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Spontaneous acceptance of mouse kidney allografts is associated with increased Foxp3 expression and differences in the B and T cell compartments

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ABSTRACT

Spontaneous acceptance of organ allografts can identify novel mechanisms of drug-free transplantation tolerance. Spontaneous acceptance occurs in both mouse kidney transplants and rat liver transplants however the early immune processes of mouse kidney acceptance have not been studied. Acceptance of C57BL/6 strain kidney allografts in fully MHC-incompatible B10.BR recipients was compared with rejection (REJ) of heart allografts in the same strain combination. Graft infiltrate and antibody deposition were examined by immunohistochemical staining. Expression of mRNA was measured by quantitative real-time PCR. Apoptosis was examined by TUNEL staining. The majority of kidney allografts were accepted long-term and induced tolerance (TOL) of donor-strain skin grafts, showing that acceptance was not due to immune ignorance. There was an extensive infiltrate of T cells in the TOL kidney that exceeded the level in REJ hearts but subsequently declined. The main differences were deposition of IgG2a antibody in REJ that was absent in TOL, more B cells infiltrating TOL kidneys and a progressive increase in the ratio of CD8 : CD4 cells during rejection. There was also significantly greater Foxp3 mRNA expression in TOL. Kidneys from $RAG^{-/-}$ donors were accepted, showing that donor lymphocytes were not necessary for acceptance. Neutralising antibodies to TGF-β administered from day 0 to day 6 did not prevent TOL. On the basis of cytokine expression and apoptosis there was no evidence for immune deviation or deletion as mechanisms of acceptance. In accord with the findings of spontaneous acceptance of liver allografts in rats, the main difference between mouse kidney TOL and heart REJ was in the B cell compartment. The major difference to rat liver allograft acceptance was that apoptosis of infiltrate did not appear to play a role. Instead, increased Foxp3 expression in TOL kidneys implies that regulatory T cells might be important.

1. Introduction

Spontaneous acceptance of transplanted organs in the absence of any treatment of either donor or recipient occurs in a very few transplant models but can give valuable insights into mechanisms that promote transplant tolerance. Spontaneous acceptance of liver allografts has been investigated in pigs [1], rats [2] and mice [3]. In rats and mice, several factors contribute to liver acceptance including donor leukocytes transferred with the liver [4–7], the large size of the liver [8] and the liver's unique vasculature [9]. Liver acceptance in the rat model is associated with rapid and abortive immune activation of recipient T cells [10,11] which then die by apoptosis [12–14]. These findings in rats have been generally supported by a mouse liver transplant model, where extensive activation and exhaustion of graft-reactive CD8 T cells was followed by their inactivation by deletion and anergy [15,16].

Kidney allografts in some mouse models are also spontaneously accepted without requirement for immunosuppression. This was first reported over 30 years ago for kidneys transplanted between mice with a major histocompatibility complex (MHC) class I disparity [17,18]. This has subsequently been confirmed in several donor/recipient combinations mismatched at both the class I and class II loci [19,20], although it is highly strain-dependent [15]. The immunological basis for acceptance has not been extensively researched and there are few reports that examine the underlying mechanism, especially in the early induction period. Examination of murine

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin; MHC, major histocompatibility complex; REJ, rejection (C57BL6 to B10.BR heart transplant); TGF, transforming growth factor; TOL, tolerance (C57BL6 to B10.BR kidney transplant).

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kidney allograft recipients at 60 days after transplant showed that TGF-β is involved in maintenance of tolerance [20]. By 150 days after transplant the main mechanism of maintenance is expression of indoleamine dioxygenase by "regulatory" dendritic cells [21]. Despite these findings in established kidney transplant acceptance, the immediate post-transplant immune events that lead to these outcomes are not known.

The mechanisms contributing to kidney allograft acceptance could include deletion, anergy, immune deviation, ignorance or immune regulation of alloreactive T cells. Immune deviation is characterised by low levels of Th1 cytokines such as IL-2 and IFN-γ. Immunological ignorance involves the absence of an immune response and is identified by the rejection of secondary grafts of donor strain in first-set tempo. Regulation involves a preponderance of cells that suppress potentially alloreactive T cells. A number of cell populations are capable of immune regulation, the best characterised being CD4+ CD25+T cells [22] that express the forkhead box p3 (Foxp3) transcription factor [23]. The aim of this study was to characterise the early immune changes that occurred in spontaneous acceptance of kidney allografts by comparing it with rejection of heart allografts in the same strain. The pattern of graft infiltration, expression of cytokines and Foxp3 in graft and recipient spleen, donor cell migration and apoptosis of infiltrating cells was compared between TOL kidney and REJ heart grafts.

2. Materials and methods

2.1. Organ transplantation

C57BL/6 (H-2^b) mice were used as donors and B10.BR (H-2^k) were used as recipients. In some experiments C57BL/6 $Rag^{-/-}$ animals were used as donors. All were from the Animal Resources Centre, Perth, Western Australia. The Institutional Animal Care Ethics Committee of Royal Prince Alfred Hospital approved all experiments. The kidney transplant technique was based on the method of Han et al. [24] with modification. Briefly, the donor kidney was removed and the ureter was dissected close to the bladder. After removal of the left recipient kidney, the aorta and vena cava of the donor kidney were anastomosed end-to-side with the recipient aorta and vena cava with 10–0 nylon sutures. The donor ureter end was tied with a 5–0 suture and implanted into the recipient bladder by pulling the suture through the back wall to the front. The ureter was fixed at the ureter implant site by suturing periureteral tissue to the bladder back wall with four 10–0 sutures. Excess ureter was cut at the bladder front wall and after retraction of the ureter the hole was closed with one 10–0 suture. The remaining native kidney was removed at 4 days post-transplantation. Heterotopic heart transplants in mice were implanted as an auxiliary graft similar to the procedure described for rats [25]. Heart graft function was monitored by daily palpation while kidney graft rejection was indicated by significant morbidity and death and confirmed histologically. In some experiments the recipient was treated with a neutralising antibody to transforming growth factor β (TGF-β) clone 1D11.16.8 (Bio Express, West Lebanon, NH), 200 μg purified antibody per day i.p. on days 0, 2, 4 and 6.

2.2. Immunohistochemical staining

A three-step indirect immunoperoxidase method was used, similar to previously-described methods [26]. Primary rat monoclonal antibodies reactive with the following mouse antigens were used: KT3, T cells (CD3ε); YTS169.4, CD8 cells; F4/80, macrophages; LO-MG1-2, IgG1; LO-MG2a-9, IgG2a; LO-MM-9, IgM (all from Serotec, Oxford UK). Antibodies RA3-6B2, B Cells (B220, CD45R) and GK1.5, CD4 cells were from BD Biosciences (San Diego, CA). Diluent for all antibodies was 5% normal swine serum in PBS. The second- and third-step reagents were rabbit antibody to rat immunoglobulin conjugated to horseradish peroxidase, 1:50 dilution (Dako, Carpinteria, CA) and goat antibody to rabbit immunoglobulin conjugated to horseradish peroxidase, 1:100 dilution (Zymed, South San Francisco, CA) respectively. Frozen sections of 6 μ were air-dried, fixed in acetone and incubated with primary antibody. After rinsing and washing in PBS, sections were incubated with second antibody in diluent plus 20% normal mouse serum then washed in PBS before incubation with third antibody, washing and colour development with diaminobenzidine substrate. After air-drying, the sections were counterstained with Mayers haematoxylin and counted with the aid of an eyepiece graticule as previously described [26].

2.3. Real-time PCR for cytokine mRNA expression

The methods for RNA preparation and cDNA synthesis [26] and for quantitative real-time PCR analysis [27] have been previously described. Briefly, total RNA was prepared by acid phenol-guanidine extraction and 1 μg was reverse-transcribed using Superscript II (Invitrogen, Carlsbad CA). Aliquots of cDNA were stored at −70 °C prior to amplification in quantitative PCR. PCR reaction mixtures contained Universal master-mix (Applied Biosystems, Foster City CA), cDNA and gene-specific primers and Taqman probe (Applied Biosystems). Primer and probe sequences for cytokines were: IL-2 forward, CAG GAT GCT CAC CTT CAA ATT TT; IL-2 reverse, CGC AGA GGT CCA AGT TCA TCT; IL-2 probe, 6FAM TTG CCC AAG CAG GCC ACA GAA TTG Tamra; IL-4 forward, CGG AGA TGG ATG TGC CAA AC; IL-4 reverse, CGA GCT CAC TCT CTG TGG TGT T; IL-4 probe, 6FAM CCT CAC AGC AAC GAA Tamra; IFN-γ forward, CAG CAA CAG CAA GGC GAA A; IFN-γ reverse, CTG GAC CTG TGG GTT GTT GAC; IFN-γ probe, 6FAM TCA AAC TTG GCA ATA CTC ATC AAT GCA TCC T Tamra; Granzyme B forward, AAG TCA TCC CTA TGG TAA AAT GCA T; Granzyme B reverse, CTT ACT CTT CAG CTT TAG CAG CAT GAT; Granzyme B probe, 6FAM CCC ACC CAG ACT ATA ATC CTA AGA CAT TCT CCC A Tamra; Foxp3 forward, TTG GCC AGC GCC ATC TT; Foxp3 reverse, TGC CTC CTC CAG AGA GAA GTG; Foxp3 probe, 6FAM CAG CTG CTG CTC CAG-minor-groove-binding, non-fluorescent quencher (MGBNFQ); T cell Receptor _ constant region (TRBC) forward, CTA GCA GGA TCT CAT AGA GGA TGGT; TRBC reverse, CAA ACC TGT CAC ACA GAA CAT CAG; TRBC probe, 6FAM CCA CAG TCT GCT CGG C MGBNFQ. Sequences for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers and probe have been previously published [27] and were completely homologous with the mouse gene. Cytokine expression was quantified using a cDNA standard, which consisted of a four-fold serial dilution of a sample with high-level expression. Expression of cytokine cDNA was corrected for differences in quality of RNA using GAPDH expression according to the formula: Cytokine expression/GAPDH expression $\times 10^3$. To ensure reproducibility of results, cDNA synthesis and PCR amplification was performed twice for each sample and the results correlated by linear regression analysis. The average of the two results was used for further analysis. Foxp3 expression was normalised to the extent of T cell infiltration by the formula: Foxp3 expression/TRBC expression.

2.4. Flow cytometry

Migration of donor leukocytes to recipient lymphoid tissues was assayed by single-colour flow cytometry similar to previously-described methods [10]. The recipient spleen was disrupted to form a single-cell suspension and stained to identify cells expressing donor antigen using primary antibody mouse anti-H-$2K^b$ conjugated to biotin (BD Biosciences). The secondary antibody was streptavidin conjugated to allophycocyanin (Molecular Probes, Eugene, OR). A total of 30,000 cells was analysed on a FacsCalibur flow cytometer with Cellquest software (BD Biosciences, Immunocytometry Systems, Mountain View CA).