Basic Science Reports

Display of Fas Ligand Protein on Cardiac Vasculature as a Novel Means of Regulating Allograft Rejection

Nadir Askenasy, MD, PhD*; Esma S. Yolcu, PhD*; Zhiliang Wang, MD; Haval Shirwan, PhD

Background—Fas ligand (FasL) is a potent death-inducing molecule with important functions in immune homeostasis and tolerance to self-antigens. The complex biological activities of FasL and its inefficient expression using conventional gene transfer approaches limit its use for immunomodulation to prevent allograft rejection. We have recently generated a chimeric FasL with core streptavidin (SA-FasL) with potent apoptotic activity and designed a novel approach to display it on the surface of several cell types via biotinylation. We herein tested whether SA-FasL can also be displayed on vascular endothelial cells in the heart and examined its effect on graft survival after transplantation into syngeneic and allogeneic hosts.

Methods and Results—SA-FasL was efficiently displayed on the vasculature of BALB/c hearts with a half-life of 9 days in vivo. Transplantation of hearts displaying SA-FasL into syngeneic hosts resulted in indefinite graft survival without detectable toxicity to the grafts and hosts. In contrast, transplantation of allogeneic C57BL/10 hearts displaying SA-FasL into BALB/c recipients resulted in graft rejection, but in a delayed fashion as compared with control hearts (mean survival time=17.4±5 versus 9.6±1 days). Allograft survival was further extended to 21±2.6 and 24±3 days (P<0.05) by intravenous treatment of graft recipients with 1 dose of SA-FasL–decorated donor splenocytes on days 2 and 6 after transplantation, respectively.

Conclusions—This study shows for the first time that exogenous proteins can be displayed on the endothelium of solid organs for therapeutic purposes. This approach provides a convenient and rapid means of displaying exogenous proteins on the surface of cells, tissues, and solid organs, with broad research and therapeutic implications. (Circulation. 2003;107:r41–r47.)

Key Words: immunology ■ proteins ■ apoptosis ■ transplantation ■ gene therapy

The critical role that the Fas/Fas ligand (FasL) interaction plays in immune homeostasis, self-tolerance, and immune privilege in vital organs1–3 led to attempts to use these molecules to prevent allograft rejection and induce tolerance to solid organ grafts.4–9 Conceptually, the presentation of alloantigens to T cells in the presence of FasL may lead to the apoptosis of activated cells and may block alloreactive responses.10–12 The process of activation-induced cell death (AICD) involves interaction of FasL with the Fas receptor expressed on the surface of activated lymphocytes, which initiates a cascade of events that culminates in the activation of caspases and cell apoptosis.13,14 Several studies demonstrated effective blockade of alloreactive responses and survival of allogeneic liver, kidney, thyroid, and pancreatic islets using Fasl expressed via genetic modifications as an immunomodulatory approach.4–6,15–17 In contrast, transgenic expression of wild type (wt)FasL in pancreatic β-cells and hearts resulted in their accelerated rejection by allogeneic hosts.7–9 The hyperacute rejection of these allografts was accompanied by heavy inflammatory infiltrates, which were likely recruited into the grafts by the soluble FasL, generated from the membrane-bound form by matrix metalloproteinases.18–20 Soluble FasL has dual functions that include an anti-apoptotic effect, which is caused by competition with the membrane-bound FasL for Fas binding, and chemotaxis of neutrophils, which initiates and perpetuates inflammation.21–23 Additional factors, such as the expression level of FasL, the kinetics of expression, the nature of the microenvironment where FasL is expressed, and the lack of mechanisms that regulate FasL function in various tissues may contribute to the opposing effects of FasL on the immune responses, ie, immune activation versus immune blockade.3,12

We reasoned that the transient cell-surface display of a non-cleavable form of FasL that lacks the metalloproteinase cleavage site and exerts a more potent apoptotic function than wtFasL might facilitate its effective use as an immunomodu-
latory molecule to regulate allograft rejection. Furthermore, because the clinical application of gene therapy is limited by the low efficiency of gene delivery systems, the modest capacity to control gene expression, difficulties in targeting to the desired tissues, and safety issues, we have recently developed a novel method, designated as ProtEx, that allows for rapid and durable display of exogenous proteins on the surface of isolated cells. This technique relies on the generation of chimeric proteins with core streptavidin/avidin and their attachment to biotinylated cells under physiological conditions. In this study, we tested whether a chimeric molecule composed of the extracellular portion of rat FasL lacking the metalloproteinase site and of core streptavidin (SA-FasL) can be effectively displayed on the heart graft vasculature, and tested the effect of SA-FasL on graft survival in syngeneic and allogeneic hosts. Selective display of FasL on the surface of vascular endothelium may be particularly relevant to the efficient use of this molecule to prevent heart rejection because the endothelium is resistant to Fas/FasL-mediated apoptosis, whereas cardiomyocytes are extremely sensitive to this apoptotic pathway. We report here for the first time that cardiac vasculature can be modified with an exogenous chimeric protein under conditions of hypothermic extracorporeal preservation without detectable toxicity, and such grafts showed indefinite survival in syngeneic and prolonged survival in allogeneic hosts.

Methods

Animals
B10 (C57BL/10Sn, H-2b) and BALB/c (H-2b) mice were purchased from Jackson Laboratories (Bar Harbor, Me), housed in a pathogen-free facility, and treated in accordance with the National Institutes of Health Guide for the Care and Use of Animals under supervision of the Institutional Animal Care and Use Committee.

Heart Preparation
Mice (8 to 10 weeks old) were anesthetized by intraperitoneal injection of ketamine/xylazine (100/10 μg/g), and 20 μL of heparin sulfate was administered. Aortas of the donor hearts were cannulated and hypothermic cardiac arrest was induced by infusion of ice-cold medium. Retrograde coronary perfusion at normothermia (37°C) was performed in a Langendorff preparation using double-walled, water-jacketed containers and tubing to maintain constant temperature.

The hearts were paced at 8 Hz, diastolic pressure was set at 5 to 10 mm Hg, and the left ventricular developed pressure (LVDP) was determined as the difference between the peak systolic and diastolic pressures against rat FasL (PharMingen; 1:50 dilution of 0.5 mg/mL solution), followed by perfusion with KH solution. Tissues were snap-frozen at −80°C and sectioned with a Polycut S Microtome using a heated knife. Images were acquired at magnifications of 10× to 20× using a charge-coupled device camera (Princeton Instruments) and processed to simulate the real hues.

Decoration of Splenocytes With SA-FasL
Spleens were harvested from B10 donors and processed for single cell suspension. Splenocytes were then biotinylated in 15 μmol/L of freshly prepared EZ-Link Sulfo-NHS-LC-Biotin for 30 minutes at room temperature. Cells were washed twice and resuspended in PBS supplemented with 50 to 100 ng SA-FasL per 107 cells/mL. After incubation on ice for 20 minutes with gentle mixing, the cells were washed extensively and analyzed in flow cytometry for the cell surface SA-FasL protein using MFL-4 mAb conjugated to FITC. Four million irradiated cells (2000 rad) were injected intravenously into graft recipients on days 2 or 6 after transplantation.

Statistical Analysis
Data are presented as means±SD for each experimental protocol. The experimental variables were evaluated for reproducibility by 1-way ANOVA. Differences between the experimental protocols were evaluated with a post hoc Scheffe test, with P<0.05 selected for level of statistical significance.

Results

Modification of Cardiac Endothelium With Biotin and Exogenous Proteins Without Detectable Toxicity
Modification of organs with exogenous proteins for therapeutic purposes is contingent on the preservation of organ function after manipulation. We first evaluated the effect of
Modification of heart endothelium with biotin. The images present the surface of tissue cryosections as observed by fluorescence microscopy (Axioplan, C. Zeiss) at 10×. Hearts (n=5) were perfused at hypothermia with Krebs-Henseleit solution (KH) at a rate of 0.05 mL/min in 5 stages: 4 minutes KH, 8 minutes KH containing 5 μmol/L biotin, 4 minutes KH, 8 minutes KH containing 0.5 μg/mL streptavidin-FITC, and 4 minutes KH. Tissues were frozen and then sectioned. a, Brightfield image of the frozen tissue surface. b, Fluorescence of streptavidin-FITC bound to the biotinylated endothelium is demonstrated on the surface of the section. The image was pseudocolored to simulate the real hues. A cutoff arteriole shows no fluorescence (blue arrow), and reflected endothelium injured by the sectioning process shows bright fluorescence (yellow arrowheads). Measurements of the fluorescence intensity showed 70±3 arbitrary units (at the location demarcated 4 to 6) as compared with a background fluorescence of 7.5±3 U (at the location demarcated 1 to 3). c, The trajectory of the coronary artery is demarcated in superposition of the fluorescence (b) image over the brightfield image (a). d, Unmodified hearts perfused with streptavidin-FITC served as controls without a detectable signal.

We next assessed the feasibility of displaying SA-FasL on the surface of B10 hearts perfused at normothermia (protocol 1). Control hearts (n=5) had baseline LVDP of 89±6 mm Hg and coronary flow rates of 1.4±0.1 mL/min. Biotinylation and decoration with streptavidin-FITC (Figure 1), S2 proteins, or SA-FasL (n=5 in each group) resulted in an insignificant decrease in LVDP to 81±5 mm Hg, without detectable changes in coronary flow rates.

We next tested whether hearts manipulated ex vivo at hypothermia can function after transplantation into syngeneic hosts. Hearts that were perfused with KH solution only survived indefinitely in syngeneic BALB/c mice without detectable acute or chronic toxicity (n=7; mean survival time [MST] >100 days; Table 1). Control hearts that were modified with biotin and decorated with the SA-FasL protein (protocol 2) also survived indefinitely in syngeneic recipients (n=7; MST >100 days). Importantly, SA-FasL on the graft vasculature was not toxic to the recipient liver as determined by the normal levels of alanine aminotransferase (25±9 IU/mL) and alkaline phosphatase (48±13 IU/mL) measured on days 7 and 21 after transplantation. Furthermore, livers from these animals did not show significant immunostaining for apoptosis as compared with naïve animals (data not shown).
The long-term survival of hearts decorated with SA-FasL in syngeneic hosts led us to test if such hearts are refractory to rejection when transplanted into allogeneic recipients. B10 hearts were decorated with SA-FasL (protocol 2) and transplanted into allogeneic BALB/c hosts disparate for minor and major histocompatibility antigens. All the grafts in this group (n = 7) showed statistically significant (P < 0.01) prolonged survival, with MST = 17.4 ± 5 days as compared with unmanipulated control allografts (n = 7; MST = 9.6 ± 1 days) and allografts modified with biotin and S2 proteins (n = 6; MST = 8.6 ± 1.4 days; Table 1).

To provide evidence that the observed graft prolongation was due to FasL-induced apoptosis in Fas-expressing alloreactive lymphocytes, aortic endothelial cells decorated with SA-FasL were co-incubated with A-20 cells (a Fas+ mouse B lymphoma line) for 18 hours. Apoptosis was determined using propidium iodide and annexin V-FITC in flow cytometry in 4 independent cultures. There was >40% cell death of A-20 cells in cultures containing SA-FasL–decorated endothelial cells as compared with <5% cell death in cultures containing biotinylated endothelial cells with and without core streptavidin as control (Figure 3). Furthermore, we did not observe significant endothelial cell death in cultures containing SA-FasL as compared with control cultures (data not shown). This is consistent with previous studies demonstrating that endothelial cells are resistant to FasL-mediated killing.

Extended Endothelial Display of SA-FasL In Vivo in the Absence of Neutrophil Infiltration

The rejection of allogeneic grafts displaying SA-FasL might have been caused by the loss of protein from the endothelium, which resulted in the restoration of alloreactive immune responses over time, and/or by the recruitment of inflammatory infiltrates. To assess the persistence of SA-FasL on graft vasculature, hearts were excised at different times after transplantation and stained with FITC-MFL-4 mAb. The fluorescence intensity ratio (versus background) decreased from 9.3 ± 2.2 before transplantation to 5.7 ± 1.4 and 3.2 ± 0.7 on days 6 and 12 after transplantation, respectively (Figure 4). A linear curve fit showed a half-life of 9 days for SA-FasL on vascular endothelium, suggesting that the shear stress of blood flow in the graft vasculature did not significantly contribute to the turnover of biotin-streptavidin complex from the endothelial surface. Persistence of SA-FasL protein for weeks on the endothelium is consistent with our previous observations that SA-FasL may persist on the cell surface for weeks in vitro and in vivo.

To assess whether the grafts were rejected because of early chemotactic recruitment of neutrophils, we performed a histological analysis of the grafts. In unmanipulated and S2 protein-treated hearts on day 6 after transplantation, there were extensive signs of acute rejection, including disruption of the endothelial lining of blood vessels, infiltration of inflammatory cells (perivascular cuffing), and deterioration of the structural organization of myofibers in the subepicardial and subendocardial regions, reflecting onset of mild to moderate ischemia (Figure 5b). In marked contrast, SA-FasL–decorated hearts were largely devoid of these histopathological changes when analyzed at this time (Figure 5c). These hearts, however, showed evidence of inflammation on day 12 after transplantation, similar to but less intensive than the unmanipulated or S2 controls at 6 days after transplantation (Figure 5d). Notably, the perivascular infiltrate was predominantly lymphocytic, with no significant presence of polymorphonuclear cells. Taken together, these data suggest that the prolonged heart survival is caused by a delay in acute rejection, which reappeared with the loss of SA-FasL from the endothelium.

### Table 1. Display of SA-FasL on Heart Vasculature Results in Prolonged Graft Survival in Allogeneic Hosts

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Recipient</th>
<th>Donor</th>
<th>Treatment</th>
<th>Mean Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>7</td>
<td>BALB/c</td>
<td>BALB/c</td>
<td>...</td>
<td>&gt;100</td>
</tr>
<tr>
<td>B</td>
<td>7</td>
<td>BALB/c</td>
<td>BALB/c</td>
<td>Perfusion*</td>
<td>&gt;100</td>
</tr>
<tr>
<td>C</td>
<td>7</td>
<td>BALB/c</td>
<td>BALB/c</td>
<td>Biotin-FasL†</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D</td>
<td>7</td>
<td>BALB/c</td>
<td>B10</td>
<td>...</td>
<td>9.6 ± 1</td>
</tr>
<tr>
<td>E</td>
<td>6</td>
<td>BALB/c</td>
<td>B10</td>
<td>Biotin-S2‡</td>
<td>8.6 ± 1.4</td>
</tr>
<tr>
<td>F</td>
<td>7</td>
<td>BALB/c</td>
<td>B10</td>
<td>Biotin-FasL†</td>
<td>17.4 ± 5.6</td>
</tr>
</tbody>
</table>

*Cardiac grafts were transplanted after perfusion at hypothermia with Krebs Henseleit solution.
†Hearts perfused with biotin and SA-FasL.
‡Hearts perfused with biotin and S2 proteins.
§P < 0.01 vs group D.

Figure 3. Endothelial cells decorated with SA-FasL induce apoptosis in Fas+ A-20 lymphoma cell line. A-20 cells were co-incubated with biotinylated cells (a, None), cells decorated with core streptavidin (b, SA), and cells decorated with SA-FasL (c, FasL) for 18 hours. The cells were then harvested and analyzed for apoptosis using PI and annexin V-FITC, and A-20 cells were selected by gating on B220 positive cells (anti-B220 mAb-APC) with the use of flow cytometry.

Immunomodulation of Graft Recipients With Donor Splenocytes Decorated With the SA-FasL Protein Extends Graft Survival

The apparent late onset of acute rejection in the presence of SA-FasL on vascular endothelium led us to test whether...
additional immunomodulation of graft recipients with splenocytes expressing SA-FasL further delayed rejection. Injection of naïve (n=5) or biotinylated donor splenocytes treated with S2 proteins (n=5) on day 2 after transplantation into recipients of unmanipulated heart allografts did not affect the tempo of graft rejection (MST=8±1.4 days and 9.5±2 days, respectively; Table 2). Acute allograft rejection was also unaffected by injection of donor splenocytes decorated with SA-FasL on day 2 after transplantation of unmanipulated hearts (n=5; MST=10.2±1.3 days), implying that the direct display of SA-FasL on the heart endothelium may be critical to the prolonged survival. Consistent with this notion, injection of SA-FasL decorated splenocytes into the recipients of SA-FasL–decorated hearts on day 2 after transplantation resulted in prolonged allograft survival to 21±2.6 days (n=7). This rejection time was further extended to 24±3 days when donor splenocytes decorated with SA-FasL were injected 6 days after transplantation (n=7; P<0.05 versus MST=17.4±5 days for SA-FasL–decorated hearts). By contrast, injection of the recipients of SA-FasL–decorated hearts with donor unmanipulated splenocytes 2 days after transplantation abolished the SA-FasL–mediated prolonged survival (n=5, MST=10.8±2 days, P<0.05 versus MST=17.4±5 days for SA-FasL–decorated hearts only).

Discussion

We present a novel approach that exploits the direct display of exogenous proteins on vascular endothelium of solid organs for immunotherapeutic purposes. This approach is unique and differs in several aspects from conventional methods that utilize gene transfer or systemic administration of biologics for immunomodulation. First, recombinant proteins are directly displayed on the vascular endothelium to avoid the difficulties associated with gene transfer approaches, such as the inefficiency of gene transfer, limited control over the levels of expression, and lack of safety. Second, the display of proteins is efficiently performed within a short period of time under conditions of extracorporeal organ preservation similar to those used in the clinic. Third, the proteins are selectively introduced into the target tissues or organs to achieve a localized effect, which may prove more efficacious as compared with systemic immunomodulation. Fourth, the high selectivity of direct protein display on the graft endothelium may avoid toxicities that otherwise would be associated with the introduction of proteins into the parenchymal tissue. For example, the toxicity of FasL for smooth and cardiac muscles is negated when the expression of this death molecule is restricted to the endothelium.

Using FasL as an immunomodulatory molecule, we have demonstrated that the direct display of a non-cleavable isoform on cardiac vascular endothelium results in statistically significant prolongation of graft survival in allogeneic hosts. We did not observe infiltration of neutrophils in hearts
displaying the chimeric protein, which suggests that SA-Fasl is not chemotactic for polymorphonuclear cells, as shown in studies that used wtFasL\(^{1,23}\) and other forms of non-cleavable Fasl.\(^{32}\) Furthermore, the selective display of SA-Fasl on the graft vascular endothelium may also prevent intimal hyperplasia, and may therefore ameliorate the development of transplant-associated vasculopathy. Previous reports showed that the expression of wtFasL in vascular endothelial cells results in T cell apoptosis\(^{33}\) and reduces inflammatory cell infiltrates,\(^{34,35}\) and both wt and non-cleavable Fasl limit intimal hyperplasia.\(^{36,37}\)

The studies presented herein mainly provide proof-of-concept for protein-based therapy. Optimization of SA-FasL concentration and the timing of injection of decorated donor splenocytes remain to be further evaluated for the effective use of SA-FasL as a tolerogenic molecule. The inability of SA-FasL to completely prevent graft rejection may be due to the emergence of alloreactive memory cells from non-lymphoid tissues when the concentration of SA-FasL on graft vasculature decreased below the effective threshold.\(^{35,38-40}\) Although SA-Fasl in our chimeric protein lacks the putative metalloproteinase site and as such persists on the graft vasculature with a half-life of 9 days in vivo, this may not be sufficient for effective elimination of a large pool of peripheral alloreactive lymphocytes. This notion is consistent with our observations that additional post-transplant immunomodulation of graft recipients with donor splenocytes decorated with SA-FasL further prolongs graft survival. In contrast, immunomodulation with unmanipulated donor splenocytes negates the protective effect exerted by SA-FasL on graft vasculature, plausibly by effectively activating a large pool of peripheral alloreactive lymphocytes that shifts the immunological balance toward rejection. These observations suggest that repeated injection of SA-Fasl–decorated donor cells into graft recipient before or after transplantation may serve as an effective approach to induce tolerance. T cells become extremely sensitive to apoptosis by Fasl/Fasl interaction after going through several rounds of antigenic challenge, approximately 3 days after the initial antigenic stimulation.\(^{38}\) Consistently, repeated pretransplant manipulation of graft recipients with donor dendritic cells or macro-

### TABLE 2. Immunomodulation With SA-Fasl\(^{[en]}\)Decorated Donor Splenocytes Further Extends the Prolonged Survival of Hearts Displaying SA-Fasl on Graft Vasculature

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Recipient</th>
<th>Donor</th>
<th>Heart Treatment</th>
<th>Mean Survival Time, d</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>BALB/c</td>
<td>B10</td>
<td>Unmanipulated (day 2)</td>
<td>8±1.4</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>BALB/c</td>
<td>B10</td>
<td>S2 (day 2)†</td>
<td>9.5±2</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>BALB/c</td>
<td>B10</td>
<td>Fasl (day 2)‡</td>
<td>10.2±1.3</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>BALB/c</td>
<td>B10</td>
<td>Biotin-Fasl*</td>
<td>Unmanipulated (day 2)</td>
</tr>
<tr>
<td>E</td>
<td>7</td>
<td>BALB/c</td>
<td>B10</td>
<td>Biotin-Fasl*</td>
<td>Fasl (day 2)‡</td>
</tr>
<tr>
<td>F</td>
<td>7</td>
<td>BALB/c</td>
<td>B10</td>
<td>Biotin-Fasl*</td>
<td>Fasl (day 6)‡</td>
</tr>
</tbody>
</table>

*Heart grafts displaying SA-Fasl.
†Splenocytes decorated with S2 proteins.
‡Splenocytes decorated with SA-FasL.
§P<0.005 vs group C; P<0.05 vs 17.4±5 MST for hearts displaying SA-Fasl (Table 1, group F).

phages genetically modified to express wtFasL was shown to lead to donor-specific immune non-responsiveness.\(^{16,17}\)

Immunomodulation by FasL expression involves selective deletion of alloreactive immune cells, predominantly of CD4\(^+\) and CD8\(^+\) T cell phenotypes, that express Fas on activation\(^{41-43}\) and are prone to AICD\(^{10-12}\) both in vivo and in vitro.\(^{16,17}\) A similar proapoptotic effect was shown for wtFasL expressed on the surface of endothelial cells and vascular endothelium,\(^{33-36}\) and we demonstrate that endothelial cells and splenocytes decorated with non-cleavable Fasl are even more potent as a trigger of apoptosis in Fas-positive cells.\(^{25}\) The display of FasL either on the graft itself or in its immediate vicinity aims to create a site of immune privilege that simulates a physiological defense mechanism present in vital organs.\(^{1-3}\) Importantly, recent studies have demonstrated the critical role of localized responses in immune regulation and raise the question whether such responses are not only more efficacious, but also specific, the 2 important characteristics of the adaptive immune system.\(^{39,40}\) We have recently demonstrated that co-transplant of pancreatic islets and SA-Fasl–decorated splenocytes under the kidney capsule of allogeneic hosts results in the prevention of graft rejection by non-systemic immunoregulatory mechanisms.\(^{25}\) Our present findings demonstrate the importance of local defense by expression of the Fasl chimeric protein on the surface of vascular endothelium to achieve prolonged graft survival.

This study demonstrates for the first time that heart grafts can be directly manipulated to display exogenous proteins on the vascular endothelium under extracorporeal preservation conditions used in the clinics, with no significant toxicity. This method of cell membrane modification with biotin and decoration with proteins chimeric with streptavidin is rapid, practical, and safe as compared with protein expression by gene transfer approaches. Furthermore, localized immune protection with protein display not only avoids adverse side effects associated with systemic therapy, but may also prove more efficacious because the target tissue is the site where the effector immunological reactions take place. Hearts modified to display SA-Fasl on the vascular endothelium had prolonged survival in allogeneic hosts, demonstrating the immunomodulatory potential of this novel approach. Inasmuch as FasL-mediated apoptosis primarily affects the proinflamma-
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Fas Ligand Protein Extends Heart Graft Survival


48. Askenasy et al Fas Ligand Protein Extends Heart Graft Survival
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