Renoprotective Effects of Cotransplanted Allogeneic Testicular Sertoli Cells in a Renal Acute Rejection Model in Rats

Hai-xing Mai,1 Lei Yu,2 Li-jun Chen,3 Nan Qu,3 Li Zhao,3 Ya-Ling Wang,3 Jian-Tao Li,3 Hua You,3 Xu Zhang1

Abstract

Objectives: We sought to study the renoprotective effect of cotransplanted allogeneic testicular Sertoli cells on renal acute rejection in rats.

Materials and Methods: A renal acute rejection model using kidneys from Sprague-Dawley (n=30) transplanted into Wistar rats (n=30) was constructed. The rats were randomly divided into 3 groups: (1) the cyclosporine group, which was treated with daily hypodermic injections of cyclosporine (15 mg/kg) after transplant, (2) the Sertoli cells group with cell suspension (n = 2 × 10^6 cells) into the subcapsular space of the renal graft, and (3) the control group, which received no posttransplant intervention. Graft function was measured based on serial serum creatinine. Graft histology was examined at 10 days posttransplant, and survival duration was recorded.

Results: Serum creatinine was significantly higher in the Sertoli cells and cyclosporine groups than in the controls. Survival duration was significantly longer in the Sertoli cells (19.50 ± 4.3 d) and cyclosporine groups (21.50 ± 5.9 d) than in the controls (14 ± 3.1 d). Allografts in the control group exhibited typical severe acute rejection, including widespread interstitial infiltration with tubulous, patchy necrosis and hemorrhage, severe glomerulitis with extensive capillary occlusion caused by endothelial swelling, and intimal arteritis in the cortex. Findings of acute rejection were less in the Sertoli cells and cyclosporine groups.

Conclusions: Sertoli cell implantation is an effective method for increasing survival duration in rat renal transplant, and it has potential as a new alternative to cyclosporine immunosuppression.

Key words: Renal transplant, Fas-L, Sertoli cells, Immune privilege, Cell transplant

Introduction

Rejection adversely impairs the long-term survival of kidney grafts. The induction of immune tolerance has always been considered an optimal solution to transplant rejection. For years, allogeneic tissues transplanted into the testis have not been rejected by recipients. This result was deemed to be associated with the expression of Fas/FasL by testicular Sertoli cells. In the present study, Sertoli cells were cultured in vitro, and the amplified cells were transplanted into the subcapsular space of rats. Additionally, serum creatine levels, posttransplant survival duration, and pathological manifestations of the renal graft were examined in the presence of the canonical immunosuppressive agent cyclosporine (CsA). This study sought to evaluate the safety and immunomodulation of Sertoli cells, in addition to the feasibility of minimizing the use of immunosuppressive drugs by lowering the immune response or induction of immune tolerance by Sertoli cells.
Materials and Methods

Testicular Sertoli cell donors and cell isolation
Male Sprague-Dawley rats, weighing approximately 100 g, and 17 days to 22 days old, were purchased from the Institute of Laboratory Animal Medicine at the Chinese PLA Academy of Military Medical Sciences. Collagenase type I, trypsin, DNase, RPMI 1640, and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Sigma (Sigma-Aldrich Corp. St. Louis, MO, USA). Purified anti-FasL and anti-Fas monoclonal antibodies were from PharMingen International (PharMingen, San Diego, CA USA). Dulbecco’s modified Eagle’s medium-F12 culture media and fetal bovine serum were from (Invitrogen Corporation, Carlsbad, CA, USA). Trypsin (0.25%), collagenase (0.1%), HEPES (15 mmol/L), insulin (69.45 μmol/L), transferrin (0.0615 μmol/L), and epidermal growth factor (EGF, 2.5 μg/L) were all purchased from (Sigma, St. Louis, MO, USA). Tris-HCl (20 mmol/L) and SABC p-PG kit were from Boster (Wuhan, China). Finally, the freezer centrifuge (400R) and CO₂ incubator were manufactured by Heraeus (Heraeus Sepatech GmbH, Osterode, Germany).

Transplant animal model
Healthy, clean, adult male Sprague-Dawley rats (n=30) and Wistar rats (n=30), weighing 250 g to 300 g, were purchased from the Institute of Laboratory Animal Medicine at the Chinese PLA Academy of Military Medical Sciences. All the animals were housed and bred in a specific pathogen free environment.

Sertoli cell isolation
A previously described, a protocol was used to isolate the Sertoli cells. Bilateral testes were recovered from rats on postnatal day 20. The tunica albuginea was removed, and the seminiferous tubules were segmented into 1 mm. The segmented tissues were digested in 0.2% collagenase and 0.1% trypsin in a water bath shaker at 37°C for 30 minutes. The dissociation was terminated using an equal volume of DMEM/F12, containing 20% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 3 mmol/L GlutaMAX. The dissociation solution was centrifuged at 1000 rpm for 5 minutes. The supernatant liquid was discarded and replaced with DMEM/F12.

Sertoli cell culture in vitro
Freshly isolated cell suspension was plated onto poly-L-lysine-coated 12-well plates and incubated at 37°C under a 5% CO₂ atmosphere. The culture media were completely refreshed every 3 days (Figure 1).

Characterization of Sertoli cells in vitro
FasL protein was used for the immunohistochemical characterization of the Sertoli cells, whereas Hoechst 33342 was used to label the cell nuclei. The purity of the Sertoli cells was defined as the ratio of the FasL-positive cell number to the total cell number. The immunohistochemical protocol was described, as...
follows. On day 2 of the culture, the cell samples were fixed for 10 minutes in freshly prepared 4% paraformaldehyde, rinsed 3 times in PBS for 5 minutes. The cells were blocked for 20 minutes with goat serum at room temperature. FasL was incubated with the primary rabbit anti-rat polyclonal antibodies (1:100) at 37°C for 3 hours. After rinsing 3 times in PBS for 5 minutes, a secondary FITC-labeled goat anti-rabbit IgG (1:50) was added to detect the primary antibodies at 37°C for 30 minutes, followed by rinsing 3 times in PBS for 5 minutes. The cell nuclei were counterstained with 5 μg/mL Hoechst 33342 at 37°C for 30 minutes, followed by rinsing 3 times in PBS for 5 minutes. Ten visual fields were randomly selected under a fluorescent microscope to examine the FasL and Hoechst33342 staining. The FasL+ cells represented the Sertoli cells, whereas the Hoechst 33342-stained cell nuclei represented the total number of cells. The FasL+ cells and total cells were counted for each visual field (Figure 2A and 2B). The purity of the Sertoli cells = the number of FasL+ cells (Figure 2A)/the total number of cells (Figure 2B) × 100%.

Surgical procedures
The surgical procedures were performed following previously reported methods. After inducing anesthesia, the donor animals are placed in a supine position (Figure 3A). A median longitudinal abdominal incision is made. The left kidney, ureter, aorta, and inferior vena cava are identified. The bladder is exposed, and a 0.3-cm–diameter bladder flap is made into the entry of the ureter. The inferior mesenteric artery, along with the aorta and inferior vena cava in proximity with renal vessels, is mobilized, followed by removal of the adventitia. The inferior mesenteric artery, along with the aorta and inferior vena cava 1.0 cm below the renal pedicle, are each ligated. The aorta above the inferior mesenteric artery is clamped (Figure 3B). The inferior vena cava above the left renal vein is cut open, and an infusion needle is connected to a 20-mL syringe containing 4°C lactated Ringer’s solution at a constant infusion rate of 1 mL/min is inserted through the distal end of the abdominal aorta. After infusing with approximately 10 mL of the solution, the left kidney becomes yellowish. After the infusion, the left kidney, along with the ureter, are recovered and preserved in HC-A solution. The recipient animals are anesthetized and positioned using the same protocol. The left renal vessels are mobilized, and the left kidney is removed. A silicon rubber cushion is placed into the renal fossa (Figure 3C). The kidney graft is placed onto the cushion and chilled on ice during vascular anastomosis (Figure 3D). After that, we observe the appearance of vascular anastomosis patency and reperfusion with microscopic and visual (Figure 3E, Figure 3F). The renal arteries are anastomosed end-to-end (6 to 8 stitches), and the renal veins are similarly anastomosed (approximately 10 to 12 stitches). The vascular clamp is released after completing the renal vascular anastomoses. In the presence of urine

Figure 2. FasL-Labeled (A) and Hoechst 33342-Counterstained (B) Sertoli Cells on Day 2 of Culture (×400)

Figure 3. The Surgical Procedure
(A) Mobilization of donor renal vessels and ureters; (B) infusion of kidney graft; (C) appearance of pretransplant kidney graft; (D) appearance of posttransplant kidney graft; (E) microscopic appearance of vascular anastomosis showing patency and reperfusion; (F) visual appearance of patent and reperfused vascular anastomosis.
outflow from the ureteral orifice, the bladder and the ureter are anastomosed. A 4 mm-diameter incision is made at the bladder base, and the incision is closed with 5 to 6 uninterrupted full-thickness stitches on each side using 6-0 noninvasive sutures. After completing the suture, the right renal vessels are ligated, and the right kidney is removed.

Testicular Sertoli cell transplant
Cell suspensions were injected into the subcapsular space of the renal graft. The capsule was closed layer by layer. The cell suspensions were reconstituted into $2 \times 10^6$ cells per liter before the injection. The recipient animals were divided into 3 groups: group A (n=10 male Wistar rats) received left subcapsular injections of 0.5 mL of Sertoli cell suspension; group B (n=10 male Wistar rats) received left subcapsular injections of 0.5 mL of normal saline; and group C (n=10 male Wistar rats) received 15 mg/kg subcutaneous CsA injections daily. One rat was found dead on postoperative day 3, whereas the remaining 29 rats were housed and bred routinely. On day 10 after transplant, the rats were killed. The kidney grafts were fixed in 10% formaldehyde and sectioned for hematoxylin-eosin staining and microscopic examination. The remaining rats were kept for observation of survival days. Serum creatine levels were assayed on postoperative days 3, 5, and 10. The duration of animal survival was documented to characterize the immunomodulatory activity of the Sertoli cells.

Pathological examination
The diagnostic criteria for acute rejection (AR) were referred from The Banff 97 working classification of renal allograft pathology. Acute rejection was defined as the presence of interstitial inflammatory cell infiltration, tubulitis, and endarteritis under light microscopy. The diagnosis of AR was further confirmed in the presence of concomitant peritubular capillary vasculitis, glomerulitis, and interstitial edema. The severity of AR was classified into 6 grades in line with extent of interstitial inflammatory cell, as follows: 10% (normal, occasional interstitial inflammatory cell infiltration); 10%-25% (borderline, small focal interstitial inflammatory cell infiltration); 25%-50% (grade 1, focal interstitial inflammatory cell infiltration with concomitant glomerulitis, endarteritis, and peritubular capillary vasculitis); 50%-75% (grade 2, multifocal interstitial inflammatory cell infiltration with concomitant glomerulitis, endarteritis, and peritubular capillary vasculitis); > 75% (grade 3a, disseminated interstitial inflammatory cell infiltration with concomitant glomerulitis, endarteritis, and peritubular capillary vasculitis); and grade 3b (in the context of 3a, hemorrhage, infarction, and fibroid necrosis were present in vessels). The severity of AR was semiquantitatively rated as 0 = normal, 1 = borderline change, 2 = grade one, 3 = grade two, 4 = three a, and 5 = three b.

Western blotting analysis
The expression of Fas-L was detected by the Western blot test. The total protein was prepared from the transplanted tissues 7 days after the kidney transplant operation. All the tissue divided into 4 groups, (1) for the Sertoli cotransplanted kidney graft; (2) for the CsA-treated kidney graft; (3) for the control kidney graft; (4) for the normal kidney tissue. Fifty micrograms of protein were electrophoresed on a 7.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking with 0.2% Tween in Tris-buffered saline-T containing 5% milk at room temperature for 2 hours, the membranes were incubated for 2 hours at room temperature with rabbit polyclonal anti-FasL (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membrane was washed 3 times with Tris-buffered saline-T and incubated with horseradish peroxidase-conjugated secondary antibody for 2 hours at room temperature. The signal was detected by the addition of enhanced chemiluminescence reaction solution (KPL, Inc., Gaithersburg, MD USA).

Statistical analyses
All data are expressed as means ± SD. The intergroup differences were compared through either a t test, a 1-way analysis of variance, or with SPSS software (SPSS: An IBM Company, version 10.0, IBM Corporation, Armonk, New York, USA).

Results
Primary recovery of Sertoli cells
The Sertoli cells were generally adherent, and most of the cells were attached at 24 hours of culture. The cells presented round and oval morphologies. A substantial number of spermatogenic cells were
floating in the culture media. At 48 hours onwards, the cells had more cytoplasm and became less refractive. On days 3 to 4, the volume of the Sertoli cells increased, resulting in narrowing of the intercellular space. On days 4 to 6, the Sertoli cells were confluent in a monolayer. The ratio of cytoplasm to nucleus was 7:1 to 9:1, the Sertoli cells were polygonal, and their morphology and cytoplasmic area remained unchanged thereafter (Figure 1).

Characterization of Sertoli cells in vitro
The staining outcomes of the FasL immuno-fluorescence and Hoechst 33342 on day 2 of the culture are shown in Figures 2A and B. The FasL positive cells represented the Sertoli cells, whereas the Hoechst 33342 labeled the cell nuclei. The purity of Sertoli cells is expressed as the mean ± SD (95.64% ± 2.76%) (Table 1).

Rat kidney transplant model
Out of the 30 rats that underwent kidney transplant, 29 rats survived. The operative duration was 35 minutes for the donor and 65 minutes for the recipient. The lengths of warm and cold ischemia were approximately 1 minute and 40 minutes. The whole process of transplant lasted for 90 minutes to 130 minutes. The overall surgical success rate was 97%, but 1 animal died of massive blood loss from renal venous anastomosis (Figure 3).

Histologic study of kidney grafts
Ten days after transplant, the kidney grafts from the control group exhibited the typical AR, which manifested as extensive interstitial inflammatory cell infiltration, patched hemorrhagic necrosis, severe glomerulitis secondary to extensive capillary obliteration from edematous endothelial cells, cortical endarteritis, and mesenchymal infiltrative and hemorrhagic tubulitis in most cases (Figure 4A). Acute rejection was also present in the CsA (Figure 4B) and Sertoli cell treatment groups (Figure 4C). However, interstitial inflammatory infiltration was significantly alleviated; the majority of glomeruli and arteries exhibited no evident or minimal pathological change, and the tubules did not show any marked tubulitis or protein deposition (Figure 4D). Sertoli cells implanted into the renal graft subcapsular space grow well (Figure 4D). The FasL express of transplant tissue will show on the Western blot test (Figures 5). We also observed the tissue of Sertoli cell cotransplanted kidney graft (Figure 6). The Sertoli cells can protect kidney graft from AR (Figures 6B and 6C), we can see the kidney tissue has no evidence of rejection (Figures 6B and 6C). Among kidney tissue, we can only see some evidence of acute renal tubule injury. But the transplant of Sertoli cell still can cause some immune response of body (Figure 6A); that means the Sertoli cell cannot totally remove the body’s immune response.

Fasl express in the kidney tissue
As we can see from the Western blot test, group 1, which stands for the Sertoli cotransplanted kidney graft, has more Fas-L protein expression; the 3 and 4 groups, which stand for the control kidney graft and the normal kidney tissue, have no Fas-L protein. The 2 that stand for the CsA-treated kidney graft, have much less Fas-L protein expression.

Table 1. Serum Creatine Outcomes (Mean ± SD, mg/dL)

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 3 (Mean ± SD)</th>
<th>Day 5 (Mean ± SD)</th>
<th>Day 10 (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CsA group</td>
<td>1.10 ± 0.18</td>
<td>1.68 ± 0.42</td>
<td>3.46 ± 0.35</td>
</tr>
<tr>
<td>Sertoli cell group</td>
<td>1.26 ± 0.16</td>
<td>1.75 ± 0.31</td>
<td>2.26 ± 0.67</td>
</tr>
<tr>
<td>Control group</td>
<td>1.17 ± 0.17</td>
<td>1.95 ± 0.29</td>
<td>4.16 ± 0.75</td>
</tr>
</tbody>
</table>

Figure 4. Histologic Appearance of Grafts
(A) Control kidney graft (×200), (B) cyclosporine-treated kidney graft (×200), (C) Sertoli cotransplanted kidney graft (×200), and (D) Sertoli cells implanted into the renal graft subcapsular space.

Figure 5. Western Blot Test for the Transplant Tissue
Lane 1: the Sertoli cotransplanted kidney graft; lane 2: the cyclosporine-treated kidney graft; lane 3: the control kidney graft; lane 4: normal kidney tissue.
Serum creatine assay and posttransplant survival of kidney grafts

The serum creatine levels on postoperative days 3, 5, and 10 are shown in Table 1. The comparisons between the Sertoli cell treatment group and CsA-treated group over time revealed no significant differences on days 3, 5, and 10 ($P > .05$). In contrast, the 2 groups showed serum creatine levels significantly different from the control group on days 5 and 10 ($P < .05$). The duration of posttransplant survival of the control versus the Sertoli cell treatment versus the CsA-treated rats were 14.5 ± 3.1 vs 19.5 ± 4.3 vs 21.5 ± 5.9 ($P < .05$). No statistically significant difference in survival duration was identified between the Sertoli cell treatment group and CsA-treated group.

Discussion

Extensive studies have shown that Sertoli cells are less immunogenic but immunomodulatory.4-13 In cotransplanted with allogeneic cells, Sertoli cells not only are immune privileged, but also assist fibromyoblasts and islet cells in escaping immune rejection, which allows the long-term survival of the grafts.14-19 Such privilege is thought to be associated with the FasL expressed on the surface of Sertoli cells. FasL and Fas are present on the cell surface in the form of membrane or soluble proteins, which mediate apoptosis. FasL and Fas are reportedly closely associated with autoimmune diseases, and the immune tolerance of tumors and grafts.1,20,21 However, the renoprotective effects of Sertoli cells on kidney grafts have been rarely studied.

In the current study, the Sertoli cell treatment recipients survived a mean duration of 19.5 ± 4.3 days, which is not significantly different from the CsA-treated rats (21.5 ± 5.9 d; $P > .05$) but was significantly different from the control group ($P < .05$). These findings indicate that the cotransplant of Sertoli cells is equivalent to low-dose CsA. Comparison of the serum creatine levels over time showed no significant difference among the 3 groups on day 3 of the transplant ($P > .05$), whereas, on days 5 and 10, the Sertoli cell treatment group and the CsA treatment group were significantly different from the control group ($P < .05$). On day 10, kidney grafts from the control group exhibited typical AR, but the AR was less serious in the CsA treatment group and Sertoli cell treatment group. These findings correspond with the differences in survival duration.

Posttransplant serum creatine levels, survival durations, and pathologic manifestations of kidney grafts were systematically examined. The results showed that renal subcapsular cotransplant of Sertoli...
cells is beneficial in terms of pathologic outcome, the serum creatine levels in the recipients, and the duration of survival, which were similar to low-dose CsA immunosuppression. However, the immunity of recipients, the response of inflammatory molecules, and the exact sites and mechanisms of action remain to be investigated. Moreover, whether the experimental results derived from rodents are applicable to human patients is still unknown. The study will help improve the protection of allogeneic kidney transplants and enable organ transfection with FasL gene, as a promising modality for cell graft protection.

References