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Dilly M. Little
Lynn D. Haynes
Tausif Alam
James G. Geraghty
Hans W. Sollinger
Debra A. Hullett

Does transforming growth factor β1 play a role in the pathogenesis of chronic allograft rejection?

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D.M. Little (✉)
Oxford Transplantation Centre,
Nuffield Department of Surgery,
The Churchill Hospital, Oxford, UK
Fax: + 44–1865–225–616

L.D. Haynes · T. Alam · J.G. Geraghty · H.W. Sollinger · D.A. Hullett
Division of Transplantation,
Department of Surgery,
University of Wisconsin,
600 Highland Avenue, Madison,
WI 53792–7375, USA

Abstract To investigate the potential role of Transforming Growth Factor β1 (TGFβ1) in the pathogenesis of chronic allograft rejection, we studied TGFβ1 expression in a rat aortic allograft model. mRNA and protein expression of total and endogenously active TGFβ1 were analysed in infra-renal orthotopic aortic syngeneic and allogeneic grafts and matched with the histological appearances of the grafts, 2, 4 and 12 weeks post-transplantation. Serum levels of TGFβ1 were also measured. The level of TGFβ1 m RNA and protein expression appeared highest 2 and 4 weeks following transplantation in both syngeneic and allogeneic grafts, with significantly elevated levels of mRNA expression in the 2 week allograft specimens. These timepoints correlate histologically with maximal inflammatory cell infiltration of the grafts. By 12 weeks post-transplantation, TGFβ1 mRNA expression is reduced in allogeneic grafts compared to syngeneic grafts. However, detectable levels of total and endogenously active TGFβ1 protein levels in the allografts exceed those measured in the syngeneic grafts at this time point. These results demonstrate the complex expression pattern of this growth factor during the progression of chronic rejection and suggest an aetiological link between TGFβ1 and the process of accelerated graft atherosclerosis.

Key words Chronic rejection · Transforming growth factor β1 · Extracellular matrix · Intimal hyperplasia · Smooth muscle cell

Introduction

Chronic rejection is a significant cause of long-term allograft dysfunction and loss [3, 5, 13]. The obstructive vascular lesion characteristic of this complex process progresses through endothelial cell activation, medial smooth muscle cell migration and progressive neointimal hyperplasia to eventually result in vascular obliteration.

In addition to its potent effects on cell proliferation and differentiation, TGFβ1 plays an active role in wound healing, tissue repair, and regulation of the immune response [26, 30]. It provides a link between the processes by which cells or tissues respond to injury and initiate repair. In an immunological process such as chronic rejection, the early release of cytokines and growth factors including TGFβ1 from activated endothelial cells sets in motion a cascade of events that ultimately may determine the outcome of the allograft. Recent studies have demonstrated that TGFβ1 gene and protein expression is increased in human atherosclerotic lesions [19], particularly in lesions of luminal restenosis. TGFβ1 protein has also been detected in neointimal segments of balloon-injured rat carotid arteries after mechanical injury [16, 38] and in aortic segments of hypertensive rats [27]. While most models of vascular mechanical injury producing atherosclerosis implicate TGFβ1 in promoting or perpetuating the atherogenic

process, direct etiological links between TGFβ1 and the vascular lesions of chronic allograft rejection have not been defined. In one of the few studies examining the possible direct etiological role of TGFβ1 and chronic rejection, Waltenberger et al. [35, 36], demonstrated elevated levels of TGFβ protein and mRNA in chronically rejecting rat cardiac allografts. They concluded that this increased level of TGFβ, which occurred at a delayed stage in the time-course of chronic rejection, represents a mechanism of "endogenous immunosuppression" by graft infiltrating lymphocytes, prolonging the overall survival time of these allografts. This concept of a beneficial immunosuppressive effect of TGFβ1 is further supported by the documented prolongation of subcutaneous cardiac allograft survival in mice administered recombinant human TGFβ1 intraperitoneally [34]. However, direct evidence linking this growth factor to the development of accelerated graft atherosclerosis in a standardised vascular model of chronic allograft rejection is lacking. Using a rat model of chronic aortic allograft rejection, we have studied the temporal expression of TGFβ1 gene and protein in the transplanted vasculature, as the process of chronic rejection evolves.

Materials and methods

Infra-renal orthotopic aortic transplantation. In a modification of the previously described method, [17] we performed orthotopic allotransplantation of approximately 1 cm infra-renal aortic segments in MHC disparate, high responder rat strains, ACI ($RT1^a$) to Lewis ($RT1^l$) (Harlan Sprague-Dawley, Indianapolis, USA). All rats were maintained according to NIH guidelines and project protocols were approved by the University of Wisconsin Animal Care Committee. Syngeneic, (Lewis to Lewis) aortic transplants were performed as controls. At least 6 allogeneic and 4 syngeneic transplants were performed for each time interval examined. Following sacrifice of the animals, aortic grafts were retrieved 2, 4, and 12 weeks post-transplantation. A 5 mm mid-section of each graft was formalin fixed, (10% buffered formalin) and the remainder of the graft was immediately snap-frozen in liquid nitrogen. The formalin fixed grafts were paraffin embedded and 6 µM cross-sections were stained with haematoxylin and eosin. Histological sections were examined under fluorescent light (412 nm) where the intima is readily distinguished from the medial layer by autofluorescence of the internal elastic lamina. Using a computer digital analysis system (Image 1, Universal Image Inc., Calif., USA), the mean intimal area of each graft was determined by tracing the internal elastic lamina and intimal – lumen interface. The intimal area was then calculated by subtracting the nonoccluded luminal area from the area inside the internal elastic lamina. Three readings were obtained for each graft to obtain an average area, expressed as squared microns. The mean intimal area (μM^2) for each experimental group examined (2, 4, 8, 12, and 24 weeks post-transplantation) is represented graphically in Fig. 1.

Blood was drawn from animals at the time of aortic graft retrieval 2, 4, and 12 weeks post-transplantation. Serum was extracted from whole clotted blood following centrifugation and immediately stored at –70 °C.

Reverse Transcription – Polymerase Chain Reaction (RT-PCR). RNA extraction was performed from snap-frozen, homogenised aortic samples by the single step method of acid guanidium thiocyanate phenol chloroform extraction [4]. Six allogeneic and 4 syngeneic samples were analysed for each of the three time points post-transplantation of 2, 4, and 12 weeks. mRNA from three "native" untransplanted aortas was also reverse transcribed for control purposes. The quantity of RNA obtained and the level of protein contamination in each sample was estimated by measuring absorbance of the sample at wavelengths of 260 nm and 280 nm, respectively. The mRNA was reverse transcribed into cDNA using Superscript reverse transcriptase and oligo dT followed by RNA*ase* H treatment, under conditions recommended by the manufacturer (Gibco BRL, Life Technologies, N. Y., USA). Oligonucleotide primers for TGFβ1 were designed from the published rat TGFβ1 sequence [21]. These primers amplify a cDNA fragment of 297 base pairs (bp) length, extending from 1267 bp to 1564 bp of the gene sequence.

Sense 5' C TTC AGA TCC ACA GAG AAG AAC TGC (25 Mer)

Antisense 5' CAC GAT CAT GTT GGA CAA CTG CTC C (25 Mer)

cDNA equivalent to 200 ng of untranscribed RNA was amplified in a volume of 50 µl using 1.25 U *Taq* DNA polymerase (Perkin Elmer, Roche Inc., N.J., USA), 10 mM Tris HCl (pH 8.3), 50 mM potassium chloride, 0.2 mM dNTP (Phamacia Biotech, N.J., USA), 1.2 mM magnesium chloride, 0.5 µM of each of the two primers in *HOT* Start tubes. All PCR reactions included a positive and negative control sample – in the case of the positive control, cDNA from mitogen activated (*Concavalin A*) activated rat lymphocytes was used as template cDNA, while in the negative control, RNAase free water was substituted instead of template cDNA. The following cycle parameters were employed: denaturing 94 °C for 60 s; annealing 63 °C for 60 s and extension 72 °C for 60 s. The oligonucleotide primers for the ribosomal protein S26, amplifying a 326 bp fragment of this gene located between 51 bp and 377 bp [11]were used as an internal standard.

Sense 5' TCG TGC CAA AAA GGG CCG (18 Mer)

Antisense 5' GCT CCT TAC ATG GGC TTT GGT GG (23 Mer)

Amplification conditions for these primers were identical to those described above. To calculate the ratio of TGFβ1/S26 in the linear response range, amplification of cDNA was employed over a range of three different cycles:- TGFβ1: 26, 28 and 30 cycles, S26: 22, 24 and 26 cycles, using a Perkin-Elmer Cetus Thermal Cycler.

High Performance Liquid Chromatography and Operating Conditions. Quantitative analysis of PCR products was performed using High Performance Liquid Chromatography (HPLC) as previously described [9]. The HPLC system used in this study consisted of the Hewlett Packard 1050 Series Quaternary pump, autosampler and VW detector. The analytical column was a TOSOHaas TSK-GEL DEAE-NPR column. Each sample consisted of 60 µl of 25 mM Tris-HCl (pH 9.0) and 40 µl of PCR product. Ninety-five microlitres of this total volume was injected. The two solutions used for the mobile phase were: Reservoir A: 1 M NaCl and 25 mM Tris HCl (pH 9.0) and Reservoir B: 25 mM Tris-HCl (pH 9.0). The gradient programmme employed was as follows: 25%–44% A in 30 s, 44%–52% A in 5 min, 52%–54% A in 2 min, 54%–60% A in 17 min, 60%–100% A in 1 min, 100% A for 2 min, 100%–25% A in 13 min, then 25% A for 10 min. The total injection-to-injection time was 50 min. The column was operated at ml/min at room temperature and the detector was set at

260 nm. Quantitation of the PCR products was obtained following integration of the peak area. The ratio of TGFβ1 / S26 was determined for each sample by dividing the peak area obtained when TGFβ1 cDNA was amplified at 26 cycles by the peak area value obtained when S26 cDNA was amplified at 22 cycles. This was repeated when TGFβ1 cDNA was amplified at 28 cycles and S26 cDNA was amplified at 24 cycles and finally, when TGFβ1 cDNA was amplified at 30 cycles and S26 was amplified at 26 cycles. From the three figures thus generated, a mean value for each individual sample was calculated. The mean TGFβ1 : S26 ratio (± SEM) for each experimental group is represented graphically in Fig. 2. Following amplification, 5 μl of each PCR product was also analysed on a 2% agarose gel, stained with 0.1 μg/ml ethidium bromide. TGFβ1 amplification products from syngeneic and allogeneic aortic samples at the time intervals 2, 4, and 12 weeks post-transplantation were assessed. The TGFβ1 amplification signal intensities were measured using a computer digital system. A digital image of the gel following electrophoresis was made, reversed, and the intensity of the signals measured by digital analysis using an arbitrary gray scale (white = 1; black = 256) to calculate the mean signal intensity for each group (Table 1).

Immunocytochemistry. Six allogeneic and 4 syngeneic grafts, allocated to one of the three post-transplantation time points examined, (2, 4, and 12 weeks) and 3 untransplanted "native" aortas were subjected to immunological analysis using primary antibody directed against the active form of the TGFβ1 protein: Sections (6 μM) of aortic grafts were deparaffinised in xylene (× 2) and hydrated in graded alcohol solutions (100%; 95%; 70%; 50%; 0%). Non-specific protein binding was blocked by incubation with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) (10 mM sodium phosphate pH 7.3, 150 mM NaCl) solution. A primary chicken anti-human TGFβ1 antibody (20 μg/ml diluted in 0.2% BSA / PBS) (R&D Systems, Minneapolis Minn., US) was applied to the sections overnight at 4 °C. Isotype controls were provided by incubating the sections with a Chicken IgY antibody, (Promega, Wis., USA) at the same concentration. The slides were then washed in PBS, prior to quenching of endogenous peroxidase activity with 3% hydrogen peroxide in water for 30 min. The secondary antibody, a horseradish peroxidase (HRP) conjugated rabbit anti-chicken IgG (Sigma ImmunoChemicals, St. Louis, Mo., USA) was added at a dilution of 1:1000 in PBS for 60 min. 3,3 diaminobenzidine tetrahydrochloride (DAB) (Sigma ImmunoChemicals, St. Louis, Mo., USA) was used as the substrate chromogen and allowed to develop for 10 min. The sections were briefly counterstained with haematoxylin.

Enzyme Linked Immunoabsorbant Assay (ELISA). A TGFβ1 ELISA System kit to measure soluble active TGFβ1 was obtained from Promega, Madison, Wis., USA. EIA / RIA A/2 plates (Costar, Cambridge, Mass., USA) were coated overnight at 4 °C with 25 μl monoclonal anti-TGFβ1 IgG primary antibody (1:1000 dilution in carbonate buffer, pH 9.7). After blocking with 1X block buffer at 37 °C for 35 min, 25 μl of sample (diluted in the sample buffer provided) was added to the wells. A standard curve was generated by performing 2-fold serial dilutions of the standard TGFβ1 antigen (diluted 1 ng/ml to 62.5 pg/ml). The plate was incubated for 2 h at room temperature (RT) with shaking and then washed extensively with washing buffer (0.05% Tween 20 in PBS) and PBS. Rabbit anti-TGFβ1 antibody (25 μl/well at 1:1000 dilution) was added and incubated at RT (1 h, with shaking). Following further washing, the anti-rabbit- HRP antibody conjugate (25 μl/well at 1:1000 dilution) was added for a further hour at RT with shaking. A colour reaction was developed by the addition of 25 μl of the chromogen substrate TMB (3,3',5,5'tetramethylbenzidine)/hydrogen peroxidase to each well. Colour development was stopped after approximately 4 min by the addition of 25 μl 1 M phosphoric acid to each well. The difference in absorbance was measured at 450 nm on a EL_X 800NB plate reader (Biotek Instruments Inc., Vt., USA). To measure total TGFβ1 in the serum samples, acid activation was performed as follows: 1 μl of 1 N HCl was added to 50 μl of the serum (diluted 1:5 in PBS) sample, and incubated at RT for 15 min. The acid was then neutralised by the addition of 1 μl 1 M NaOH. Acid-activated samples were assayed after a further 1:100 dilution in sample buffer.

A modified ELISA technique was performed on the harvested aortic grafts. Following retrieval 2, 4, and 12 weeks post-transplantation, each graft sample was immediately frozen in liquid nitrogen and ground to a fine powder using a mortal and pestle. The powdered tissue was stored at −70 °C. Prior to performing the ELISA, the powdered graft was solubilised by homogenisation in 100 μl of a 2X lysis buffer (4% Triton X100, 10 mM Tris (pH 8), 150 mM NaCl, 2 μg/ml soybean trypsin inhibitor, 200 μM each of N-α-p tosyl-l-lysyl chloromethylketone (TLCK), p-tosyl-l-arginine methyl ester (TAME), tosyl-l-phenylalanylchloromethane (TPCK) and 20 μM phenylmethane sulphonyl flouride (PMSF) (Sigma, Immuno Chemicals, St. Louis, MO, USA)) at 4 °C. After 30 min, samples were centrifuged and the supernatants removed for analysis. The supernatants were diluted to a final concentration of 1:10 in sample buffer prior to addition to the plate. Acid activation was performed on samples of the supernatants as described above. These samples were diluted to a final concentration of 1: 50 before addition to the plate. A standard curve was generated by performing serial dilutions of the standard antigen (diluted 1 ng/ml to 62.5 pg/ml) in sample buffer with 0.2% detergent added.

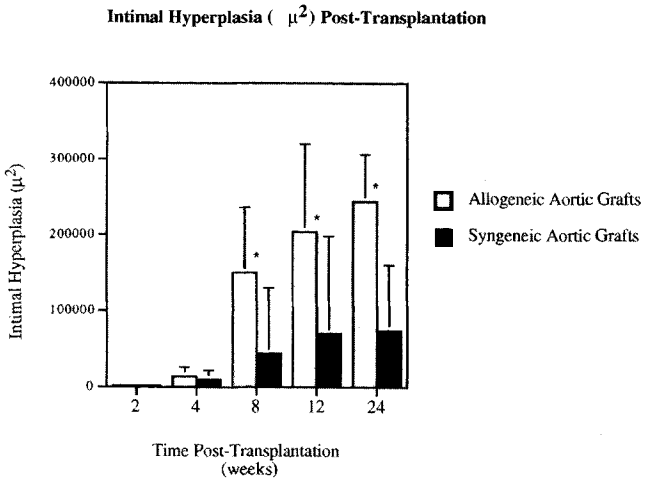
Protein Assay. Because of the small size of the graft samples, it was not possible to accurately estimate the weight of the tissue analysed. However, to prevent variability due to discrepancy in sample quantity, the total protein concentration of each sample was determined by a modification of the Micro BCA protein assay (Pierce, Rockford, Ill., USA) as previously described [6]. Samples that yielded undetectable levels of total protein were excluded from TGFβ1 analysis.

Table 1 Mean signal intensity of TGFβ1 amplification products. Signal intensity data were analysed using ANOVA followed by Fisher's protected least significant difference procedure. A *P* value less than 0.05 was considered significant

Group	Mean signal intensity (± SD)	P^a	P^b
Normal	101.8 ± 19		
12 week allogeneic	97.55 ± 16	0.84	0.0015
12 week syngeneic	156.7 ± 27	0.0138	
4 week allogeneic	173.9 ± 42	0.0022	0.3899
4 week syngeneic	191.0 ± 21	0.0006	
2 week allogeneic	185.0 ± 44	0.0006	0.6860
2 week syngeneic	177.0 ± 13	0.003	

[a] A statistically significant difference was noted between mean signal intensity in all groups except the 12-week allogeneic group and the native aortas

[b] When the mean signal intensities between time-matched allogeneic and syngeneic grafts at a given time-point post-transplantation was compared, a significant difference was detected only at the 12-week interval



* Intimal Hyperplasia in Allogeneic v's Syngeneic Grafts $p < 0.05$

Fig. 1 The development of neointimal hyperplasia (area μm^2) in aortic allogeneic and syngeneic grafts post-transplantation. * Intimal hyperplasia in allogeneic vs syngeneic grafts $P < 0.05$

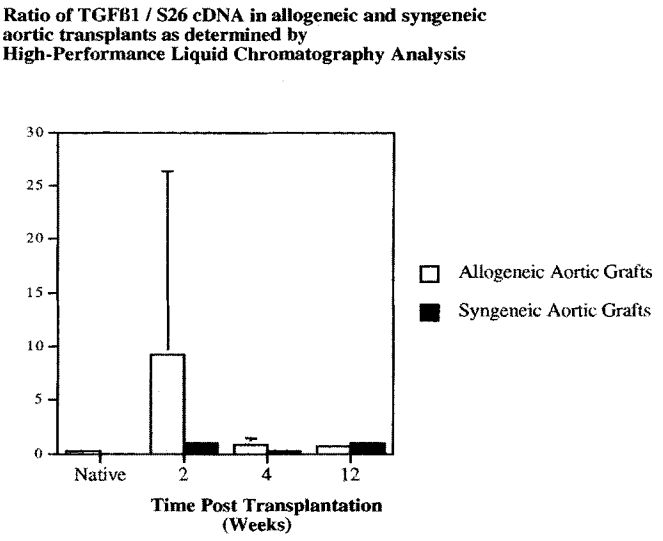


Fig. 2 Ratio of TGFβ1:S26 cDNA in allogeneic and syngeneic aortic transplants as determined by quantitative high pressure liquid chromatography demonstrating an increased level of TGFβ1 cDNA in the early allogeneic grafts which declines with time post-transplantation

Statistical Analysis. The mean concentration of both endogenously active and total TGFβ1 present in serum was calculated for each experimental group, and statistical analysis was carried out using a two-tailed Student's t-test to compare groups. Signal intensity data were analysed using ANOVA followed by Fisher's protected least significant difference procedure. A P value less than 0.05 was considered significant.

Results

Infrarenal orthotopic aortic transplantation

Using this rat model of infra-renal orthotopic aortic transplantation, the histological features of chronic rejection manifested by accelerated intimal hyperplasia and medial cell depletion are consistently reproduced by 12 weeks post-transplantation in the allografts. In contrast, syngeneic grafts maintain cellular integrity in the medial layer and develop minimal intimal hyperplasia (Fig. 1). In the early phase (2 weeks) post-transplantation, allogeneic grafts undergo acute cellular rejection, characterised by dense inflammatory cell infiltration predominantly localised to the adventitial layer of the graft. A milder cellular infiltration of the syngeneic grafts at this time-point is attributed to the normal response to surgical trauma.

TGFβ1 gene expression in transplanted aortic grafts

In the experimental group used to determine the level of TGFβ1 mRNA, 1 animal in the 4-week allogeneic group died in the second week post-transplantation and was therefore excluded from analysis. Two samples in the 12 week syngeneic group were found to have low signal intensity when amplified with both the S26 internal standard and TGFβ1 primers. This finding was therefore attributed to sub-optimal RNA preparation, and replacement transplants were performed, bringing the total number of animals in this group to 7.

As can be seen in Fig. 2, the ratio of TGFβ1 : S26 was increased in allogeneic transplants compared to time-matched syngeneic aortic grafts, harvested 2 weeks post-transplantation (9.2 ± 7.0 vs 1.05 ± 0.1). By 4 weeks post-transplantation, the differences in TGFβ1 gene expression between allogeneic and syngeneic grafts is less evident and has returned to levels observed in native aortic tissue. However, the TGFβ1: S26 ratio remains higher in allogeneic grafts compared to syngeneic grafts (0.89 ± 0.5 vs 0.26 ± 0.2). These time-points correlate histologically with maximal inflammatory cell infiltration of the grafts and presumably maximal expression of the TGFβ1 gene.

By 12 weeks post-transplantation, the pattern of the TGFβ1 expression is actually reversed, with the observed ratio of TGFβ1 : S26 expression in the syngeneic grafts actually exceeding that observed in the allografts (0.97 ± 0.5 vs 0.55 ± 0.19) at this time point. This observation was again confirmed by measurement of the gel electrophoresis TGFβ1 signal intensity (Table 1) allogeneic grafts (97.55 ± 16.5) vs syngeneic grafts (156.7 ± 27). This difference reached statistical significance with a P value of 0.0015. This post-transplantation time point is associated histologically with a well-estab-

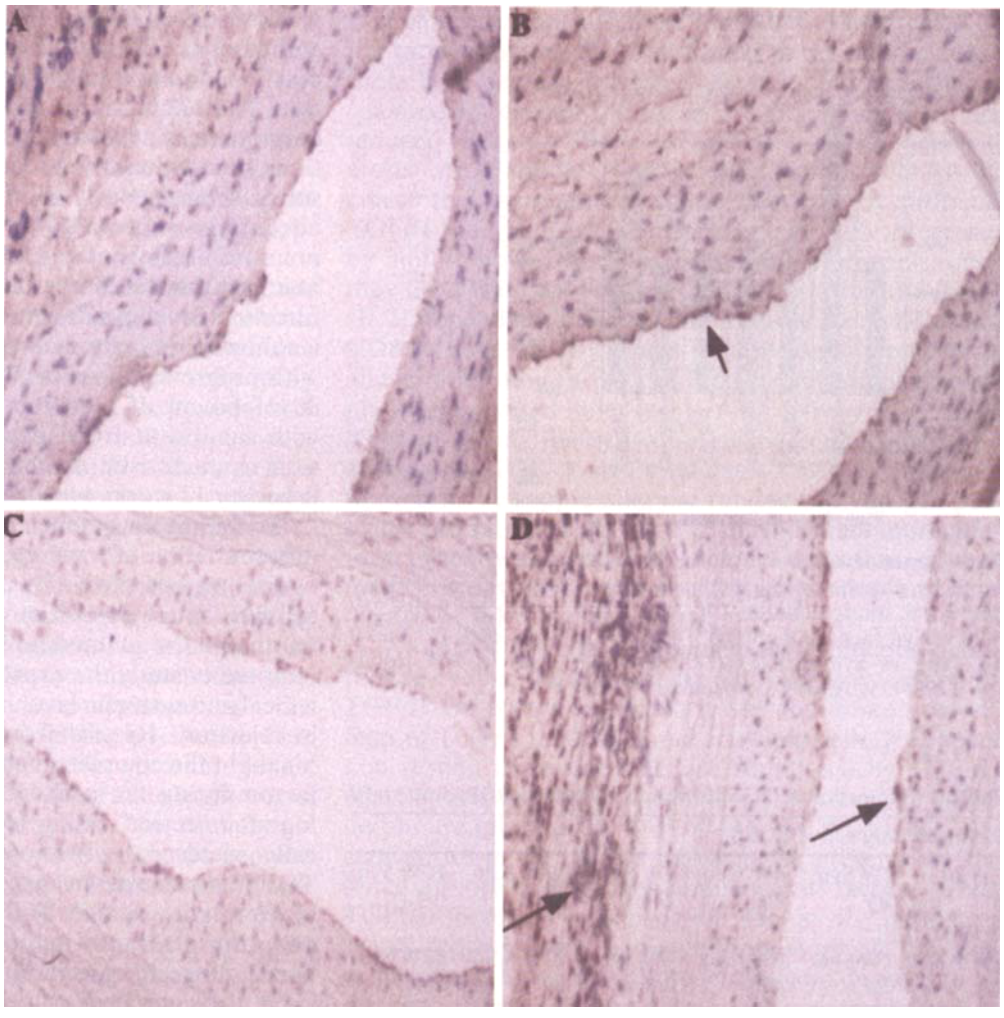


Fig. 3 Immunohistochemical staining of paraffin embedded sections of a 1-month syngeneic graft (**B**) and a 3-month allogeneic graft (**D**) stained with anti-TGFβ1 antibody which specifically detects the active form of the protein. The negative isotype controls for each section are shown in **A** and **C**

lished neointimal layer in the allogeneic grafts and with a reduced number of smooth muscle cells in the medial layer [28].

Localisation of active TGFβ1 expression by Immunocytochemistry. Analysis of TGFβ1 immunocytochemistry performed on the transplanted aortic grafts demonstrated specific staining for the active form of TGFβ1 protein in the endothelial layer of the grafts at all time points. This staining was also predominant in the adventitial layer of allografts. In the 12 week allografts, high levels of TGFβ1 staining were noted in those cells of the neointima adjacent to the lumen of the vessel, but not throughout the entire neointima (Fig. 3).

ELISA of TGFβ1 protein expression in serum and transplanted grafts. Results of TGFβ1 serum analysis derived from three independent experiments are represented in Fig. 4. The level of endogenously active TGFβ1 was significantly elevated in the serum of animals with allogeneic grafts at 2 weeks post-transplantation compared to syngeneic controls ($P = 0.004$). However by 4 weeks post-transplantation, the serum levels of endogenously active TGFβ1 have declined and are similar in both syngeneic and allogeneic animals. At the 12-week-time point, the level of endogenously active TGFβ1 is actually lower in the allogeneic animals compared to the syngeneic animals ($P = 0.019$). At this time point, a distinct neointimal layer has developed in the allografts. In contrast, the total level of TGFβ1 in serum shows little variation between allogeneic and syngeneic animals at any of the time-points examined.

When the level of TGFβ1 protein was examined by ELISA in the actual graft tissue, detectable TGFβ1 levels (> 62.5 pg/ml) were found in both graft types [allogeneic (6 of 6) and syngeneic (2 of 4)], 2 weeks post-transplantation (Table 2). Intergroup variability in the 2 week syngeneic grafts was due to variations in the level of TGFβ1 in the grafts, rather than the level of total protein analysed. By 4 weeks post-transplantation, de-

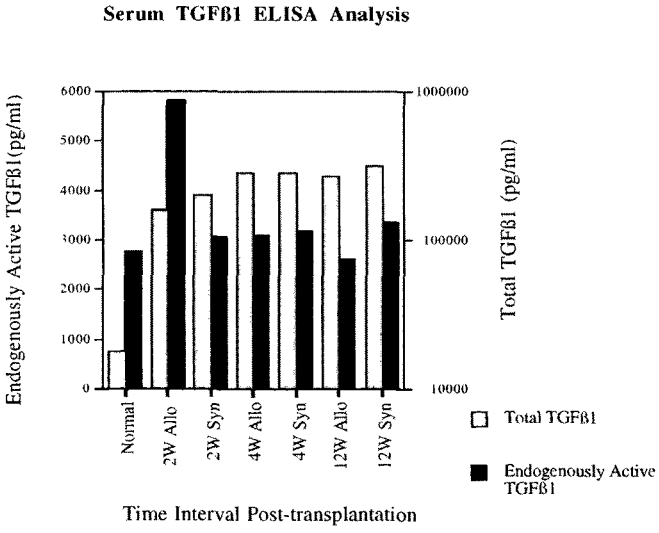


Fig. 4 Serum levels of total and endogenously active TGFβ1 measured by enzyme-linked immunosorbent assay 2,4, or 12 weeks post-aortic transplantation. 2W Allo vs Syn (total) $P < 0.085$; 2W Allo vs Syn (active) $P < 0.004$*; 4W Allo vs Syn (total) $P < 0.974$; 4W Allo vs Syn (active) $P < 0.821$; 12W Allo vs Syn (total) $P < 0.288$; 12W Allo vs Syn (active) $P < 0.019$*

Table 2 Expression of TGFβ1 protein in transplanted aortic grafts

Endogenously active TGFβ1					
2W Allo	2W Syn	4W Allo	4W Syn	12W Allo	12W Syn
6/6[a]	2/4	6/6	2/4	6/6	0/4
Total TGFβ1					
2W Allo	2W Syn	4W Allo	4W Syn	12W Allo	12W Syn
6/6	2/4	4/6	2/4	6/6	1/4

[a] The number of animals in each experimental group/total number of animals in each group with detectable levels (> 62.5 pg/ml) of either endogenously active or total TGFβ1 in their transplanted grafts as determined by ELISA. Allogeneic grafts had more readily detectable levels of TGFβ1 at all time points examined compared to time matched syngeneic controls

tectable TGFβ1 levels had decreased in both groups, being found in 4 of 6 allogeneic, compared to only 2 of 4 syngeneic transplants. At 12 weeks post-transplantation, this trend was still evident, with detectable levels of total and endogenously active TGFβ1 levels in 6 of 6 allografts. Only 1 of 4 syngeneic grafts analysed had detectable levels of active TGFβ1 at this time point. The level of both total and active TGFβ1 in non-transplanted native aortas was non-detectable by this assay.

Discussion

The infrarenal aortic transplant model is unique among experimental animal models of chronic rejection in that it allows specific focus on the immunological targeting and response to injury by the vessel wall itself – a process fundamental to the development of chronic rejection. Without immunosuppression, these transplants undergo a short acute inflammatory episode (acute rejection) predominantly affecting the adventitia which resolves spontaneously within the first month. This acute adventitial inflammation is significantly more pronounced in allogeneic grafts compared to that of syngeneic grafts and is characterised by dense lymphocytic infiltration. In allografts, the acute inflammatory episode is followed by a chronic type of inflammation associated with progressive loss of SMC from the media, and the development of a distinct concentric neointimal layer with significant ECM deposition. This histological lesion characteristic of chronic rejection is fully established by 12 weeks post-transplantation.

TGFβ1 has a potent effect on the proliferation and differentiation of a variety of cell types and is also recognised as one of the most potent stimulators of extracellular matrix deposition [1, 20, 26, 30]. Because of its unique role as an immunomodulatory agent, we have attempted to study the expression of this growth factor at a local and systemic level in this animal model of chronic rejection. Results from this series of experiments highlight the complex expression patterns of this growth factor during the pathogenesis of chronic vascular allograft rejection. Using techniques designed to specifically detect the biologically active form of the TGFβ1 molecule, we have demonstrated elevated levels of circulating active TGFβ1 within the first 2 weeks post-transplantation in the serum of animals undergoing aortic allograft transplantation (Fig. 4). This time interval is also associated with increased mRNA and protein levels of the growth factor at a local level within the grafts themselves, predominantly in the allografts. At the later time interval of 4 weeks post-transplantation, circulating levels of TGFβ1 and mRNA appear to equilibrate in the allogeneic and syngeneic environments. By 12 weeks post-transplantation, serum levels of TGFβ1 in the syngeneic animals actually exceed those found in animals with allografts. However, within the grafts, levels of both total and bioactive TGFβ1 protein are sustained at a detectable level in 6 of 6 of the allografts examined 12 weeks post-transplantation, compared to only 1 of 4 syngeneic grafts. Conversely, the mRNA expression is decreased in the allogeneic grafts, compared to the syngeneic grafts at this time point.

The apparent dichotomy between the levels and actions of TGFβ1 present in the systemic circulation, and that present at a local level, has been previously described: In an experimental arthritis model, local intra-articular administration of TGFβ1 drives the inflammatory response, whereas systemic administration inhibits the same response [31]. The exact mechanism underlying this observation remains unclear. However, it has

been suggested that TGFβ1 can exert a gradient effect: high levels of the growth factor at a specific site attracts inflammatory cells to the area and activates them. Conversely, relatively high levels of circulating TGFβ1 may reduce local inflammation by drawing inflammatory cells out of a localised area into the vasculature. Translated into the context of this model of chronic allograft rejection, high local levels of TGFβ1 in the immunologically activated allogeneic graft may act as a chemoattractant for more inflammatory cells, thereby perpetuating immune-mediated insult to the allograft. In animals with syngeneic grafts, the relatively higher circulating levels of TGFβ1 may draw inflammatory cells away from the graft site into the circulation. However, it still remains unclear why serum levels of endogenously active TGFβ1 remain elevated in animals with syngeneic grafts some 12 weeks post-transplantation, compared to control animals that did not undergo transplantation.

The source of TGFβ1 present within the recently transplanted aortic grafts (2 and 4 weeks post-transplant) is likely to be partially derived from circulating cells, especially platelets, and infiltrating inflammatory cells attracted to the site of the immune response [30]. However, by 12 weeks post-transplantation, the TGFβ1 mRNA levels in the allografts is reduced. This is in contrast to the detectable protein levels (> 62.5 pg/ml) in these grafts which remain higher than in the time matched syngeneic grafts. This finding initially appears to be contradictory. However, the neointimal layer with its associated extracellular matrix is well established in allogeneic grafts at this time point post-transplantation. Previous studies have demonstrated the ability of extracellular matrix components, particularly the proteoglycans, decorin, endoglin and betaglycan to bind the latent TGFβ1 protein and act as a reservoir for the molecule [25]. We postulate that binding of TGFβ1 to these proteoglycans in the extracellular matrix may initiate a negative feedback effect on protein TGFβ1 synthesis and therefore cause mRNA levels in the graft to decline. This "negative feedback loop" would be more evident in allografts which have well-developed neointimal layers. Hence, in 12-week-allografts, detectable levels of TGFβ1 protein remain higher but mRNA levels are reduced, compared to syngeneic grafts which do not exhibit a well defined neointima. This reservoir of TGFβ1 in the neointima may in turn act to attract further inflammatory cells into these allogeneic grafts, thereby potentiating the immune response and driving exracellular matrix production.

More detailed examination of the role of TGFβ1 in the immune response of chronic rejection reveals a paradox – on the one hand TGFβ1 appears to function as a "pro-inflammatory" molecule, exerting potent chemotactic stimulus for neutrophils, T cells, monocytes and fibroblasts which respond to femtomolar concentrations of the growth factor. This is associated with up-regulation of endothelial adhesion molecules, also induced by the growth factor [32, 33]. Moving to the allograft site, these cells become activated as they encounter higher (picomolar) concentrations of TGFβ1. Monocytes are induced to secrete fibroblast growth factor, tumour necrosis factor, and interleukin 1 (IL1) and fibroblasts increase their synthesis of extracellular matrix proteins. TGFβ1 then appears to down-regulate these processes by inhibiting the functions of inflammatory cells, once they have been activated. TGFβ1 inhibits interleukin 2 (IL2)-dependent T cell proliferation of both CD4 and CD8 subsets [10, 26]. It also suppresses differentiation and proliferation of natural killer (NK) cells and lymphokine activated killer cell (LAK) activity [34]. Apart from modifying the activation of macrophages via IL1 expression, TGFβ1 also modulates the cytotoxicity of these cells by suppressing the release of hydrogen peroxide and nitric oxide [29, 37]. Further immunoregulatory properties of TGFβ1 include inhibition of thymocyte proliferation and B cell proliferation and production of IgM and IgG [26, 35].

While it is difficult to reconcile these obviously conflicting events, the stimulatory and inhibitory effects of TGFβ1 are partly accounted for by the differential effects of TGFβ1 on resting and activated cells. It appears that resting immature cells are stimulated by TGFβ1, whereas activated representatives of the same cell populations are inhibited by TGFβ1 [31, 33]. Also local concentrations of the growth factor in the presence or absence of other cytokines influence its action. Thus TGFβ1 serves as a conversion factor or "biological switch" converting an active inflammatory site into one of persistent low-grade alloreactivity and deposition of extracellular matrix.

While TGFβ1 can function either as an agonist or antagonist of cell proliferation and inflammation, it consistently and potently acts on cells, particularly fibroblasts, to induce the deposition of extracellular matrix. TGFβ1-action on extracellular matrix deposition is mediated through its effect on synthesis [7, 14, 24], enzymatic degradation of extracellular matrix, [7, 12] and expression of extracellular matrix receptors on cells [8]. In the context of wound healing and repair, extracellular matrix deposition has obvious benefits. However, unopposed deposition of extracellular matrix, as occurs in atherosclerosis or in chronic rejection, implicates TGFβ1 as perpetuating this pathological process.

Therefore, as both an immunosuppressive and potent pro-inflammatory molecule, TGFβ1 orchestrates events vital to the initiation, progression and resolution of immune-mediated inflammatory responses. Attempts by other workers to influence the outcome of chronic allograft rejection by either altering the expression of TGFβ1 gene, [2, 15, 18, 22] direct administration of exogenous TGFβ1 to transplanted animals, [23] or blocking TGFβ1 receptor sites with antibodies, [38] have re-

sulted in widely varying results: claims that TGFβ1 ameliorates and exacerbates the development of intimal hyperplasia in various transplantation models serve to underline the complex role of this growth factor in the pathogenesis of chronic rejection.

In our experimental aortic model of chronic rejection, the early expression of both total and endogenously active TGFβ1 in the allogeneic grafts and the significantly elevated early (2 week) levels of active TGFβ1 levels in the serum of allotransplanted animals may be an important influencing factor in the ultimate development of chronic rejection – a process which displays dysfunction in many areas putatively under the influence of this growth factor. While bearing in mind the complexity of such a process as chronic allograft rejection, and the fact that TGFβ1 is but one of a myriad of cytokines and growth factors potentially involved, it seems reasonable to suggest an etiological link between TGFβ1 and chronic rejection. However, the authors fully accept that the content of this work is primarily descriptive and in no way proves the etiological link between TGFβ1 and the development of chronic allograft rejection. In an attempt to improve our understanding of the exact mechanism of this complex process, we have alterated the local expression of the TGFβ1 gene following aortic transplantation, using adenoviral vectors and anti-sense gene technology.

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References

1. Border WA, Noble NA (1994) Transforming growth factor β in tissue fibrosis. N Engl J Med 331: 1286–1292
2. Border WA, Noble NA (1997) TGF-β in kidney fibrosis: a target for gene therapy. Kidney Int 51: 1388–1396
3. Cecka JM (1994) Outcome statistics of renal transplants with an emphasis on long-term survival. Clin Transpl 8: 324–327
4. Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
5. Cook D (1987) Clinical Transplantation. UCLA Tissue Typing Laboratory, Los Angeles, pp 277
6. DeVito LD, Sollinger HW, Burlingham WJ (1990) Adsorption of cytotoxic anti-HLA antibodies with HLA class I immunosorbant beads. Transplantation 49: 925–931
7. Edwards DR, Murphy G, Reynolds JJ, Whitham SE, Docherty AJ, Angel P, Heath JK (1987) Transforming growth factor modifies the expression of collagenase and metalloproteinase inhibitor. EMBO J 6: 1899–1904
8. Ignotz RA, Massangue J (1987) Cell adhesion protein receptors as targets for transforming growth factor-β action. Cell 51: 189–197
9. Katz ED, Dong MW (1990) Rapid analysis and purification of polymerase chain reaction products by high-performance liquid chromatography. Biotechniques 8: 546–555
10. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, Sporn MB, Fauci AS (1986) Production of transforming growth factor β by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med 163: 1037–1050
11. Kuwano Y, Nakanishi O, Nabeshima Y, Tanaka T, Ogata K (1985) Molecular cloning and nucleotide sequence of DNA complementary to rat ribosomal protein S26 messenger RNA. J Biochem 97: 983–992
12. Laiho M, Saksela O, Keski OJ (1987) Transforming growth factor-β induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to exogenase urokinase. J Biol Chem 262: 17467–17474
13. Land W (1989) Kidney transplantation – state of the art. Transplant Proc 21: 1425
14. Lawrence WT, Norton JA, Sporn MB, Gorschboth C, Grottendorst GR (1986) The reversal of an Adriamycin induced healing impairment with chemoattractants and growth factors. Ann Surg 203: 142–147
15. Lefebvre B, Formstecher P, Lefebvre P (1995) Improvement of the gene splice overlap (SOE) method. Biotechniques 19: 186–187
16. Majesky MW, Lindner V, Twardzik DR, Schwartz SM, Reidy MA (1991) Production of transforming growth factor β1 during repair of arterial injury. J Clin Invest 88: 904–910
17. Mennander A, Tiisala S, Halttunen J, Yilmaz S, Paavonen T, Hayry P (1991) Chronic rejection in rat aortic allografts – an experimental model for transplant arteriosclerosis. Art and Thromb 11: 671–680
18. Nabel EG, Shum L, Pompili VJ, Yang Z, San H, Shu HB, Liptay S, Gold L, Gordon D, Derynck R, Nabel GJ (1993) Direct transfer of transforming growth factor β1 gene into arteries stimulates fibrocellular hyperplasia. Proc Natl Acad Sci USA 90: 10759–10763
19. Nikol S, Isner JM, Pickering JG, Kearney M, Leclerc G, Weir L (1992) Expression of transforming growth factor-β1 is increased in human vascular restenosis lesions. J Clin Invest 90: 1582–1592
20. Nilsen-Hamilton M (1990) Current topics in development biology. 24 Academic Press, New York, pp 95–136
21. Qian SW, Kondaiah P, Roberts A, Sporn MB (1990) cDNA cloning by PCR of rat transforming growth factor β-1. Nucleic Acids Res 18: 3059
22. Qin L, Chavin KD, Ding Y, Woodward JE, Favaro JP, Lin J, Bromberg JS (1994) Gene transfer for transplantation. Prolongation of allograft survival with transforming growth factor-beta 1. Ann Surg 220: 508–518
23. Raju GP, Belland SE, Eisen HJ (1994) Prolongation of cardiac allograft survival with transforming growth factor-β1 in rats. Transplantation 58: 392–396

24. Roberts AB, Sporn MB, Assosian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci AF (1986) Transforming growth factor β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. Proc Natl Acad Sci USA 83: 4167–4171
25. Ruoslahti E, Yamaguchi Y (1991) Proteoglycans as modulators of growth factor activities. Cell 64: 867–869
26. Ruscetti FW, Palladino MA (1991) Transforming growth factor-β and the immune system. Progress in Growth Factor Research Great Britain 3: 159–175
27. Sarzani R, Brecher P, Chobanian AV (1989) Growth factor expression in aorta of normotensive and hypertensive rats. J Clin Invest 83: 1404–1408
28. Stoltenberg RL, Geraghty JG, Steele DM, Kennedy E, Hullett DA, Sollinger HW (1995) Inhibition of intimal hyperplasia in rat aortic allografts with cyclosporin. Transplantation 60: 993–998
29. Tsunawaki S, Sporn MB, Ding A, Nathan C (1988) Deactivation of macrophages by transforming growth factor-β. Nature 334: 260–262
30. Wahl SM (1992) Transforming growth factor beta (TGFβ) in inflammation: a cause and a cure. J Clin Immunol 12: 61–74
31. Wahl SM (1994) Transforming growth factor β: The good, the bad, and the ugly. J Exp Med 180: 1587–1590
32. Wahl SM, McCartney-Francis N, Allen JB, Dougherty EB, Dougherty SF (1990) Macrophage production of TGF-β and regulation by TGF-β. Ann NY Acad Sci 593: 188–196
33. Wahl SM, McCartney-French N, Mergenhan SE (1989) Inflammatory and immunomodulatory roles of TGF-β. Immunol Today 10: 258–260
34. Wallick SC, Figari IS, Morris RE, Levinson AD, Palladino MA (1990) Immuno-regulatory role of transforming growth factor-β (TGFβ) in development of killer cells: comparison of active and latent TGF-β1. J Exp Med 172: 1777–1784
35. Waltenberger J, Miyazono K, Funa K, Wanders A, Fellstrom B, Heldin CH (1993) Transforming growth factor-β and organ transplantation. Transplant Proc 25: 2038–2040
36. Waltenberger J, Wanders A, Fellstrom B, Miyazono K, Heldin CH (1993) Induction of transforming growth factor-β during cardiac allograft rejection. J Immunol 151: 1147–1157
37. Warwick-Davies J, Lowire DB, Cole PJ (1995) Selective deactivation of human monocyte functions by TGF-β. J Immunol 155: 3186–3193
38. Wolf YG, Rasmussen LM, Ruoslahti E (1994) Antibodies against transforming growth factor-β1 suppresses intimal hyperplasia in a rat model. J Clin Invest 93: 1172–1178