Donor-Specific Tolerance in a Rat Renal Allograft

CTLA4Ig Gene Transfer Prolongs Survival and Induces Donor-Specific Tolerance in a Rat Renal Allograft

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Abstract. Organ transplantation requires lifelong antirejection therapy, which carries the risk of infection and cancer. A revolutionary approach is to transduce the organ graft with immunomodulatory genes to render them tolerated with no need of systemic immunosuppression. Prolonged allograft survival was achieved by adenovirus-mediated transduction of the cold-preserved kidney with sequences encoding CTLA4Ig, a recombinant fusion protein that blocks T cell activation. Organ expression of the transgene was achieved associated with mild infiltration of mononuclear cells in the transplanted kidney. Mixed lymphocyte reaction as well as the production of both Th1 and Th2 cytokines were reduced. Thus, the gene transfer technique to prolong graft survival is indeed effective and safe and can induce donor-specific unresponsiveness. Pending appropriate large animal testing, ex vivo genetic manipulation of the organ before surgery may hopefully represent a major step forward in human transplant medicine.

Current antirejection drugs definitely prolong survival of transplanted organs, at least in the short term, but invariably reduce systemic immunity, which in turn increases the risk of infections and cancer. In fact, if modern drugs were to improve graft survival up to 30 yr, as much as 100% of graft recipients would most likely develop a cancer (1). The future of transplant medicine is therefore crucially dependent on whether incompatible organ allografts can indeed be tolerated in humans in a donor-specific manner with little or no systemic immunosuppression. The possibility of actually achieving such a goal rests on gene transfer approaches, originally designed as a strategy to replace altered genes in inherited diseases, but now increasingly recognized as an appropriate tool to deliver therapeutic proteins. Relevant to transplant medicine are genes with immunomodulatory properties whose activity can be restricted at target organ level, thus leaving the immune system unaltered (2). Based on data that T cells activated to express Fas—a cell surface molecule belonging to the tumor necrosis factor family—undergo apoptosis upon engagement with Fas ligand, the gene transfer technique has been attempted targeting such system. Thus, transplantation of allogeneic islets with FasL-transfected syngeneic myoblasts protected the graft from rejection and maintained normoglycemia in diabetic mice (3). However, possibly due to the complexity of the diverse pathways involved in Fas/Fas ligand interactions, others have found the opposite to the extent that (i) induction of FasL expression in pancreatic islets promoted granulocytic destruction of islets and (2) transplantation of FasL-expressing islets from transgenic mice resulted in their rapid destruction (4). We recently succeeded in transfecting the kidney before transplantation with adenoviral constructs encoding reporter genes (5), which showed a good transfection efficiency. Here, we investigated whether survival of the recipient could be prolonged by ex vivo transfection of cold preserved kidneys with an adenoviral vector encoding murine CTLA4Ig (AdmCTLA4Ig). We also studied whether such an approach could reduce in vitro responsiveness to donor antigens. This recombinant fusion protein is homologue to the naturally occurring molecule CD28—a major T cell costimulatory receptor—which competes for binding to the CD28 ligand B71 and B72 (6). Costimulatory signals delivered by this and other T cell surface molecules engaged to their counter receptors on antigen-presenting cells (APC) are required to complete T cell activation in response to transplant antigens. In vitro antigen stimulation in the absence of costimulation leads to functional unresponsiveness or clonal anergy (7,8).

Previous studies from our laboratory and others have shown that intraperitoneal administration of CTLA4Ig can prolong allogeneic and xenogeneic solid organ engraftment (9–11). Furthermore, muscle cells engineered to secrete CTLA4Ig co-transplanted with pancreatic islets significantly improved graft survival in diabetic mice without the need of immunosuppressive intervention (12). Moreover, rat recipients of cold preserved liver adenovirally transduced with sequences encoding CTLA4Ig had an unusually prolonged graft survival accompanied by donor-specific unresponsiveness (13). Can the above results be generalized to transplanted organs other than liver?
In answering that, one should consider that there are many reports in the literature of animals that can spontaneously tolerate liver allograft with no immunomodulation at all and very prolonged survival (14). This is probably the result of the peculiar immunologic privilege offered by the liver as opposed to organs like heart or kidney. Prolonged survival of rat cardiac allograft to 20 to 40 d compared to a mean of 6 d in controls has, however, also been achieved with adenoviral CTLA4Ig gene transfer in recent experiments (15). No attempts have been made so far with kidney allograft.

**Materials and Methods**

**Animals**

Adult male Brown Norway (BN), Lewis, and Sprague Dawley (SD) rats (Charles River Italia, Calco, Italy) weighing 260 to 280 g were used. Animal care and treatment were conducted in accordance with national and international laws and policies (European Economic Community Council Directive 86/609, OJL 358, December 1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). All animals were allowed free access to standard rat chow and tap water.

**Experimental Design**

Kidney allograft was studied here in high responder combination of Brown Norway (BN, RT.1 n ) to Lewis (RT.1 l). Two different experimental groups were given CTLA4Ig as a plasmid DNA (100 μg, group I) or in the form of replication-deficient adenoviral construct AdmCTLA4Ig (group II). Two additional groups of rats were injected with an adenovirus encoding the reporter gene β-galactosidase (Adβgal) (group III) or with saline (group IV) and used as controls. Left kidney from BN rats was injected ex vivo with 150 μl of a solution containing plasmid CTLA4Ig cDNA (100 μg) or AdmCTLA4Ig (1.7 × 10⁹ plaque-forming units [pfu]) or Adβgal (1 × 10⁹ pfu) or saline through the renal artery at 4°C. After 30 min, kidneys were transplanted in a Lewis recipient animal. There was no surgical mortality.

Animals were followed for survival. Renal function was evaluated in all animals. Histology, immunohistochemistry, and CTLA4Ig time course gene expression were evaluated in animals injected with the adenoviral vector and sacrificed at predetermined times (4 and 7 d from allotransplantation, n = 3) and in long-surviving transplanted animals (>50 and >80 d). Syngeneic transplants (sacrificed at 4, 7, and 14 d from surgery, n = 3) were used for comparison. An additional group of three healthy animals was used as controls.

**Injection and Transplant Procedure**

Left kidney from BN (RT.1 n ) rats was removed, and ex vivo gene transfer was performed during cold incubation for 30 min after injection of plasmid DNA or adenovirus through the renal artery, in a final volume of 150 μl. The recipient animal (Lewis, RT.1 l) was anesthetized with leptoform (1 mg/kg body wt, intramuscularly) and prepared by removal of the left kidney. End-to-end anastomoses were created between the recipient and the donor renal artery and the renal vein. The vascular clamps were released as soon as the vascular anastomoses were completed, with an ischemia time of 20 to 30 min. Donor and recipient ureters were attached end-to-end.

**Adenoviral Vectors**

The replication-deficient adenoviral vector containing the cDNA for the *Escherichia coli* lacZ gene coding for the enzyme β-galacto-

sidase was obtained according to a technique described previously (16). The adenoviral vector AdmCTLA4Ig contains an expression cassette encoding the murine CTLA4 gene coupled to the mouse Ig sequence, replacing the viral E1 region and most of the E1B region, rendering the virus replication-defective. The construction and propagation of the vector were described previously (13).

**RNA Isolation and Reverse Transcription-PCR Analysis**

To evaluate the expression of the transgene, total RNA was extracted from the kidney specimens by the guanidinium isothiocyanate/cesium chloride procedure. Reverse transcription (RT) reaction was performed using reagent and protocols from SuperScript preamplification system for first-strand cDNA synthesis (Life Technologies Italia) in a final volume of 20 μl, starting from 5 μg of total RNA. The PCR reaction was done with 1 μl of DNA template from the RT reaction, 23 pmol of forward and reverse primers, 200 μM dNTP, 1.5 μM MgCl₂, 1 × GeneAmp II PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), and 1 U AmpliTaqGold (Perkin-Elmer, Norwalk, CT) in a final volume of 25 μl. The following primer sequences were used: forward 5′-TGTCCTACGATTCACAGG-3′, reverse 5′-CAT-GAAGTCGACAGCATCCA-3′. The PCR reaction was performed in a Perkim-Elmer 9700 thermal cycler by 40 cycles of denaturation (94°C, 30 s), annealing (55°C, 1 min), and extension (72°C, 1 min) with pre-PCR heat step of 10 min. PCR products were analyzed by electrophoresis in 1% agarose gel with 1 μg/μl ethidium bromide added for ultraviolet visualization.

**Mixed Lymphocyte Reaction**

Mixed lymphocyte reaction (MLR) was performed as described previously (17). Lymphocytes were isolated from lymphonodes of Lewis tolerant and Adβgal-treated animals, and 1 × 10⁵ cells were mixed with 1 × 10⁶ allogeneic spleen cells from BN and SD rats that had been irradiated (4000 rad) in vitro, and cultured in tissue culture plates in 2 ml of culture medium, RPMI-1640 supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 0.05 mM 2-mercaptoethanol, 1 mM pyruvic acid, and 20% fetal bovine serum. After 6 d in culture, 200 μl of each tube was transferred in triplicate in 96-well round-bottomed plates and pulsed for 18 h with tritium-thymidine (1 μCi/well, Amersham International, Buckinghamshire, United Kingdom). Cells were then harvested and their H-thymidine uptake was measured with a β counter. In each experiment, control combinations of Lewis lymphocytes and irradiated Lewis spleen cells were performed. The stimulation index (SI) was calculated using the following formula:

\[
SI = \frac{CPM \text{ in allogeneic MLR}}{CPM \text{ in control combination}}.
\]

where CPM is counts per minute.

**Routine Histology**

Kidney specimens were fixed for 6 h in Dubosq-Brazil and dehydrated in alcohol. After paraffin embedding, 3-μm sections of the blocks were cut and stained with periodic acid-Schiff reagent. Tubular (atrophy, cast, and dilation) and interstitial (fibrosis and inflammation) changes were graded on a scale of 0 to 4+: 0, no changes; 1+, changes affecting <25% of the sample; 2+, changes affecting 25 to 50% of the sample; 3+, changes affecting 50 to 75% of the sample; 4+, changes affecting 75 to 100% of the sample. All renal biopsies were analyzed by the same pathologist who was blinded to the nature of the experimental groups.
**Immunohistochemical Analysis**

Mouse monoclonal antibodies were used for the detection of the following antigens: (1) ED1 antigen present in the rat monocytes and macrophages (Chemicon, Temecula, CA); (2) a rat MHC class II antigen monomorphic determinant (OX6) (Serotec, Oxford, United Kingdom); (3) CD4 cell surface glycoprotein, a 55-kD molecule expressed by helper T cells, thymocytes, and macrophages (W3/25) (Serotec), and (4) rat CD8 cell surface glycoprotein expressed by T suppressor cells (OX8) (Serotec). All antigens were analyzed by indirect immunofluorescence technique. The tissue fragments were frozen in liquid nitrogen. Tissue sections (3 μm thick) were cut using a Mikrom 500 O cryostat (Walldorf, Germany). The sections were blocked with phosphate-buffered saline (PBS)/1% bovine serum albumin, incubated overnight at 4°C with the primary antibody (ED1, 14 μg/ml; OX6, 5 μg/ml; W3/25, 40 μg/ml; OX8, 1:100), washed with PBS, and then incubated with Cy3-conjugated donkey anti-mouse IgG antibodies (affinity-purified, absorbed with rat IgG, 5 μg/ml in PBS; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. For each marker, the number of cells was counted in at least 10 randomly selected high-power microscope fields (×400) for each animal.

**Analytical Data**

Creatinine was measured weekly by a Reflotron creatinine test (Boehringer Mannheim) on whole blood collected from the tail vein of anesthetized animals.

**Statistical Analyses**

Data are given as mean ± SEM. Survival data were analyzed by PROC LIFETEST of SAS 6.12, and multiple comparisons between groups were assessed by the log-rank test. MLR results were analyzed by one-way ANOVA and immunohistochemical results by the non-parametric Kruskal-Wallis test for multiple comparisons. Statistical significance was defined as P < 0.05.

**Results**

Animals given CTLA4Ig plasmid DNA showed a renal survival time slightly more prolonged than control animals of group III receiving kidneys transduced with an adenovirus encoding Adβgal (P < 0.05) (Table 1). In AdmCTLA4Ig-treated animals of group II, survival was dramatically prolonged (P < 0.01 versus groups III and IV; 57.3 ± 24.3 versus 6.6 ± 0.5 and 7.4 ± 0.5 d; P < 0.05 versus group I; 10.8 ± 4.7 d). Serum creatinine, taken as a marker of renal function, was measured weekly in transplanted animals. Animals of group I showed a strong elevation in the creatinine levels 7 d after transplantation (5.12 ± 0.27 mg/dl). For comparison, serum creatinine of an additional group of syngeneic animals undergoing transplantation after AdmCTLA4Ig transfection and studied at day 7 after surgery was 0.56 mg/dl. In AdmCTLA4Ig-transduced allografted animals of group II, serum creatinine at 7 d was 3.04 ± 0.82 mg/dl and decreased thereafter remaining markedly stable with time (day 35: 2.5 ± 0.6 mg/dl, n = 5; day 50: 2.5 ± 0.8 mg/dl, n = 4; day 75: 2.2 mg/dl, n = 2; day 95: 1.6 mg/dl). AdmCTLA4Ig-transfected allografted animals developed mild proteinuria averaging 50 ± 1 mg/d at day 45 with respect to the value of syngeneic animals receiving AdmCTLA4Ig that was 16 ± 3 mg/d at the same time period. At day 90, protein excretion rate did not show any further increase.

RT-PCR was used to evaluate CTLA4Ig mRNA time course expression in ad hoc performed groups of syngeneic and allogeneic grafts both transduced with AdmCTLA4Ig and sacrificed at different times. In syngeneic animals, the transgene was detectable at high levels at 4 and 7 d and rapidly declined to undetectable levels 14 d after transplantation. In allogeneic animals, the CTLA4Ig mRNA was only detectable 4 d after transplantation (Figure 1). Differences in transgene distribution between normal and diseased kidney have been suggested by Zhu et al., who found that in the context of ischemia-reperfusion injury, gene transfer occurred two to four times more efficiently than in normal kidney parenchyma. Moreover, in the same study the use of vasodilators led to more uniform distribution of the transduced gene than without, again suggesting that vascular permeability/tissue integrity may interfere with transduction efficiency (18).

To determine whether AdmCTLA4Ig treatment could actually induce systemic unresponsiveness, the functional status of T cells was evaluated by MLR in vitro in AdmCTLA4Ig-transduced allografted animals of group II and in Adβgal animals of group III. Stimulation index of Lewis rats of group III receiving a graft transduced with the reporter gene β-galactosidase was 13.1 ± 0.6, which was significantly (P < 0.01) higher compared to that of AdmCTLA4Ig-transduced allografted animals of group II (mean stimulation index 4.1 ±

**Table 1. Survival of renal allografts**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Plasmid CTLA4Ig DNA</td>
<td>5</td>
<td>8, 19, 10, 9, 8b</td>
</tr>
<tr>
<td>II</td>
<td>AdmCTLA4Ig</td>
<td>6</td>
<td>67, &gt;80, &gt;45, &gt;50, 19, &gt;100c,d</td>
</tr>
<tr>
<td>III</td>
<td>Adβgal</td>
<td>5</td>
<td>6, 7, 7, 7, 6</td>
</tr>
<tr>
<td>IV</td>
<td>Saline</td>
<td>5</td>
<td>7, 7, 8, 7, 8</td>
</tr>
</tbody>
</table>

* The symbol > denotes that the kidney was still functioning at the time the rats were sacrificed for mixed lymphocyte reaction study, histologic examination of the kidney, and immunohistologic evaluation of cellular subpopulations.

b P < 0.05 versus group III.

c P < 0.01 versus groups III and IV.

d P < 0.05 versus group I.
0.3). Stimulation index of cells taken from both Adβ-gal and AdmCTLA4Ig animals in response to Sprague Dawley third-party stimulator cells was always instead very high, i.e., 20.1 ± 1.2 and 10.8 ± 0.9, respectively (Figure 2).

Studies were subsequently performed to establish whether rat kidney infected with the replication-defective adenoviral vector encoding CTLA4Ig had either pathology evidence of parenchymal injury or leukocyte infiltration. Neither syngeneic- nor allogeneic-grafted animals had evidence of renal parenchymal injury at days 4, 7, and 14 (data not shown). Allografted animals taken at day 50 and day 80 after transplantation only showed mild signs of glomerulosclerosis accompanied by tubular necrosis and tubulointerstitial damage and occasionally vessel wall thickening. All animals given AdmCTLA4Ig before either syngeneic or allotransplantation had evidence of mononuclear cell infiltration since day 4 from surgery. The finding of infiltration of inflammatory cells in animals infected with replication-defective virus is consistent with our previous observation in rats given a syngeneic graft after they were transduced with an adenoviral construct encoding β-galactosidase (5). We undertook a detailed immunohistologic evaluation of syngeneic and allografted animals studied at different time intervals (Table 2) to evaluate the degree of cell infiltration either in response to viral proteins or to transplant antigens. Subpopulations of mononuclear cells and dendritic cells were also quantified (mean ± SEM) as an additional clue for distinguishing the two conditions and were compared to infiltrating cells in control animals. The number of infiltrating cells was significantly higher in syngeneic transplanted animals (*P < 0.01) than in controls. A further significant increase (*P < 0.01) was found in allografted versus syngeneic animals. As expected, compared with syngeneic animals—whose parenchymal infiltration of inflammatory cells can be attributed solely to the inflammatory response against viral proteins—in allografted animals there was persistence of mononuclear cell infiltrate with a higher absolute amount of monocytes/macrophages, CD4+, CD8+, and MHC+ cells despite no signs of vasculitis and tubulitis characteristic of untreated rejecting rats. This finding may be taken as evidence of some form of immunomodulatory response to alloantigenic challenge in immune incompatible graft virally infected with Adβ-gal.

### Table 2. Analysis of mononuclear cell subpopulations in the kidneys of syngeneic and allotransplanted rats transduced with AdmCTLA4Ig and in control rats

<table>
<thead>
<tr>
<th>Group</th>
<th>ED1+</th>
<th>CD4+</th>
<th>CD8+</th>
<th>OX6+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8 ± 2</td>
<td>18 ± 4</td>
<td>3 ± 1</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Syngeneic*</td>
<td>19 ± 5</td>
<td>28 ± 4</td>
<td>8 ± 3</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>7 days</td>
<td>25 ± 3</td>
<td>28 ± 4</td>
<td>6 ± 2</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>14 days</td>
<td>30 ± 8</td>
<td>32 ± 5</td>
<td>7 ± 3</td>
<td>33 ± 7</td>
</tr>
<tr>
<td>Allogeneic*</td>
<td>80 ± 22</td>
<td>81 ± 8</td>
<td>26 ± 7</td>
<td>55 ± 18</td>
</tr>
<tr>
<td>4 days</td>
<td>96 ± 17</td>
<td>88 ± 5</td>
<td>34 ± 7</td>
<td>64 ± 16</td>
</tr>
<tr>
<td>&gt;50 days</td>
<td>105 ± 16</td>
<td>95 ± 9</td>
<td>34 ± 8</td>
<td>89 ± 13</td>
</tr>
<tr>
<td>&gt;80 days</td>
<td>82 ± 14</td>
<td>89 ± 10</td>
<td>44 ± 9</td>
<td>102 ± 7</td>
</tr>
</tbody>
</table>

* P < 0.01 versus control.

b P < 0.01 versus syngeneic.
Discussion

In the present study, we explored the possibility that transducing graft with relevant genes could produce mediators capable of locally suppressing alloimmune response. The results documented that delivering into kidney allograft sequences that encode proteins blocking CD28/B7 costimulatory interaction effectively prolongs graft survival and induces sustained unresponsiveness to donor antigens. This model allowed prolonged survival in a highly reactive rat strain combination without the need for systemic immunosuppression. While plasmid DNA was enough for liver graft to be tolerated (admittedly with quite a different experimental approach), here plasmid DNA encoding CTLA4Ig was never capable of prolonging kidney allograft survival in the present experiments.

Despite the prolonged renal graft survival, rats transfected with AdmCTLA4Ig had elevated serum creatinine as early as 7 d after surgery. This could have been the result of an inflammatory response to viral protein, which also precludes long-term gene expression, as documented previously (19,20). However, syngeneic animals given the same vector have normal renal function, which serves to rule out the above possibility. On the other hand, the inflammatory cell infiltrate of the syngeneic transplanted rats transfected with AdmCTLA4Ig was almost exclusively of monocyte/macrophage type, one of the cellular components of the innate immune response that does not require costimulatory signals to be activated (21). Unlike allografted animals exposed to AdmCTLA4Ig, the cellular infiltrate includes CD4+, CD8+, and MHC+ cells in addition to monocytes/macrophages. Those cells generate molecules including interleukin-4 (IL-4), IL-6, IL-8, and IL-10, which, in addition to their property of locally modulating immune response, also have vasoactive properties that ultimately could contribute to renal dysfunction by affecting glomerular hemodynamics. Of note, renal function remained quite stable during the observation period in animals with long-term gene expression, as documented previously (19,20). Despite the prolonged renal graft survival, rats transfected with AdmCTLA4Ig were treated with recombinant protein activity so as to safely and effectively transduce the graft. This could have been the result of an inflammatory response to viral protein, which also precludes long-term gene expression.

The extraordinarily possible of performing organ transplantation without the need for systemic immunosuppression now requires large animal testing, which should verify the actual feasibility of transducing relevant genes at the local organ level. If gene transfer with CTLA4Ig will allow expression of recombinant protein activity so as to safely and effectively control rejection, the future of transplant medicine will change substantially.

Acknowledgment

We are greatly indebted to Dr. Andrew E. Gelman (University of Pennsylvania, Philadelphia, PA) for providing the adenoviral vector AdmCTLA4Ig and to Dr. Ingegerd Hellstrom (Bristol Myers Squibb, Seattle, WA) for CTLA4Ig plasmid DNA. We also acknowledge Dr. Elena Gagliardini for immunohistology experiments.

References


