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A Critical Role of TRAIL Expressed on Co-Transplanted Hepatic Stellate Cells in Prevention of Islet Allograft Rejection

Horng-Ren Yang, MD^{2,3}, Ching-Chuan Hsieh, MD¹, Lianfu Wang, MD², John J Fung, MD., PhD.², Lina Lu, MD^{1,2}, and Shiguang Qian, MD^{1,2}

¹Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH

²Department of General Surgery, Cleveland Clinic, Cleveland, OH

³Department of Surgery, China Medical University Hospital, Taiwan

Abstract

Hepatic stellate cells (HSCs) have demonstrated a strong T-cell inhibitory activity. In a mouse islet transplantation model, co-transplanted HSCs can protect islet allografts from rejection. The involved mechanism is not fully understood. We showed in this study that expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), an important apoptosis-inducing ligand, on HSCs was crucial in protection of islet allografts since HSCs derived from TRAIL knockout mice demonstrated less inhibitory activity towards T-cell proliferative responses, and substantially lost their capacity in protecting co-transplanted islet allografts from rejection, suggesting that TRAIL mediated T cell apoptotic death is important in HSC-delivered immune regulation activity.

Introduction

Since we developed liver transplant model in mice using microsurgery technique,¹ substantial achievements have been made in better understanding of immunological and molecular mechanisms by which liver allografts are spontaneously accepted across major histocompatibility barriers without the requirement for immunosuppressive therapy.^{2–5} Obviously because various monoclonal antibodies and transgenic strains have been well developed, many definitive studies to determine the involved factors become realistic. Interestingly, although liver allografts are accepted, hepatocytes transplants in the same combination are promptly rejected,^{6,7} suggesting a crucial role of liver nonparenchymal cells (NPC) in protecting parenchymal cells from immune attacks. We have demonstrated that hepatic stellate cells (HSCs), well known to participate in liver fibrogenesis, markedly inhibit T cell responses *in vitro* and protect co-transplanted islets allografts from rejection in a mouse islet transplant model.^{8,9} B7-H1, an inhibitory molecule of the B7 family, plays an important role in the immunosuppressive activity of HSCs. However, blockade of B7-H1 ligation by addition of anti-B7-H1 mAb can only partially reverse the HSC-induced inhibition of T cell proliferation,⁸ suggesting that other molecule (s) may contribute to the immunosuppressive effect of HSCs.

TNF-related apoptosis-inducing ligand (TRAIL) is a type II membrane protein of the TNF superfamily.¹⁰ As a newly identified TNF family member, TRAIL-induced apoptosis is mainly restricted to tumor cells and virus infected cells.^{11–13} There is accumulating evidence demonstrating that TRAIL-induced apoptosis is not limited to the transformed cells, but may

also be induced in primary cells. For instance, in contrast to resting B cells that are resistant to TRAIL-mediated killing, plasma cells generated in a T cell dependent immune response become sensitive to TRAIL-induced apoptosis, indicating that TRAIL can also induce apoptosis in immune cells.¹⁴ Parallel to the FasL/Fas system, TRAIL is also constitutively expressed in different organs, suggesting that it could be implicated in the immune surveillance. Thus, TRAIL expression in placenta has been associated with the maintenance of immune privilege.¹⁵ It has been reported that TRAIL also has an effect on immune cell functions in autoimmune diseases.^{16–19} Gene transfer of TRAIL-encoding adenoviruses is able to significantly ameliorate clinical signs of arthritis via induction of apoptosis in activated T cells and synoviocytes.²⁰ The role of TRAIL in regulation of immune response in transplantation has not been extensively investigated. In this study, we demonstrated that, upon activation, HSCs upregulated expression of TRAIL. HSCs derived from TRAIL knockout (KO) mice induce less T cell apoptosis and are less effective in protecting co-transplanted islet allografts compared to HSCs derived from wild type (WT) mice.

MATERIALS AND METHODS

Mice

Male C57BL/6 (B6; H2^b) and BALB/c (H2^d) mice were purchased from Jackson Laboratory (Bar Harbor, ME). TRAIL KO (B6 background) mice were provided by Amgen (Seattle, WA). All animals were maintained in the specific pathogen-free facility at Lerner Research Institute in Cleveland Clinic, provided with Purina Rodent Chow (Ralston Purina, St. Louis, MO) and tap water ad libitum, and were used at 8–12 weeks of age following the guidelines of the National Institutes of Health.

Isolation and Culture of HSC

HSCs were isolated from mouse livers as previously described.^{8,9} Briefly, the liver was perfused via the subhepatic vena cava with 20 ml Ca²⁺ and Mg²⁺-free Hank's balanced salt solution (Mediatech, Herndon, VA), followed by perfusion with 1 ml collagenase IV (1 mg/ml, Sigma-Aldrich, St. Louis, MO). The liver was then removed, meshed, and agitated in collagenase IV (1 mg/ml) at 37 °C for 40 min. Cell suspension was filtered through a nylon mesh. HSCs were then enriched by Percoll density gradient centrifugation, and cultured in RPMI-1640 medium (Mediatech Inc., Herndon, VA) supplemented with 20% fetal bovine serum in 5% CO₂ in air at 37°C for 7 to 14 days. The purity of isolated HSC ranged from 90% to 95% as determined by desmin immunostaining and the typical light microscopic appearance of the lipid droplets as described previously.^{8,9} To further activation, HSCs were exposed to IFN- γ (100 U/ml) for the last 24 h culture.

Culture of Dendritic Cells

Bone marrow cells isolated from mouse femurs and tibias were lysed of red blood cells, cultured in RPMI-1640 medium containing 10% v/v heat-inactivated fetal calf serum, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 20 μ mol/L 2-mercaptoethanol, and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin) in the presence of mouse recombinant granulocyte-macrophage colony-stimulating factor (4 ng/mL) and interleukin (IL)-4 (1,000 U/mL) (both from Schering-Plough, Kenilworth, NJ). Nonadherent cells were released spontaneously from the proliferating cell clusters. These cells were then harvested, washed, and resuspended for further study.

Islet Transplantation

Diabetes was induced in recipients with a single intraperitoneal injection of streptozotocin (STZ) (180 mg/kg, Sigma-Aldrich, St. Louis, MO). Only the mice with blood glucose

exceeding 350 mg/dL were used in experiments. Islets were isolated from donor pancreata by collagenase digestion, and separated on a Ficoll gradient (Type 400, Sigma-Aldrich). Briefly, the donor mice were anesthetized with isoflurane. The abdomen was shaved, and attached to the operating board with elastic restraints and the abdomen prepped with betadine. After ensuring adequate anesthesia, a transverse incision was made in the abdomen. The pancreas was carefully dissected under the surgical microscope, and perfused via bile duct with 3 ml collagenase IV (1 mg/ml). The pancreas was removed for isolation of cells. The islets were purified by hand picking. 300 islets alone or mixed with 3×10^5 HSC were aspirated into polyethylene tubing (PE-50), pelleted by gentle centrifugation, and placed under renal capsule of the recipient mice with microsurgery technique. Transplantation was considered successful when blood glucose returned to and remained normal (≤ 150 mg/dL) for 4 days after transplantation. The first day of two consecutive readings of blood glucose greater than ≥ 350 mg/dL was defined as the date of graft failure. No immunosuppressive agents were administered throughout the experiment.

Quantitative Real-time PCR

Total RNA was extracted from HSCs with TRIzol reagent (Invitrogen Life Technologies). A 1.0- μ g aliquot of RNA was treated with deoxyribonuclease I following the manufacturer's manual (Invitrogen Life Technologies), and reverse-transcribed into cDNA with random primers and superscript II reverse transcriptase using manufacturer's protocol (Invitrogen Life Technologies). qPCR was performed by using an Applied Biosystems 7500 Fast Real-Time PCR System in duplicate with SYBR Green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. PCR consisted of 40 cycles of 95°C for 15 s and 60°C for 60 s. A final cycle (95°C, 15 s, 60°C) generated a dissociation curve to confirm a single product. The cycle quantity was determined with a standard curve, and expression levels were normalized to 18S. Primer sequences of TRAIL were forward, GCA GGT TAA GAG GCA ACT GTA TCA; reverse, TGG GCA AGG GAG GAG TAC TTA G.

T cell proliferation assay

One-way mixed leukocyte reaction (MLR) assay was performed in triplicate in 96-well, round-bottom microculture plates (Corning, Corning, NY). Proliferation of nylon wool-eluted B6 spleen T cells (2×10^5 /well in 200 μ L) was elicited by gamma-irradiated (20 Gy; X-ray source) BALB/C dendritic cells (DC) at a T:DC ratio of 10:1. To examine the role of HSCs in inhibition of T-cell proliferation, graded doses of gamma-irradiated (40 Gy) HSCs were added into the culture at the beginning of the culture. Cultures were maintained in RPMI-1640 complete medium for 3 to 4 days in 5% CO₂ in air. [³H]-thymidine (1 μ Ci/well) was added for the final 18 hours, and incorporation of [³H]-thymidine into DNA was assessed via liquid scintillation counting. Results were expressed as mean counts per minute (cpm) $\pm$ SD.

Flow cytometric analysis

Anti-CD3, -CD4, -CD8 mAbs were purchased from BD PharMingen (San Diego, CA). Apoptotic T-cells were identified using Apoptosis Detection Kit (BD, PharMingen) according to manufacturer's instructions. Briefly, the T-cells were washed and resuspended in annexin V binding buffer and stained with Annexin V-FITC for 15 min at room temperature in the dark. Cells were analyzed within 1 h on a FACSCalibur cytometer (BD Biosciences). Data processing was accomplished with FlowJo software (Tree Star, Ashland, OR).

Islet and HSC Co-Transplantation

Diabetes was induced in recipients with a single intraperitoneal injection of streptozotocin (STZ, 220 mg/kg body weight, Sigma-Aldrich, St. Louis, MO). Non-fasting blood glucose levels were measured daily. Only the mice with blood glucose exceeding 350 mg/dl were used

in the experiments. Islets were isolated from donor pancreas by collagenase V (Sigma-Aldrich) digestion as previously described.⁹ After separation on histopaque gradient (Type 400, Sigma-Aldrich), the islets were purified by hand picking. 300 islets alone or mixed with 3×10^5 HSCs were aspirated into polyethylene tubing (PE-50, Becton Dickinson, Parsippany, NJ), and pelleted by centrifugation for 2 min, and then gently placed under an operating microscope into the subcapsular space of recipient kidney. Non-fasting blood glucose was monitored after transplantation. Transplantation was considered successful if the blood glucose returned to and remained normal (150 mg/dL) for the first 4 days after transplantation. The first day of 2 consecutive readings of blood glucose greater than 350mg/dL was defined as the date of graft rejection. No immunosuppressive agents were administered throughout the experiments.

Statistical Analysis

The parametric data were analyzed by Student's *t*-test (2-tailed). Graft survival difference between groups of transplanted animals was compared using the Kaplan–Meier log rank test. A *p* value less than 0.05 was defined as significant.

RESULTS

Expression of TRAIL on HSCs

Expression of TRAIL mRNA in HSCs was determined by quantitative real-time PCR. As shown in Fig. 1, HSCs isolated from WT B6 mice and cultured *in vitro* for 7–14 days expressed very low TRAIL. Expression of TRAIL was substantially upregulated following activation by IFN- γ . HSCs isolated from TRAIL KO mice did not express TRAIL, despite the stimulation of IFN- γ , indicating that TRAIL gene was successfully knocked out.

HSCs deficient in TRAIL demonstrate less inhibitory activity in MLR assay

To address the effect of TRAIL expressed on HSCs on influencing T-cell responses, irradiated WT HSCs or TRAIL KO HSCs (B6) were added into an allogeneic MLR culture in which B6 spleen T-cells were stimulated by irradiated BALB/c DC at a T:DC ratio of 10:1 for 3 days. At a T:HSC ratio of 10:1, an optimal ratio for HSC to exert inhibitory activity as demonstrated in our previous study (8), thymidine uptake by T-cells was markedly inhibited [Fig. 2A, 7610 ± 624 vs. 67461 ± 1834 of no HSCs (islet transplantation alone) control, $p < 0.01$]. Addition of HSCs from TRAIL KO mice demonstrated significantly less inhibition of T-cell proliferation (Fig. 2A, 27461 ± 2853 vs. 7610 ± 624 of WT HSCs $p < 0.05$), suggesting that TRAIL expressed on HSCs contributes to immune inhibitory activity of HSCs.

TRAIL-Deficient HSC Induce Less T lymphocyte Apoptosis

Cells collected from the aforementioned cultures were double stained with anti-CD3 and annexin V mAbs, and analyzed by flow cytometry. The presence of WT HSC markedly increased the incidence of CD3⁺annexin V⁺ cells compared to no-HSC (islet transplantation alone) controls (from 5.2 % to 25.4%), indicating that co-cultured HSCs induce apoptotic death in T lymphocytes. The incidence of apoptotic T cells in TRAIL KO group was reduced to 19.8% (Fig. 2B), suggesting that TRAIL is partially responsible for the induction of T-cell apoptosis.

TRAIL Deficient HSC Substantially Loss Their Protective Effect on Islet Allografts

Since TRAIL-deficient HSCs exhibited less inhibitory effect on T cell proliferation *in vitro*, it was rational to speculate that TRAIL might contribute to the protective effect of HSCs on co-transplanted islet allografts. To examine this, islets isolated from BALB/c mice were co-transplanted with HSCs derived from WT B6 or TRAIL KO mice into chemically-induced diabetic B6 recipients. Transplantation with islet allografts alone served as control. In islet

alone group (n=6), all islet allografts were rejected within 17 days, while co-transplantation with WT HSC (n=8) effectively protected islet allografts from rejection (median survival 83 days, ranging from 21 to 242 days) with 50% recipients achieved indefinite graft survival (>100 days, $P < 0.01$ compare with islet only group). Co-transplantation with TRAIL^{-/-} HSCs (n=6) also significantly prolonged islet allograft survival (median survival 24 days, compared to islet alone group, $P < 0.05$), but the protective effect was markedly less compared to WT HSC group ($P < 0.01$); none of islet allografts survived longer than 28 days, indicating that TRAIL plays a crucial role in mediating immunosuppressive activity of HSCs.

DISCUSSION

We have extensively studied the underlying mechanisms of the liver transplant tolerance since establishment of orthotopic liver transplant model in mice using microsurgery technique,¹ and revealed that the acceptance of liver allografts is not due to deletion of the allogene-specific T cell clones, but is associated with apoptosis of activated T cells.⁴ It has long puzzled us what cause T cell apoptosis in the liver. Indeed, solid organ transplantation across HLA barriers has been successfully applied in clinic for decades, but the results of somatic cell transplantation remain disappointing, implicating that the organ NPC may protect parenchymal from immune attacks. After investigating various liver NPC, we have found that HSC, the main retinoids (vitamin A) storing cells, and well known for participating in repairing and fibrosis during liver injury, have potent immune suppressive activity both *in vitro* and *in vivo* via induction of activated T cell apoptotic death.^{8,9} The immunosuppressive activity of HSCs may represent one of the mechanisms that regulate immune responses in the liver favoring tolerance rather immunogenicity. Although the underlying molecular basis remains unclear, our previous data have suggested an important role of B7-H1 expressed on activated HSCs in mediating the immunosuppressive activity.^{8,9} However, B7-H1 may not be the only responsible molecule, because neutralization of B7-H1 by anti-B7-H1 mAb can only partially reverse HSC-induced inhibition of T cell proliferation. Other molecule(s) or pathways may be involved. We have analyzed several death molecules in HSC by qPCR, showing that HSC expressed very low FasL, TNF- α , TRAIL and its receptor DR5. Following IFN- γ stimulation only expression of TRAIL was upregulated, other death-inducing molecules (TNF- α , FasL) remained low. The role of TRAIL in T cell apoptosis induced by HSC was then investigated in this study. Considering that HSC locate next to hepatocytes that are extremely sensitive to FasL mediated injury, we believe HSC are smart not to rely on Fas signaling pathway.

The present study demonstrated that HSCs deficient in expression of TRAIL exerted significant immune inhibitory activity in attenuating T-cell proliferative response in MLR assay. The attenuation of T cell proliferative response was associated with enhanced apoptosis in T lymphocytes. In keeping with the observations *in vitro*, HSCs from TRAIL KO mice largely lost their capacity in protecting co-transplanted islet allografts. TRAIL has been shown a role in regulation of immune response via tight control of immune cells to prevent any harm of tissue cells.²¹ These data presented in this study suggest that TRAIL may be involved in the immune regulatory function of HSCs, which is likely mediated by TRAIL triggered death of activated T cells.

In this study, we demonstrate that cultured HSCs express low levels of TRAIL, which is markedly upregulated after activation by IFN- γ , a key proinflammatory cytokine involved in cellular immune responses. We speculate that, in the presence of an alloresponse, HSCs are activated by IFN- γ , which is secreted mainly by activated T lymphocytes, to upregulate expression of TRAIL, which in turn exerts inhibitory effect on activated T lymphocytes. This appears to be a negative feedback loop in the liver where HSC reside, resulting in a phenomenon called "hepatic tolerance". We have used this immune regulatory property of HSC to protect islet allografts. As long as islet allografts exist, T cell responses will be continuously elicited,

and the co-transplanted HSC be continuously stimulated by the proinflammatory cytokines, such as IFN- γ . The islet allografts are thus, effectively protected. TRAIL belongs to TNF superfamily.¹⁰ Unlike other members of the TNF superfamily that interact with one or two specific receptors, TRAIL can potentially interact with five different receptors, including death receptor (DR4, DR5, decoy receptor (DcR)1, DcR2 and a soluble receptor called osteoprotegerin.¹⁶ Recombinant TRAIL has recently been reported to be a promising agent in anticancer therapy because it selectively kills tumor cells but not normal cells, leaving the host unharmed.^{22,23} The data of this study suggest that TRAIL expressed on HSCs can also kill activated T cells.

Several studies have also demonstrated that TRAIL is an inhibitor of autoimmune diseases. The blockade of endogenous TRAIL activity with soluble DR5 exacerbates experimental autoimmune encephalomyelitis, autoimmune arthritis, and type I diabetes.^{16–18} Administration of recombinant TRAIL was shown to be capable of inhibiting experimental autoimmune thyroiditis (EAT).¹⁹ Therefore, TRAIL may be involved in maintaining immune homeostasis and downregulate immune responses that lead to autoimmune inflammation. In the EAT model, it was demonstrated that TRAIL significantly decreased the number of infiltrating lymphoid cells in thyroid, suggesting a role of TRAIL in regulating T cell homeostasis.¹⁹ It has been shown that TRAIL can inhibit autoantigen-specific T cells.^{22,24} This study exhibited that TRAIL expressed on HSCs can also inhibit allo-responsive T cells, which provides new insights into the interaction between tissue cells and graft infiltrating T lymphocytes in transplant tolerance.

Abbreviations

B6	C57BL/6
DC	dendritic cell
HSCs	hepatic stellate cell
FasL	Fas ligand
IFN	interferon
KO	knockout
mAb	monoclonal antibody
MLR	mixed leukocyte reaction
NPC	non-parenchymal cells
TNF	tumor necrosis factor
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
WT	wild type

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Figure 1. TRAIL expression on HSCs

HSCs from WT B6 mice or TRAIL KO mice were cultured *in vitro* for 7–14 days. Further activation was achieved by addition of IFN- γ at 100 U/ml for the last 24 hours of culture. Total RNA were isolated and mRNA expression of TRAIL was analyzed by quantitative real-time PCR. Data are expressed as mean $\pm$ SD of three experiments.

Figure 2. TRAIL mediates immune inhibitory activity of HSCs

In a MLR culture, B6 splenic T-cells were stimulated by irradiated BALB/c DC at a T:DC ratio of 10:1 for 3 days. Irradiated HSCs from WT B6 or TRAIL KO mice were added at the beginning of the culture at a T:HSC ratio of 10:1. Without addition of HSCs was used as control. (A) T cell proliferative response was determined by [^3H]-thymidine uptake. (B) Cells were collected at the end of cultures, double stained with anti-CD3 and annexin V, and analyzed by flow cytometry, expressed as percentages of annexin V⁺ cells in CD3⁺ cell populations. The data are representative of three separate experiments.

Figure 3. Protection of islet allografts by co-transplanted HSCs requires TRAIL expression
Islets (300) from BALB/c donors were transplanted in combination with 5×10^5 HSCs from WT B6 or TRAIL KO mice into chemically-induced diabetic B6 recipients. Transplantation of islet allograft alone (none) served as control. Islet allograft survival was determined by monitoring blood glucose levels.