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Research Article

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A Critical Role for Transforming Growth Factor-β in Donor Transfusion-induced Allograft Tolerance

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Abstract

Donor-specific (DST) or nonspecific blood transfusions administered before transplantation can enhance survival of vascularized allografts both in humans and animals but the immunological mechanisms of this effect remain unclear. We have analyzed the expression and the role of endogenous TGF-β1 in a model of heart allograft tolerance, induced by pregraft DST in adult rats. We reported previously that this tolerance occurs despite a strong infiltration of leukocytes into the graft that are unable to produce both Th1- and Th2-related cytokines in vivo. Allografts from DST-treated rats express high levels of TGF-β1 mRNA and active protein. This phenomenon is correlated with the rapid infiltration of leukocytes producing high amounts of TGF-β1. TGF-β1–producing cells are virtually absent among early infiltrating cells in rejected grafts but are found at a later time point. The induction of allograft tolerance in vivo is abrogated by administration of neutralizing anti–TGF-β mAb. Moreover, overexpression of active TGF-β1 in heart allografts using a recombinant adenovirus leads to prolonged graft survival in unmodified recipients. Taken together, our results identify TGF-β as a critical cytokine involved in the suppression of allograft rejection induced by DST and suggest that TGF-β–producing regulatory cells are also involved in allograft tolerance. (*J. Clin. Invest.* 1998. 102:1920–1926.) Key words: transplantation • tolerance • suppression • TGF-β • rodent

Introduction

Molecules of the TGF-β family are known to play an important role in immunomodulation (1, 2). Their effects include: (*a*) inhibiting the proliferation and function of almost all T cell subsets, particularly by counteracting IL-2–dependent T cell proliferation by suppressing IL-2 and IL-2 receptor mRNA expression (3–5); (*b*) inhibiting the proliferation and function (IgG and IgM production) of B lymphocytes (6, 7); (*c*) inhibiting the generation of cytotoxic T cells but not their cytotoxic activity and inhibiting the generation and cytotoxicity of natural killer cells (8, 9); and (*d*) inhibiting macrophage activation and nitric oxide formation by activated macrophages (10, 11). Previous reports suggested a critical role for regulatory TGF-β–producing T cells in suppressing Th-1–mediated inflammatory responses that occur during experimental allergic encephalomyelitis in mice and rats (for review see reference 12) or colitis in mice (13). Given these important suppressive effects, TGF-β may also play a major role in the suppression of allograft rejection. Indeed, two studies showed that in vivo administration of recombinant TGF-β1 delayed allograft rejection in rodents (14, 15), and another showed that injection of a TGF-β1–encoding plasmid into mice heart allografts resulted in a significant prolongation of graft survival (16). Interestingly, it has been proposed recently that cyclosporin A could exert its immunosuppressive effect partly through inducing TGF-β production (17). Therefore, the potential role of endogenously produced TGF-β in the induction of allograft tolerance and the suppression of acute allograft rejection still needs to be investigated.

Tolerance to a heart allograft can be induced in adult rats by administering pregraft donor-specific blood transfusion (DST)[1] (18). We and others have shown that allografts in DST-treated recipients were more rapidly infiltrated by recipient mononuclear cells than in untreated animals (19, 20). That these cells were able to develop a specific cytotoxic response against donor cells in vitro argues against a model involving clonal deletion of alloreactive T cells (20–22). Furthermore, the upregulation of both Th1- (IL-2, IFN-γ) and Th2-related (IL-4, IL-10, and IL-13) cytokine mRNAs was prevented in grafts from DST-treated rats during the first week after transplantation, suggesting anergy or suppression of the whole graft-infiltrating Th population (19). We hypothesized that the defect in Th function in DST-treated allograft recipients could be mediated by the suppressive cytokine TGF-β1. Here, we report that TGF-β1 is overexpressed in allografts from DST-treated animals as compared with control ones. This early upregulation of TGF-β1 transcription is related to the rapid infiltration by leukocytes producing high levels of TGF-β1 mRNA. Induction of allograft tolerance is abrogated by in vivo neutralization of TGF-β in DST pretreated recipients and adenovirus-mediated overexpression of TGF-β1 in heart allografts

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1. *Abbreviations used in this paper:* DST, donor-specific blood transfusion; GIC, graft-infiltrating cells; HPRT, hypoxanthineguanine phosphoribosyltransferase; ISH, in situ hybridization.

prolonged survival in unmodified recipients, indicating that suppression of allograft rejection by DST is mediated by early and vigorous TGF-β production.

Methods

Animal model of DST-induced heart allograft tolerance. 8–12-wk-old male Lewis.1W (RT1.u) and Lewis.1A (RT1.a) rats served as blood and heart donors, and as blood and allograft recipients, respectively. These MHC incompatible congenic strains were obtained from the Centre d'Elevage Janvier (Le Genest-Saint-Isle, France). Blood was collected by cardiac puncture in a heparinized syringe (final concentration: 20 IU/ml). To induce tolerance, allograft recipients were transfused with 1 ml of donor blood 14 and 7 d before transplantation. Heterotopic heart grafts were performed using Ono and Lyndsey's technique (23). The grafts were evaluated daily for function by palpation through the abdominal wall. Lewis.1A recipients rejected Lewis.1W heart allografts in 6.4±1.7 d, whereas allografts in DST-treated rats survived indefinitely (18). Previous studies have shown that the suppression of acute rejection induced by DST is donor specific (18). Moreover, long-term tolerant animals (> 100 d) accepted donor type but not third party skin allografts (data not shown).

Reagents. Polyclonal chicken anti–porcine TGF-β1 Ab cross-reacting with rat TGF-β1 was purchased from R&D Systems (Minneapolis, MN). This Ab was shown previously to recognize only the active form of the protein in a colonic adenocarcinoma cell line producing either latent or active TGF-β1 (24) and was therefore used in immunohistological studies. For in vitro and in vivo studies, a neutralizing murine anti–human TGF-β mAb producing hybridoma (clone 2G7; 25) was kindly provided by Dr. K. Melief (Academish Ziekenhuis Leiden, The Netherlands). The OKT3 mAb was used as an isotype-matched control. Both 2G7 and OKT3 mAbs were purified from ascites as described previously (19). Human rIL-2 was a generous gift of Cetus Corp. (Emeryville, CA).

Immunohistology. Heart allografts were removed, immediately snap-frozen in liquid nitrogen after embedding in OCT compound (Tissue Tek; Miles Laboratories, Elkhart, IN), and then stored at −70°C until use. 5-μm cryostat sections were cut at −20°C, air-dried, fixed in acetone for 10 min at room temperature, and stored at −20°C. For TGF-β1 staining, sections were incubated overnight at 4°C with polyclonal chicken anti–porcine TGF-β1 antibody (R&D Systems) diluted to 0.125 mg/ml. Slides were then incubated with peroxidase-conjugated rabbit anti–chicken IgG for 60 min at 37°C followed by VIP (Vector, Burlingame, CA) as a substrate and slightly counterstained with hematoxylin. To compare the magnitude of active TGF-β1 staining in the infiltrate between the two groups, quantification was blindly performed using the following units: 0, no staining; +, 0–25% of positively stained graft-infiltrating cells (GIC); ++, 25–50%; +++, 50–75%; ++++, 75–100%.

Quantitative PCR. Oligonucleotides used are shown in Table I. Oligonucleotides named X.Cons encompass the X.5′ primer, four bases of the X cDNA sequence, followed by a deletion of four bases, and ending with the corresponding sequence of the X cDNA sequence (26). RNA and cDNA preparation was done as follows. Total RNA from heart allografts was purified in a cesium chloride gradient (27). 10 μg was reverse transcribed, using the Boehringer cDNA synthesis kit, and diluted in a final volume of 100 μl. Standard constructions were performed essentially as described previously (19, 26). Quantification of transcript X was performed as described (19). In brief, a constant amount of cDNA corresponding to the reverse transcription of 50 ng of total RNA was mixed with 10^6, 10^5, 10^4, 10^3, 10^2, or 0 copies of the X standard, and then amplified to saturation followed by a dye-labeled oligonucleotide elongation (run-off reaction). Run-off reaction products were electrophoresed for 7 h using an Applied Biosystem 373A DNA sequencer. The Immunoscope® software was specially devised to measure, for each detected DNA peak, both its length (with a precision of < 0.2 nucleotide) and its area (with a precision of < 5%) (26).

In situ hybridization (ISH). Detection of TGF-β1 mRNA-producing cells in allograft was performed using a TGF-β1 ISH kit (R&D Systems) and following the manufacturer's instructions. In brief, 5-μm cryostat heart allograft sections were pretreated with 0.4 μg/ml proteinase K for 10 min at 37°C and fixed in 0.4% paraformaldehyde for 20 min at 4°C. Slides were then hybridized with a cocktail of digoxigenin-labeled TGF-β1 probes (no cross-reactivity with TGF-β2 or β3) for 18 h at 37°C followed by six washes, and then incubated with an antidigoxigenin Fab fragment antibody coupled to alkaline phosphatase. Sections were then revealed with nitroblue tetrazolium and lightly counterstained with methyl-green. Positive controls consisted of poly-dT probe (data not shown) and negative controls were performed by using either no probe or TGF-β1 sense probe.

Proliferation assays. A standard one-way MLR was performed as described previously (28). In brief, spleen cell suspensions were prepared from untreated and DST-treated animals killed 5 d after transplantation. Cells were resuspended in culture medium consisting of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml of penicillin, 0.1 mg/ml streptomycin, 1 mM sodium pyruvate, and 5×10^{-5} M 2-ME. Irradiated donor-type Lewis.1W (RT1.u) or third-party Buffalo (RT1.b) spleen cells served as stimulator cells. 10^5 responder and 2×10^5 irradiated stimulator cells were plated in 96-well round-bottomed plates in triplicate in a final volume of 200 μl and cultured for 5 d at 37°C and 5% CO_2, and for the last 8 h in the presence of 0.5 μCi [^{3}H]TdR (Amersham, Les Ulis, France). The cells were then harvested on glass fiber filters and [^{3}H]TdR incorporation was measured using standard scintillation procedures (Packard Instruments, Meriden, CT).

Construction and application of a recombinant adenovirus for TGF-β. Recombinant adenovirus (Ad) was constructed, propagated, purified, and titered according to standard protocols (29) and as previously described (30). The Ad vector used for the construction of AdTGF-β was deleted of the E1 and E3 regions and carried a thermo-sensible mutation in the E2a region shown to decrease inflammation due to expression of viral proteins after in vivo adminis-

Table I. List of Oligonucleotides Used

Name	Sequence	Length of wild-type PCR products	Length of wild-type run-off products
HPRT.5′	TGCTGGATTACATTAAAGCGC	425	
HPRT.3′	CTTGGCTTTTCCACTTCGC		
HPRT.RO	GTTGACTGGTCATTACAGTAGC		100
HPRT.Cons	TGCTGGATTACATTAAAGCGCTGAAAATAGTGATAGGTCCATTCC		
TGF-β.5′	CTACTGCTTCAGCTCCACAG	320	
TGF-β.3′	TGCACTTGCAGGAGCGCAC		
TGF-β.RO	AGACAGAAGTTGGCATGGTAGC		120
TGF-β.Cons	CTACTGCTTCAGCTCCACAGAGAATGCTGTGTACGGCAGCTGTA		

tration, while extending expression of vectorized sequences (31). The TGF-β1 cDNA sequence used was mutated in cysteines 223 and 225 to serines, which results in production of only bioactive and no latent forms of TGF-β (32). The TGF-β1 cDNA was placed under the transcriptional control of the CMV promoter and 5′ of polyA sequences from SV40 in a plasmid carrying Ad5 sequences necessary for homologous recombination in 293 cells. The resulting recombinant Ad were confirmed by restriction digestion and Southern blotting of Hirt-purified DNA from 293 cells. Production of TGF-β1 from cells transduced in vitro with AdTGF-β was confirmed by ELISA (Promega Corp., Madison, WI) and by growth inhibition of mink lung epithelial cells. AdLac-Z is an E1- and E3-deleted recombinant adenovirus containing the RSV long-terminal repeat promoter, a nuclear location signal from the SV40 large T antigen, and the *Escherichia coli* Lac-Z coding sequence (33). Vectors were purified by two consecutive CsCl gradient ultracentrifugations, dialyzed against sterile buffer, aliquoted, and stored at −80°C until used. Recombinant adenoviruses were titered on 293 cells using plaque-forming unit (pfu) assays and expressed as pfu/ml.

Some minutes after reperfusion and beating of grafted hearts, hearts were clamped and recombinant Ad, diluted in sterile lactate Ringer's solution (250 μl), were slowly injected into the apex at four different points using a 30-gauge needle. After 30 s, hearts clamps were released, the abdominal wall was closed, and animals were then maintained in microisolator cages and fed ad libitum. This gene transfer procedure was perfectly tolerated and resulted in no loss of transplanted hearts.

In vivo administration of anti–TGF-β mAb. DST pretreated allograft recipients received daily intraperitoneal injection of purified 2G7 or OKT3 mAb diluted in PBS, 1 mg per injection, from day 0 to day +4 after grafting.

Statistical analysis. To compare the number of TGF-β1 mRNA copies and the score of active TGF-β1 staining between the two experimental groups, Student's *t* tests were performed and $P < 0.05$ was considered as significant. Allograft survivals were compared using the Kaplan-Meier test.

Results

TGF-β1 mRNA is strongly expressed in surviving allografts from DST-treated recipients. To analyze the level of TGF-β1 gene expression in heart allografts, we performed a quantitative RT-PCR using primers specific for rat TGF-β1. To standardize the results, we measured in parallel the expression of hypoxanthineguanine phosphoribosyltransferase (HPRT) mRNA. Fig. 1 shows that TGF-β1 mRNA accumulated earlier and more strongly in allografts from DST-treated rats than in untreated ones. The level of TGF-β1 mRNA expression in normal heart was similar to that found in rejecting allograft on day 1 (data not shown). Grafts from untreated rats showed a significantly increased (fourfold) accumulation of TGF-β1 mRNA on day 4 followed by a rapid decrease (Fig. 1). In contrast, those from DST-treated rats were characterized by a strong and sustained accumulation from day 1 ($P < 0.05$) to day 5 ($P < 0.05$), except on day 3 when a significant drop in TGF-β1 transcription was observed (Fig. 1). On day 7, levels of mRNA expression were similar and low in both situations. The level of TGF-β1 mRNA remained low in tolerated allografts analyzed at 1 and 3 mo after transplantation (data not shown).

Allografts in DST-treated recipients are early infiltrated by TGF-β1 mRNA-producing cells. To determine which cells produced TGF-β1 in allografts, we performed ISH with TGF-β1 specific antisense probes on heart graft sections from both experimental groups on days 1, 3, and 5 after transplantation

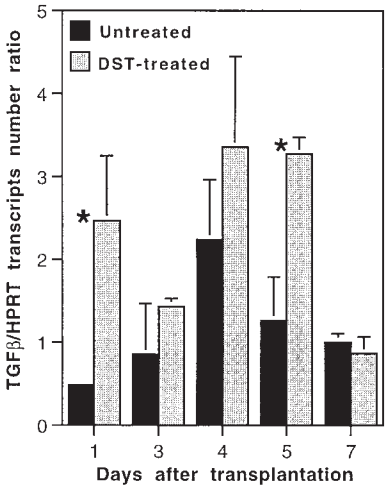

Figure 1. TGF-β1 mRNA is overexpressed in surviving allografts from DST-treated recipients. Total RNA was extracted from heart allografts from untreated (*black bars*) and DST-treated (*gray bars*) rats at different time points after grafting. Quantitative RT-PCR analysis of TGF-β1 and HPRT mRNA accumulation was performed as described in Methods. Results are expressed as means±SD from the ratio between the number of TGF-β1 and HPRT transcripts in allografts for four to six animals at each time point. *$P < 0.05$.

(Fig. 2). Negative control with a TGF-β1 sense probe did not exhibit any staining (Fig. 2 *A*). Vessel walls were positively stained in untreated and DST-treated grafts for TGF-β1 mRNA. TGF-β1 accumulation in vessels consistently increased from days 1–5 after grafting. In contrast, whereas control allograft did not exhibit TGF-β1 mRNA-producing cells outside of vessels during the first 3 d, a significant number of positive cells was observed in the hearts from DST-treated recipients at the same time (Fig. 2 *C*). These cells could be identified as GIC based on their interstitial localization and their appearance as small groups of cells. After day 3, TGF-β1 mRNA-producing cells could also be detected in control allografts; however, the staining intensity was consistently lower in these allografts than in tolerated ones (Fig. 2 *D*). Together with the results obtained by RT-PCR, these data indicate that allografts in DST-treated rats are rapidly infiltrated by a small number of cells producing high amounts of TGF-β1 mRNA, whereas these cells are absent in control grafts at the same time.

The active form of TGF-β1 protein is strongly expressed in allografts from DST-treated recipients. Posttranscriptional regulation of the secretion of TGF-β protein is a complex process and includes the production of a latent complex from which active forms of TGF-β can be liberated in vivo through proteolytic cleavage (34). To determine whether the strong upregulation of TGF-β1 mRNA expression found in DST grafts was associated with the production of active protein, we analyzed TGF-β1 protein expression in allografts using an antibody that recognizes only the active form of TGF-β1 (24). A semiquantitative analysis of active TGF-β1 expression by GIC was performed (Fig. 3 *E*). Consistent with the fact that endothelial and smooth muscle cells constitutively produce latent TGF-β1 that can be subsequently activated (35), heart vessels were found to express active TGF-β1 in both DST and control grafts (Fig. 3 *C*). As for TGF-β1 mRNA, heart grafts from DST-treated rats exhibited a strong and sustained active TGF-β1 protein expression in the interstitium and in the leukocyte infiltrate from days 1–5 (Fig. 3, *B, D,* and *E*). In contrast, control rejected allografts showed weak or no expression of active TGF-β1 protein in the infiltrate during the initial 3 d after grafting (Fig. 3, *A*, *C*, and *E*) but a strong staining was found on day 5 (Fig. 3

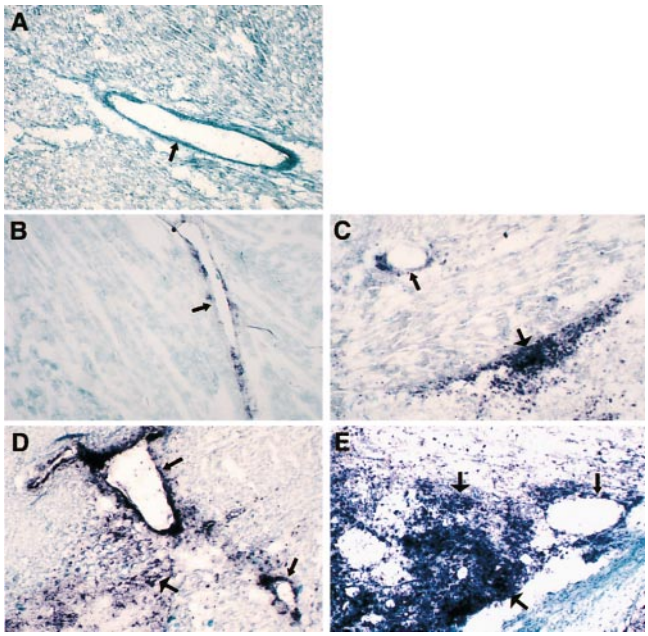

Figure 2. Heart allografts in DST-treated, but not untreated, recipients are rapidly infiltrated by TGF-β1 mRNA-producing leukocytes. Heart graft cryostat sections, pretreated with proteinase K, were incubated with a cocktail of digoxigenin-labeled TGF-β1 specific antisense probe or TGF-β1 sense probe as negative control, followed by antidigoxigenin Fab fragment antibody coupled to alkaline phosphatase and then nitroblue tetrazolium as a revealing agent (blue-brown coloration). Slides were then lightly counterstained with methyl-green. (*A*) Negative control (TGF-β1 sense probe). (*B*) Untreated, day 1. (*C*) DST-treated, day 1. (*D*) Untreated, day 5. (*E*) DST-treated, day 5. Small arrows indicate vessels and large arrows indicate the infiltrate.

E). In both groups, TGF-β1 staining decreased after day 5. Control syngeneic grafts and normal hearts showed only weak expression in vessels (data not shown), similar to that of grafts from untreated animals on day 1.

TGF-β1 is involved in the nonspecific suppression of T cell proliferative response in spleen from DST-treated allograft recipients. To analyze the potential activity of TGF-β1 in suppressing T helper function in DST-treated rats, we performed MLR using as a responder population spleen cells from DST-treated and control animals killed 5 d after transplantation and, as stimulator cells, irradiated Lewis.1W (RT1.u) or third-party Buffalo (RT1.b) spleen cells. Spleen cells from DST-treated animals showed no or very weak proliferative responses to donor cells on day 5 of culture, contrasting with the strong proliferation of those from rejecting rats (Fig. 4). The DST effect in this model has been shown to be donor specific since third-party allografts are rejected in Lewis.1W blood–transfused Lewis.1A recipients (18) and since long-term tolerant animals accepted donor type but not third-party skin allografts (data not shown). Nevertheless, the in vitro unresponsiveness of splenocytes from tolerant animals was not donor specific (data not shown). Similar posttransplantation nonspecific suppression have been described previously in different allograft tolerance models (36). When a mAb neutralizing TGF-β was added at the beginning of culture, T cells remained unresponsive (Fig. 4). However, anti–TGF-β mAb in the presence of rIL-2, but not control isotype-matched mAb, was able to restore a strong proliferative response against donor cells, comparable to that of cells from control rejecting animals, suggesting a role for TGF-β in this nonspecific suppression (Fig. 4). By contrast, the proliferative response of spleen cells from untreated rats was enhanced by rIL-2 alone (Fig. 4), but not by anti–TGF-β mAb alone or in combination with IL-2.

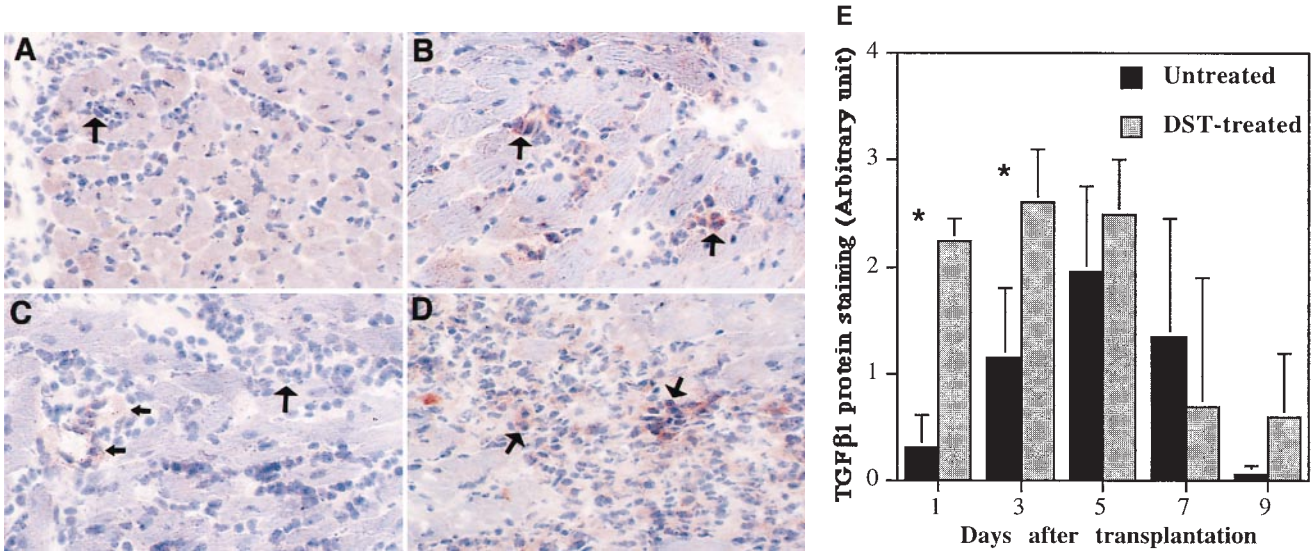

Figure 3. The active form of TGF-β1 protein is massively expressed in allografts from DST-treated rats. Heart allograft cryostat sections from control untreated (*A* and *C*) and DST-treated recipients (*B* and *D*) prepared 1 d (*A* and *B*) and 3 d (*C* and *D*) after transplantation were immunostained with an anti–TGF-β1 antibody that has been shown previously to recognize only the active form of the protein. VIP was used as a substrate for peroxidase and gives a red coloration (×400). Small arrows indicate vessels and large arrows indicate the infiltrate. (*E*) The magnitude of staining of the active form of TGF-β1 in infiltrated areas of heart graft sections was blindly scored using the following histologic grade: 0, no staining; +, 0–25% of positively stained GIC; ++, 25–50%; +++, 50–75%; ++++, 75–100%. Results are expressed as mean score±SD of four different investigators in four control (*black bars*) and four DST grafts (*gray bars*) at each time point. $^*P < 0.05$.

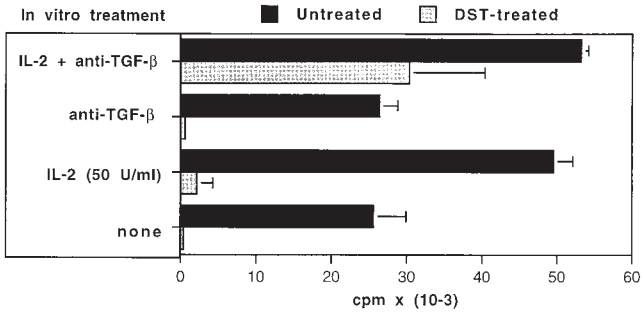

Figure 4. Anti–TGF-β mAb, in the presence of IL-2, inhibits the nonspecific suppression of proliferative response of spleen cells in DST-treated rats. MLR were performed with spleen cells from untreated (*black bars*) and DST-treated (*gray bars*) heart allograft recipients killed 5 d after the transplantation, as responder cells and irradiated donor spleen cells, as stimulator cells. [^{3}H]TdR incorporation was assessed after 5 d of culture. Error bars represent mean±SE between triplicate determinations. When indicated, rIL-2 (50 U/ml), a neutralizing anti–pan TGF-β mAb (25 μg/ml), an isotype-matched control mAb (OKT3, not shown), or rIL-2 and mAb were added at the beginning of culture.

Neutralization of TGF-β in vivo abrogates induction of allograft tolerance in DST-treated recipients. To assess the in vivo role of endogenously produced TGF-β during the induction phase of DST-induced heart allograft tolerance, DST-pretreated animals were injected intraperitoneally with a neutralizing anti–TGF-β mAb from day 0 to day +4 after the transplantation. This schedule was based on the kinetics of TGF-β1 expression observed in heart grafts from DST-treated recipients (Figs. 1 and 3). Anti–TGF-β mAb treatment abrogated allograft tolerance in 100% of animals (6/6), whereas isotype-matched control antibody had no effect on allograft survival (Fig. 5). However, allograft survival was still significantly delayed in DST-treated and anti–TGF-β treated recipients (18.8±5.8 d) as compared with untreated recipients (6.4±1.7 d, $P < 0.05$). These results indicate that endogenously produced TGF-β1 largely explains the tolerance induction observed after DST treatment.

Overexpression of TGF-β1 in heart allograft prolongs graft survival in unmodified recipients. Our results suggest that the DST effect is dependent on an early overexpression of TGF-β1 in allograft. To analyze whether overexpression of active TGF-β1 in heart allograft could induce prolongation of survival in unmodified recipients, we used an adenovirus-based gene delivery system. Heart allografts were injected in four different points with 10^{10} pfu of TGF-β1 or Lac-Z encoding adenovirus just after transplantation and allograft survival was assessed by abdominal palpation. This procedure has been shown to result in accumulation of virus-derived mRNA for TGF-β1 or β-galactosidase protein at least from day 2 after gene transfer (Anegon, I., manuscript in preparation). A more diffuse and almost complete organ transduction has been described recently using coronary adenoviruses in rabbit (37). However, as shown in Fig. 6, the overexpression of active TGF-β1 achieved using intramuscular injection was sufficient to induce a significant prolongation of allograft survival in five out of six unmodified animals, whereas Lac-Z encoding adenovirus had no effect. This strongly suggests that TGF-β1 could be a key cytokine involved in the suppression of allograft rejection in this model.

Discussion

This study indicates that endogenously produced TGF-β1 is a critical cytokine involved in the suppression of allograft rejection induced by DST pretreatment in adult rats. Unlike in control untreated recipients, heart allografts in DST-treated rats are rapidly infiltrated by recipient leukocytes that transiently produce high levels of TGF-β1. Strikingly, the major difference between our two experimental groups is that, in rejected allografts, proinflammatory cytokines (IL-2, IFN-γ, TNF-α)

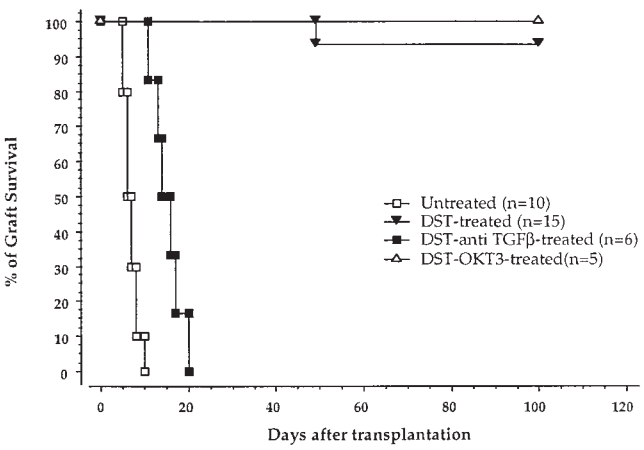

Figure 5. In vivo neutralization of TGF-β restores allograft rejection in DST-treated recipient rats. DST-treated heart allograft recipients received daily intraperitoneal injection of the neutralizing anti–TGF-β mAb 2G7 or an isotype-matched control mAb (OKT3), 1 mg per injection, from day 0 to day +4 after the transplantation. Allograft survival was assessed by daily abdominal palpation.

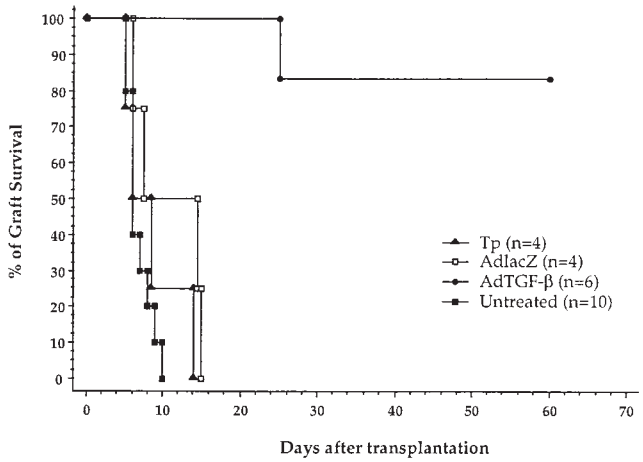

Figure 6. Overexpression of active TGF-β1 in heart allograft prolongs survival in unmodified recipients. Heart allografts were momentarily clamped and injected into the heart apex at four different points with 250 ml of Ringer's solution containing 10^{10} pfu of Ad coding for either an active form of TGF-β1 (*AdTGF-β*) or Lac-Z (*AdlacZ*) or comparable volume of virus dialysis buffer (*Tp*). Heart clamps were released after 30 s and allograft survival was assessed by daily abdominal palpation.

and to a lesser extent Th2-related cytokines (IL-4, IL-13) are also strongly upregulated after grafting, whereas TGF-β1 is, to date and despite a heavy leukocyte infiltration, the only cytokine that we have found upregulated in heart grafts from DST-treated rats (19, 38). Induction of tolerance by DST is abrogated by an anti–TGF-β mAb, suggesting that it is the early production of TGF-β after grafting which is responsible for the suppression of acute allograft rejection. The effect of DST could be reproduced in unmodified recipients by early overexpression of active TGF-β1 in allografts using recombinant adenoviruses.

TGF-β can be produced by a large variety of cells, including T cells and macrophages (34). As previously described by others (39, 40), TGF-β1 is expressed during acute allograft rejection, but TGF-β1–producing leukocytes infiltrate rejected grafts more slowly than in DST-treated rats. A previous study has shown that TGF-β–producing cells in rejected grafts are mainly infiltrating macrophages (39). A role for TGF-β in chronic allograft rejection has also been invoked because of its activity in increasing the secretion of extracellular matrix protein leading to fibrosis, a common feature of this pathology (41, 42). We believe that there is no relationship between overexpression of TGF-β induced by DST and chronic rejection in our model for the following reasons. First, heart graft in DST-treated rats survived $>$ 1 yr with no evidence of chronic rejection (data not shown). Second, overexpression of TGF-β1 in allografts from DST-treated recipients was only observed during the first week after transplantation. Third, previous studies have shown that DST have not only short-term but also long-term beneficial effects in human renal allografts (43). This also suggests that although the induction phase of allograft tolerance is TGF-β–dependent in our model, the maintenance phase could be related to TGF-β–independent mechanisms.

The early expression of TGF-β1 mRNA transcription in surviving allografts correlated with a strong expression of active TGF-β1 protein and is likely related to the rapid infiltration by a small number of cells producing high amounts of TGF-β1. These early infiltrating leukocytes are virtually absent from rejected grafts. An attempt was made to identify TGF-β1–producing GIC on heart graft sections from DST-treated recipients by confocal microscopy. However, the diffuse and extracellular staining pattern observed with the mAb recognizing the active form of TGF-β1 did not allow us to identify individual TGF-β1 producing cells clearly (data not shown). Interestingly, heart or kidney allografts are known to be more rapidly infiltrated by recipient leukocytes in DST-treated than in untreated rats (19, 20), suggesting that DST induces regulatory cells which are rapidly recruited in allograft. The present study strongly suggests that these regulatory cells, presumably T cells, produced high amounts of TGF-β1 but not other cytokines (19) upon alloantigen recognition in allografts. This pattern is reminiscent of the Th3 cells recently described in a different model of oral tolerance (12). The fact that graft passenger leukocytes are required for the induction of tolerance by DST (44 and Josien, R., et al., manuscript submitted for publication) suggests that the DST-induced regulatory cells produced TGF-β after cognate interaction with these donor antigen-presenting cells.

We showed previously that DST specifically induced in the recipient the expansion of a $CD8^+$ T cell clone bearing a Vβ18-Dβ1-Jβ2.7 rearrangement (45). This clone was detected 7 d after the first DST in a recipient and was found in GIC as soon as day 1 after grafting. Moreover, induction of allograft tolerance is abrogated when recipients are depleted of $CD8^+$ cells before the DST and before the transplantation (Douillard, P., manuscript in preparation) suggesting that DST-induced regulatory $CD8^+$ T cells are responsible for tolerance. Experiments are ongoing to determine whether this $CD8^+$ T cell clone produces TGF-β1 upon alloantigen recognition. TGF-β–producing regulatory T cells can play an important role in the suppression of several Th-1–mediated autoimmune or inflammatory diseases (46). For instance, oral administration of myelin basic protein in rodents can induce regulatory $CD4^+$ or $CD8^+$ T cells able to protect against the induction of experimental allergic encephalomyelitis via a TGF-β–dependent mechanism (12). The nonspecific suppression of T cell proliferation that we observed in spleen from DST-treated recipients and which is at least in part mediated by TGF-β is reminiscent of the TGF-β–dependent bystander suppression observed after induction of oral tolerance (47).

Although other regulatory cytokines could play a role in this model, neutralization of TGF-β alone was sufficient to abrogate induction of allograft tolerance. Moreover, adenovirus-mediated overexpression of TGF-β1 but not of IL-4 or IL-10 (Anegon, I., unpublished observation) in heart allograft was able to prolong allograft survival in nontransfused recipients. However, allograft survival in recipients treated with both DST and anti–TGF-β is still significantly delayed as compared with untreated recipients. This may be due to incomplete in vivo neutralization of TGF-β, or to additional TGF-β–independent mechanisms involved in this model of tolerance. The fact that only the combination of anti–TGF-β mAb and rIL-2 restored the antidonor proliferative response of splenic T cells from DST-treated supports this last hypothesis and suggests that T cells are also unable to produce sufficient amounts of IL-2 when stimulated with alloantigens as shown previously in a similar model (48).

In conclusion, we have identified endogenously produced TGF-β1 as a critical regulatory cytokine involved in the suppression of allograft rejection induced by DST. These results suggest that the induction phase of DST-induced allograft tolerance is an active process controlled by TGF-β–producing regulatory cells. They also provide new insights into the understanding of immunological mechanisms involved in the enhancing effect of pregraft blood transfusion on human allograft (49).

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