehyde in PBS (pH 7.4). Paraffin-embedded sections were stained with hematoxylin and eosin. Cryostat sections were fixed in 4% paraformaldehyde in PBS for 10 min, rinsed with PBS (pH 7.4), and then incubated with anti-human FasL rabbit polyclonal Ab (5 μg/ml; Q-20, Santa Cruz Biotechnology), or con- trol serum overnight at 4°C. Labeling was visualized by the avidin-biotin complex method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Results

Establishment of murine FasL transgenic mice

Among 27 newborn B6 mice, 4 were positive for the murine FasL transgene (male F₀-5.1, F₀-6, and F₀-8 and female F₀-5). Transgene expression when F₀ was crossed with normal B6 or C3H/HeJ mice was as shown in Table I. Male F₀-5.1 and female F₀-5 did not produce transgenic F₁. Male F₀-6 transmitted the FasL transgene only to female F₁, while male F₀-8 transmitted it to both male and female F₁. There were no apparent macroscopic abnormalities. Hearts of F₁ mice (F₀-8 (B6 × C3H/HeJ)) were used as donor organs in the following transplantation study, as a sufficient number of these transgenic F₁ mice were obtained.
FasL-expressing F1 allografts placed in C3H/HeJ mice as well as FasL-expressing F1 isografts placed in control and FasL-transgenic F1 mice showed massive hemorrhage, edema, and massive neutrophil infiltration as early as 1 day after transplantation. Nontransgenic heart isografts (control F1 hearts placed in control F1 mice as well as naive C3H/HeJ hearts placed in naive C3H/HeJ mice) on days 5 after transplantation showed only mild cellular infiltrates. Native FasL-expressing F1 hearts excised from FasL-transgenic F1 recipients with acceleratedly rejected FasL-expressing F1 heart isografts (day 1) looked normal on hematoxylin-eosin staining (data not shown). Fas and Igs were not detected in control F1 heart iso- and allografts (day 5) and FasL-expressing F1 heart iso- and allografts (day 1) as well as native hearts from control and FasL-expressing F1 mice (data not shown).

Table II. Survival of heart grafts

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>Graft Survival (Days)</th>
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</thead>
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<td>C3H/HeJ</td>
<td>6, 6, 7, 7, 7, 8</td>
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<td>C3H/HeJ</td>
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</tr>
<tr>
<td>F1-Tg</td>
<td>F1-non-Tg</td>
<td>1, 1, 1, 1, 2, 2</td>
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<tr>
<td>F1-Tg</td>
<td>F1-Tg</td>
<td>1, 1, 2, 2</td>
</tr>
<tr>
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<td>F1-non-Tg</td>
<td>21&lt;, 21&lt;, 21&lt;, 21&lt;, 21&lt;</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>C3H/HeJ</td>
<td>21&lt;, 21&lt;, 21&lt;, 21&lt;, 21&lt;</td>
</tr>
</tbody>
</table>

* F1, B6 × C3H/HeJ; Tg, FasL-transgenic; non-Tg, nontransgenic.

Discussion

FasL-expressing vascularized mouse heart iso- and allografts were very rapidly rejected with marked neutrophilic infiltration, edema, and hemorrhage. These data are in line with previous reports showing that syngeneic tumor cells or allogeneic pancreatic islet cells engineered to express FasL were rejected by neutrophils (4, 5).
Because FasL-expressing isografts as well as allografts but not native FasL-expressing hearts or control isografts underwent accelerated rejection, it seems that the surgical procedure itself was an initiator of graft rejection and that FasL engineered to express in heart grafts was a target of immune system of syngeneic and allogeneic recipients. Therefore, FasL expression and surgical procedure were synergistic in the induction of accelerated heart graft rejection, while allogenicity was not necessary. Reactions inducing accelerated rejection of FasL-expressing heart grafts seemed to be a local event, because FasL-transgenic recipients remained healthy after rejection and native FasL-expressing hearts of those excised at the time of accelerated rejection looked normal. Moreover, this accelerated rejection does not seem to be caused by apoptosis of heart muscle cells through Fas/FasL system or humoral rejection involving preexisting Abs, because Fas and Igs were not up-regulated in FasL-expressing heart iso- and allografts.

Differences between native hearts and isografts transplanted into syngeneic recipients might be ischemia/reperfusion, which might have triggered accelerated rejection of FasL-expressing isografts and allografts. Ischemic myocardial injury initiates an acute inflammatory response in which polymorphonuclear leukocytes are major participants, the interplaying inflammatory reactions are augmented by reperfusion and accumulating polymorphonuclear leukocytes can contribute to myocardial damage (14). The possibility that FasL-transgenic mice are not able to initiate the neutrophilic response against FasL-expressing hearts is excluded, because FasL-expressing isografts placed in FasL-transgenic recipients were also rapidly rejected.

FIGURE 3. Hematoxylin-eosin staining of F1(B6 × C3H/HeJ) heart grafts. A, Control heart (not transplanted); B, murine FasL-transgenic heart (not transplanted); C, control heart isograft (day 5 posttransplant); D, murine FasL-transgenic heart isograft (day 1 posttransplant); E, control heart allograft (day 5 posttransplant); F, murine FasL-transgenic heart allograft (day 1 posttransplant). Original magnification, ×480.
Whether allografts or tumor cells expressing FasL show graft/ tumor rejection due to the inflammatory response with the recruitment of neutrophils or prolonged allograft survival/tumor growth enhancement by inducing apoptosis in the infiltrating lymphocytes in vivo may depend on the level of FasL expression on graft/tumor cells, graft/tumor cell types, methods of gene transfer, and immune status of the host. Seino et al. (4) reported that syngeneic FasL-transfected tumor cells were rejected when transplanted s.c. or i.p., but were maintained under the kidney capsule. However, while allogeneic FasL-expressing tumor cells did not form tumors at all, FasL expression itself was not sufficient to destroy the allogeneic barrier, as previously reported (15). We have established FasL-transfected renca cells and orthotopically implanted them under the renal capsule of syngeneic BALB/c mice; we found that FasL-expressing renca tumor growth was enhanced compared with control renca tumor, via inducing apoptosis in tumor-infiltrating lymphocytes in the absence of a neutrophilic response (unpublished data).

FasL expression may contribute to the enhanced growth of human tumors under some conditions. Strand et al. reported that human hepatocellular carcinomas partially or completely lost Fas constitutively expressed by normal liver cells and might evade Fas-mediated cell killing by CTL. On the other hand, FasL-expressing HepG2 hepatoblastoma cells killed Fas+ Jurkat T cells (16). The colon cancer cell line SW620 expressing both Fas and FasL killed Jurkat T cells but did not undergo apoptosis after treatment with anti-Fas agonistic mAb (17). These reports suggested that these cancer cells are resistant to Fas-mediated T cell cytotoxicity but express functional FasL, an apoptotic death signal to activated T cells. Zeytun et al. reported a mutual killing model in which FasL+, Fas+ tumor cells, LSA and EL-4, killed Fas+ tumor-specific CTLs and were also killed by tumor-specific FasL+ CTLs. They concluded that the survival of the tumor or the host may depend on which cells can accomplish FasL-based killing more efficiently (18).

Nontransplanted native hearts of FasL-transgenic mice did not show morphologic changes, at least early in life, but when transplanted into syngeneic and allogeneic recipients, it induced marked neutrophilic reaction and was very rapidly rejected. This neutrophilic response may be due to the action of FasL itself as a cytokine or may be mediated by a neutrophilic cytokine, IL-8, as reported (5). There is a possibility that native FasL-expressing hearts become damaged in mice with time by the neutrophilic response for founder mice as well as F1, F2 mice (lines 6 and 8) looked healthy up to 1 year of age. The vascularized heart isograft and allograft rejection by neutrophils seems quite rapid compared with that of allogeneic islet cells expressing FasL. This may be because “vascularized” grafts have more favorable circumstances for inducing neutrophilic and subsequent reactions causing graft rejection due to the large blood flow. It may be necessary to find ways of controlling neutrophilic reaction/apoptosis in infiltrating lymphocytes to use FasL in clinical organ transplantation.

It is obviously useful and interesting to assess other FasL-transgenic lines in mouse heart transplantation study. We could obtain no FasL-transgenic F1 in two lines (F1-5 and F1-5.1) and only a few in one line (F1-6). When we transplanted a FasL-transgenic heart (B6) of line F1-6 into an allogeneic C3H/HeJ recipient (only one transplantation performed), the allograft was also rejected on the next day after transplantation.

Acknowledgments
We thank Ms. Kanae Sasaki for help with the mouse heart transplantation.

References