Transforming Growth Factor Beta-Induced Connective Tissue Growth Factor and Chronic Allograft Rejection[†]

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Late loss of allograft function is primarily attributed to chronic rejection (CR). There are no effective treatments for CR and the underlying cause of the disease is unknown. This study compared events that occurred within cardiac allografts placed in mice that received either anti-CD4 therapy and develop CR or anti-CD40L therapy and do not develop CR. Both TGFβ and connective tissue growth factor (CTGF), which is induced by TGFβ, were expressed in grafts with CR but were not expressed in grafts without CR. TGFβ transfection of allografts in anti-CD40L-treated recipients resulted in CTGF expression and CR. However, TGFβ transfection of syngeneic grafts did not result in CTGF expression or CR. These data indicate that TGFβ alone is insufficient to induce CR and that CTGF is required. Further, antigenic stimulation is required for TGFβ induction of CTGF. Thus, CTGF may serve as a therapeutic target for CR.

Key words: Chronic rejection, connective tissue growth factor, fibrosis, transforming growth factor beta

Introduction

Chronic rejection (CR) is the leading cause of late allograft loss, and is prevalent in cardiac, lung, renal and to

[†]This manuscript is dedicated to Dr. Charley Orosz, who passed away on August 7, 2005.

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a lesser degree, liver transplantation (reviewed in 1). CR is a progressive, irreversible disease that is characterized by deteriorating graft function, interstitial fibrosis and the occlusion of lumenal structures such as arteries and epithelial-lined conduits (reviewed in 2–4). In cardiac and renal allografts, subendothelial tissue develops in arteries forming an occlusive neointima, which is referred to as transplant-associated vasculopathy (TAV). Hence, TAV and interstitial fibrosis are commonly viewed as surrogate markers for CR. These pathologic changes of CR are distinct from those observed during acute rejection, where inflammatory cell infiltration is associated with parenchymal cell death. Rather, these changes resemble chronic tissue remodeling and/or wound repair processes that follow tissue injury (3). Despite intense investigation, the etiology of CR and associated pathologies are very poorly understood. While both alloantigen-dependent (i.e. MHC disparity, No. of acute rejection episodes) and -independent (i.e. ischemia/reperfusion injury) factors contribute to the disease process (reviewed in 5,6), reliable therapeutic targets for prevention and treatment of CR have not been identified. As summed up by Tilney and colleagues (5), 'No tests can predict the development of the process and no drugs can control or reverse it'.

TGFβ has been implicated in a number of fibrotic diseases (7–10) including CR (11). TGFβ also has numerous immunosuppressive activities that are viewed as beneficial in the settings of inflammation and transplantation (reviewed in 12). Further, TGFβ acts as a tumor suppressor for several cell types, and interference with TGFβ receptor signaling may lead to cancer of epithelial cell origin (13). Hence, long-term inhibition of TGFβ as a treatment for CR could have detrimental consequences.

CTGF is an immediate early response gene product that is induced by TGFβ (reviewed in 14,15). TGFβ, but not PDGF, FGF or EGF, has been shown to induce CTGF production from a variety of cell types, utilizing a unique TGFβ response element that is not associated with other TGFβ-regulated promoters (16). CTGF mediates many of the fibrogenic activities of TGFβ, but not its antimitogenic activity on epithelial cells (8,14,17). CTGF is mitogenic for fibroblasts and induces the production of collagen and other extracellular matrix proteins from fibroblasts and mesangial cells. While CTGF has been implicated in a variety of fibrotic diseases (18–27), CTGF has received little attention in the setting of transplantation. Hence, the current study

investigated the relationship between TGFβ-induced CTGF and CR. Our observations suggest that CTGF may provide a target for preventing CR while sparing the anti-inflammatory and anti-proliferative activities of TGFβ.

Materials and Methods

Mice

Female C57BL/6 (H-2^b) mice and BALB/c (H-2^d) mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed under specific pathogen-free conditions in the Unit for Laboratory Animal Medicine at the University of Michigan. Mice were used between 6–12 weeks of age.

Heterotopic cardiac transplantation

C57BL/6 mice were transplanted with intact BALB/c cardiac allografts, as described (28). In this model, the donor heart is anastomosed to the great vessels of the abdomen, perfused with recipient mouse's blood and resumes contraction. Transplant function was monitored by abdominal palpation.

Assessment of chronic rejection

Functioning allografts were recovered at the indicated times post-transplantation, fixed in formalin and embedded in paraffin. Sections were stained with H&E to assess myocyte viability (i.e. presence of nuclei and cross striation). As described (29), trichrome and elastin stains were used to identify collagen deposition and the presence of neointima, respectively.

Anti-CD4 and anti-CD40L therapies to prolong allograft survival

Anti-CD4 (hybridoma GK1.5, obtained from American Type Culture Collection, Manassas, VA) and anti-CD40L (hybridoma MR1, kindly provided by Dr. Randy Noelle, Dartmouth) mAb were purified and resuspended in PBS by Ligocyte Pharmaceuticals (Bozeman, MT). To transiently deplete CD4+ cells, allograft recipients were injected i.p. with 1 mg of anti-CD4 mAb on

days −1, 0 and 7 relative to transplantation (29–31). CD4+ cells begin to repopulate the periphery between 3 and 4 weeks post-transplant. For inductive anti-CD40L therapy, allograft recipients were injected i.p. with 1 mg of anti-CD40L on days 0, 1 and 2 relative to transplantation (31,32).

Adenoviral-mediated transfection of cardiac allografts

As described (33,34), cardiac allografts were transfected by perfusion via the aorta with E1/E3 deleted adenoviral vectors (5×10^8 pfu) encoding the active form of human TGFβ1 (Ad-TGFβ) or beta-galactosidase (Ad-βgal). Following perfusion, donor grafts were recovered and placed in iced Ringer's for approximately 1 h prior to transplantation. Reporter gene studies with Ad-βgal have revealed that the distribution of transgene expression within the cardiac graft is patchy, and that both cardiac myocytes and cells of the vasculature express the transgene product (33).

RNA isolation and RT-PCR

Cardiac allografts were homogenized in 1 ml TRIzol® (Invitrogen Life Technologies, Carlsbad, CA) and RNA was isolated as per manufacturer's protocol. Two μg of total RNA were reverse transcribed using a cDNA Cycle® Kit (Invitrogen Life Technologies) using oligo dT primers and AMV reverse transcriptase to generate cDNA. Human TGFβ1 (hTGFβ) primers are specific for hTGFβ and do not amplify mouse TGFβ (mTGFβ) (34). Primer sequences: hTGFβ sense 5′ GTGGAAACCCACAACGAA 3′, anti-sense 5′ GGCGGCCG-GTAGTGAAC 3′; mTGFβ sense 5′ TGGCTTCTAGTGCTGACG 3′, anti-sense 5′ GTTGCTCCACACTTGAT 3′; CTGF sense 5′ ATCCCTGCGACCCACACAAG 3′, anti-sense 5′ CAACTGCTTTGGAAGGACTCGC 3′; γ actin sense 5′ CCA-CACAGAGTACTTGCGCTCAGG 3′, anti-sense 5′ CACCCTGTGCTGCTCAC-CGAGGCC 3′. Samples were amplified using AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT) in a GeneAmp® PCR System 9700 (Applied Biosystems Inc, Foster City, CA).

Quantitative RT-PCR

CTGF primers are listed above. Collagen (pro-collagen 1a) sense 5′ TCCCTACTCAGCCGTCTGTGCC 3′, anti-sense 5′ AGCCCTCGCTTCCG-TACTCG 3′; GAPDH sense 5′ CTGGTGCTGAGTATGTCGTG 3′, anti-sense 5′
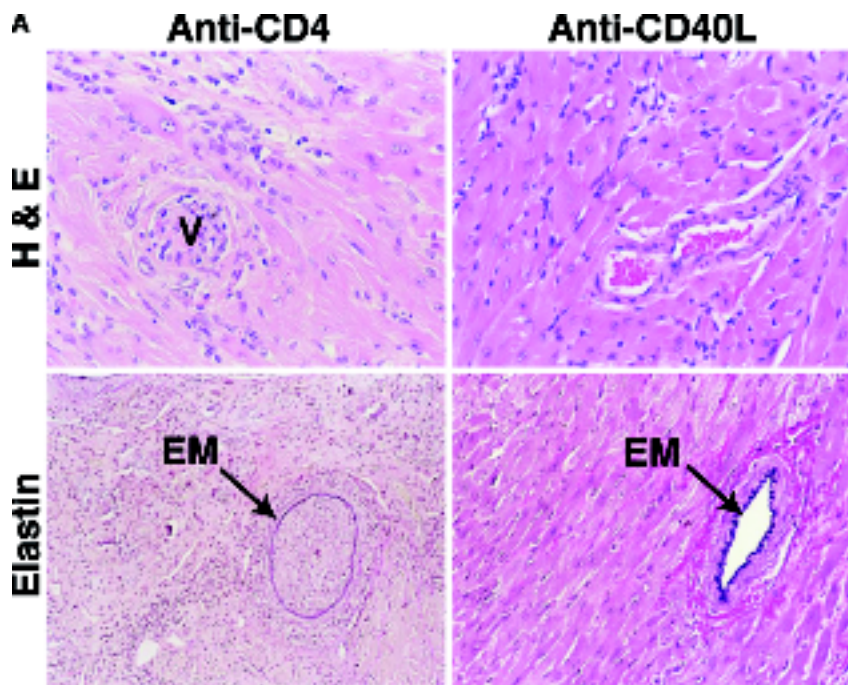

Figure 1. TGFβ and CTGF expression segregate with CR. C57BL/6 mice were transplanted with BALB/c cardiac allografts and were given inductive anti-CD4 or anti-CD40L therapy. Functioning allografts were recovered on day 60 post-transplant. **Panel A** represents a histologic assessment of CR. V = occluded vessel, EM = elastic membrane. **Panel B** depicts RT-PCR on RNA samples from 4 individual transplants per group. For mTGFβ, data are representative of 10 of 10 transplants for the anti-CD4 group and 15 of 17 (2 were weakly positive) for the anti-CD40L group. For CTGF, data are representative of 14 of 17 (3 were negative) transplants for the anti-CD4 group and 17 of 20 (3 were weakly positive) for the anti-CD40L group.
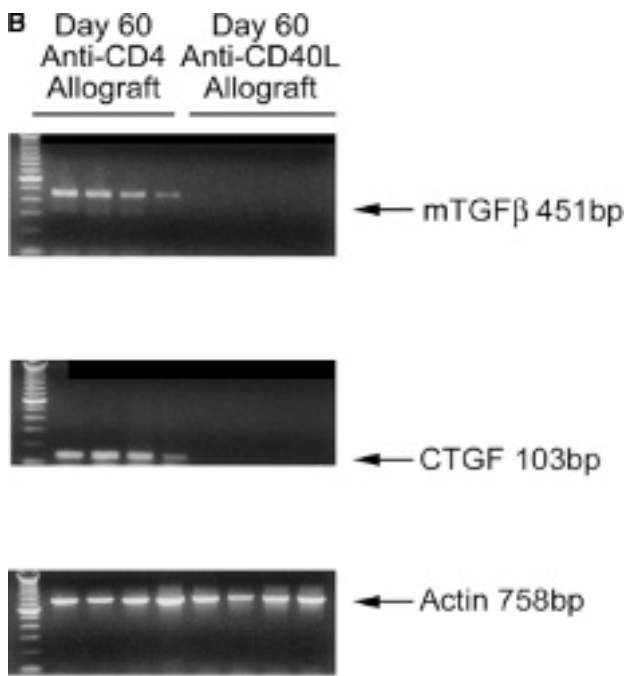
Figure 1. Continued.

CAGTCTTCTGAGTGGCAGTG 3'. Real-time PCR was performed on cDNA using a Cepheid Smart Cycler© System (Cepheid, Sunnyvale, CA) and primer binding to DNA was detected by SYBER Green I™ dye (Roche, Indianapolis, IN). Relative expression of CTGF and collagen is expressed as the exponent of the base 2 of the difference between the cycle threshold of CTGF or collagen versus GAPDH in transplant cDNA samples. Significance was determined with an unpaired t-test with Welch's correction.

Immunohistochemistry for CTGF
Allografts were paraffin embedded, sectioned, de-paraffinized and processed for antigen retrieval in citrate buffer (Biocare Medical, Concord, CA). Endogenous peroxidase was blocked using 0.3% H_2O_2/0.1% NaN_3 in dPBS for 10 min prior to standard immunohistochemistry using affinity-purified rabbit anti-CTGF (35). Biotinylated goat anti-rabbit secondary Ab (1:150, BioSource International, Camarillo, CA) was added for 30 min, followed by incubation with a 1:500 dilution of SA-HRP (BioSource) in PBS for 30 min. Sections were developed with AEC (Moss, Pasadena, CA) and counterstained with Harris hematoxylin (Fischer Scientific, Hampton, NH).

Results and Discussion

Development of CR following inductive anti-CD4 vs. anti-CD40L therapy
We have previously reported that inductive therapy with either anti-CD4 (30,31) or anti-CD40L (31,32) mAb markedly prolongs cardiac allograft survival. However, at 60 days post-transplant functioning allografts in anti-CD4-treated recipients develop TAV and exhibit interstitial collagen deposition (29), while allografts in anti-CD40L-treated recipients do not (32) (Figure 1A).

Expression of TGFβ and CTGF is associated with CR
Since TGFβ has been associated with CR (reviewed in 11), we assessed intragraft expression of TGFβ on day 60 post-transplant (Figure 1B). TGFβ mRNA was readily detectable in the allografts of anti-CD4-treated mice but was absent in the anti-CD40L- treated group. This pattern of TGFβ expression was observed in at least 10 individual transplants per group. Since CTGF is induced by TGFβ (16) and is reported to mediate the fibrotic activity of TGFβ (8,14), we assessed CTGF expression in long-term allografts (Figure 1B). CTGF expression paralleled that of TGFβ in at least 10 individual transplants per group. Thus, expression of TGFβ and CTGF segregated with the development of CR.

Allograft transfection with TGFβ results in CTGF expression and CR in anti-CD40L-treated recipients
To further explore the relationship between TGFβ-induced CTGF and CR, we transfected allografts with the active form of hTGFβ1 or βgal and transplanted them into recipients that were treated with anti-CD40L mAb. Forced expression of active hTGFβ, but not βgal, induced intragraft expression of CTGF (Figure 2A) and resulted in the development of CR (Figure 2B) in anti-CD40L-treated recipients. This pattern of hTGFβ and CTGF expression, and CR was observed in at least 20 transplants per group.

Since TGFβ induces CTGF in a variety of cell types (16), we employed immunohistochemistry to localize CTGF in hTGFβ-transfected allografts (Figure 2C). CTGF protein was readily identified in vascular endothelial cells and cells-infiltrating vessels with TAV in hTGFβ-transfected allografts, but not in uninvolved vessels in βgal-transfected grafts.

The studies above were performed between days 50 and 60 post-transplantation. We have also assessed TGFβ-induced CTGF expression at earlier time points following transplantation of Ad-TGFβ-transfected allografts into anti-CD40L-treated recipients. Intragraft expression of CTGF was readily detectable as early as day 7 post-transplantation of hTGFβ-transfected allografts. Further, fibrosis and TAV were evident by day 30 in these hTGFβ-transfected allografts (data not shown).

TGFβ transfection does not induce CTGF expression and CR in syngeneic cardiac grafts
It is well established that syngeneic grafts do not develop CR rejection to the extent that allografts do (1,5), thereby supporting a critical role for the immune system in the progression of CR. What is less clear is what role the immune system might play in TGFβ induction of CTGF and the subsequent development of CR. To this end, we assessed the impact of intragraft expression of active hTGFβ and CTGF induction in syngeneic grafts. As expected, transgene expression was readily detected in Ad-TGFβ-transfected syngeneic grafts (Figure 3A). However, CTGF was either weakly expressed or was not detectable in these
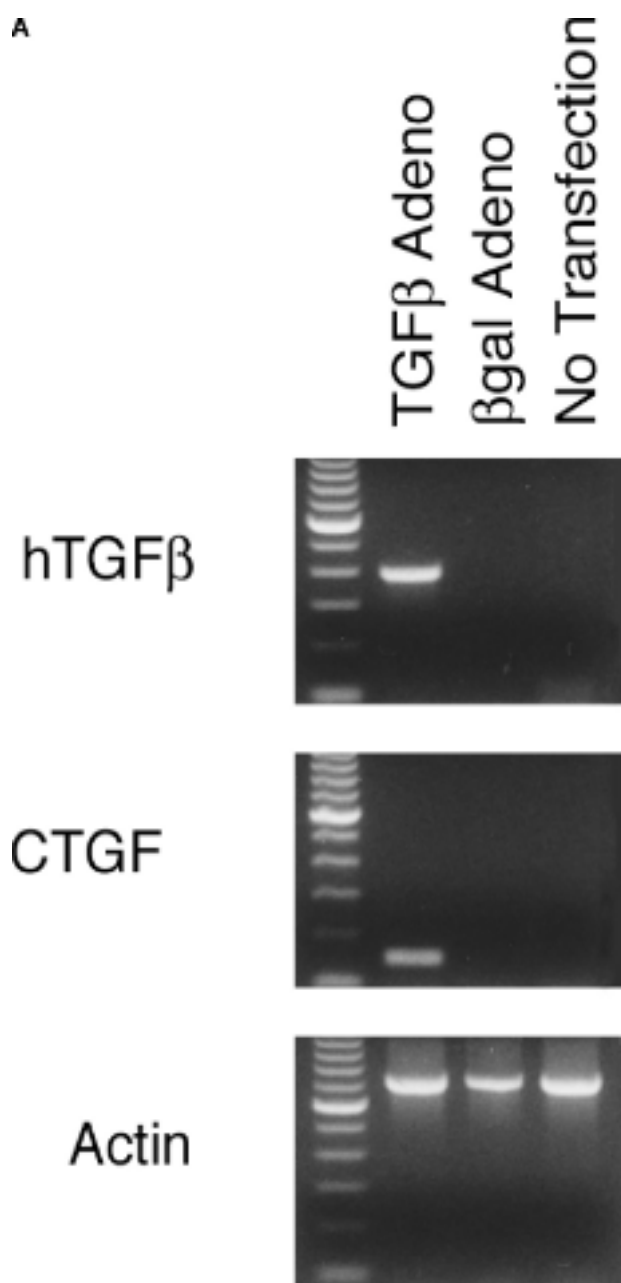
A

Figure 2. Forced expression of TGFβ induces CTGF and CR anti-CD40L-treated allograft recipients. BALB/c allografts were transfected with Ad-TGFβ or Ad-βgal and transplanted into anti-CD40L-treated C57BL/6 recipients. Functioning allografts were recovered between days 50 and 60 post-transplant. **Panel A** depicts RT-PCR for the hTGFβ transgene-induced CTGF, while **Panel B** depicts the histologic assessment of CR (200X). Data in Panels A and B are representative of >20 individual transplants for each group. **Panel C** depicts immunohistochemical localization of CTGF in TGFβ-transfected allografts and the data are representative of 12 individual transplants for the Ad-TGFβ group and 6 transplants for the Ad-βgal group.

TGFβ-transfected syngeneic grafts. To validate differences in the levels of CTGF expression in TGFβ-transfected syngeneic and allogeneic transplants, we performed real-time PCR for CTGF in these grafts (Figure 3B, left panel). Significantly less CTGF was expressed in TGFβ-transfected syngeneic transplants relative to their allogeneic counterparts. We also assessed the level of collagen type I expression in these tissues, since CTGF is known to induce collagen production (8,14) and collagen deposition is a hallmark of CR (2,3). As was the case with CTGF expression, collagen expression was significantly reduced in TGFβ-transfected syngeneic grafts when compared to allografts (Figure 3B, right panel). Importantly, the failure of active TGFβ expression to induce CTGF and subsequent collagen expression correlated with the absence of histologically defined CR in syngeneic grafts (Figure 3C). It is possible that the hTGFβ transgene is expressed at lower levels in syngeneic grafts when compared to allografts, thereby resulting in less CTFG and collagen expression. The primers we use to specifically amplify hTGFβ were designed for standard RT-PCR and are not suited for quantitative RT-PCR. Hence, we were unable to quantitatively compare expression levels of the hTGFβ transgene in syngeneic and allogeneic grafts. However, it should be noted that equally intense bands for hTGFβ were observed in Figure 2A for allografts and Figure 3A for syngeneic grafts as determined by standard RT-PCR. Further, we have reported that similar levels of βgal are expressed in Ad-βgal-transfected syngeneic and allogeneic grafts as visually determined by X-gal staining (33). Hence, we believe that the differences in CTGF and collagen expression in Ad-TGFβ-transfected syngeneic and allogeneic grafts are not likely due to differential expression of the hTGFβ transgene.

While TGFβ has been implicated in CR (11), the mechanism by which this pleiotropic cytokine contributes to the disease process has not been defined. This study reveals a strict correlation between TGFβ-induced CTGF and the development of CR. Indeed, the expression of active TGFβ in the absence of CTGF was not sufficient to drive CR (Figure 3). This is in keeping with the observation of Mori et al. (36), who reported that both TGFβ and CTGF were required to induce chronic fibrosis when injected subcutaneously. While TGFβ is the principal inducer of CTGF (8,14,15), thrombin (37,38) and hypoxia (39) have also been shown to induce CTGF. It is conceivable that allografts may be exposed to thrombin and hypoxia, which may lead to further CTGF production. It should be noted that TNFα (40), IL-4 (41), and prostaglandins and prostacyclins (42,43) antagonize CTGF. Hence, CTGF may be therapeutically inhibited for the treatment of fibrotic diseases (44), including CR. This approach would spare the anti-inflammatory activities of TGFβ, which are believed to be beneficial in the context of transplantation.

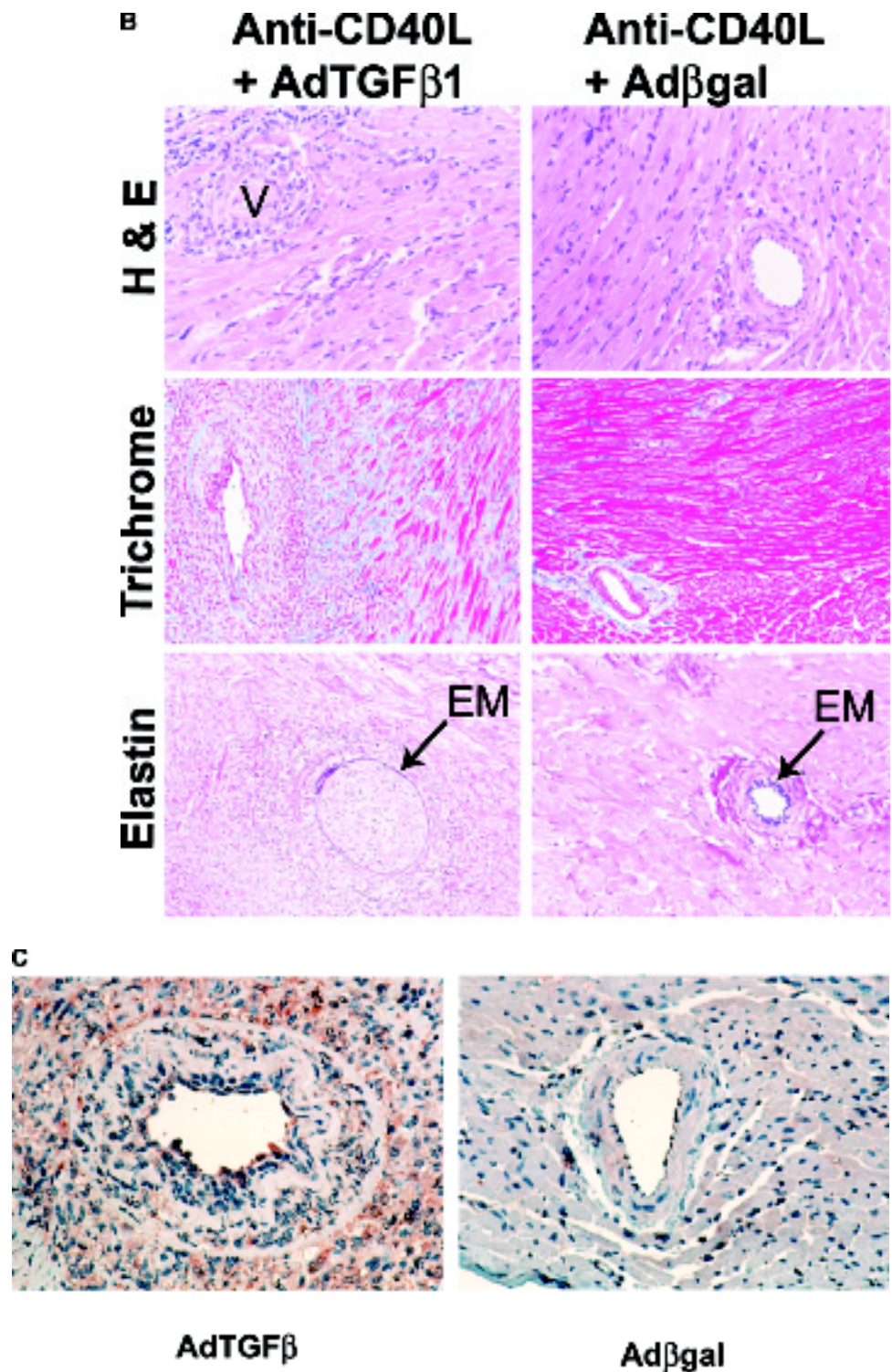The observations that TGFβ-transfected syngeneic grafts failed to express CTGF and develop CR (Figure 3) suggest

Figure 2. Continued.

that a component of the immune system is necessary to drive CTGF production in this system. It should be noted that human $\gamma\delta$, but not $\alpha\beta$ T cells have been shown to produce CTGF in response to TGFβ and IL-15 (45). In addition, NK cells, which may respond to MHC disparate allo-grafts, have recently been shown to play a role in CR (46). Further, TGFβ-transfected allografts, but not syngeneic grafts, were infiltrated by mononuclear cells (Figures 2B vs. 3C), and these graft-infiltrating cells expressed CTGF (Figure 2C). The contribution of the immune response to
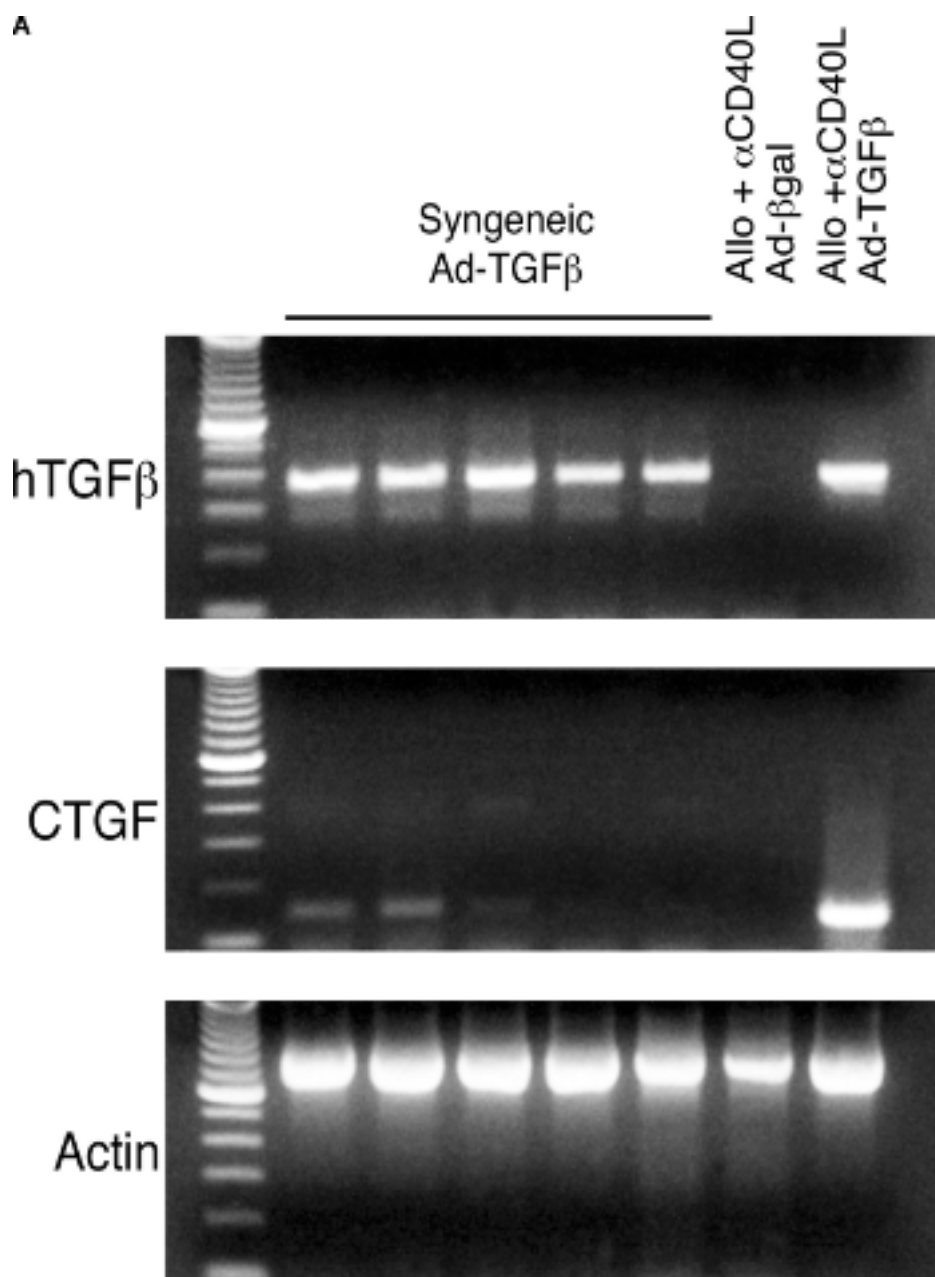
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Figure 3. TGFβ transfection of syngeneic grafts does not induce CTGF or CR. C57BL/6 grafts were transfected with Ad-TGFβ and transplanted into C57BL/6 recipients. Ad-TGFβ-transfected BALB/c allografts transplanted into anti-CD40L-treated C57BL/6 recipients served as positive controls. Functioning grafts were recovered between days 50 and 60 post-transplant. **Panel A** depicts RT-PCR results for 5 TGFβ-transfected syngeneic grafts. Note weak CTGF bands detected in 2 syngeneic grafts relative to the allograft control. **Panel B** depicts real-time RT-PCR data for CTGF (left panel) and collagen (right panel) in 5 individual transplants per group. **Panel C** depicts the absence of CR in TGFβ-transfected syngeneic grafts.

CR is well established (1,5). This study provides insight as to how elements of the immune response may do so.

Acknowledgment

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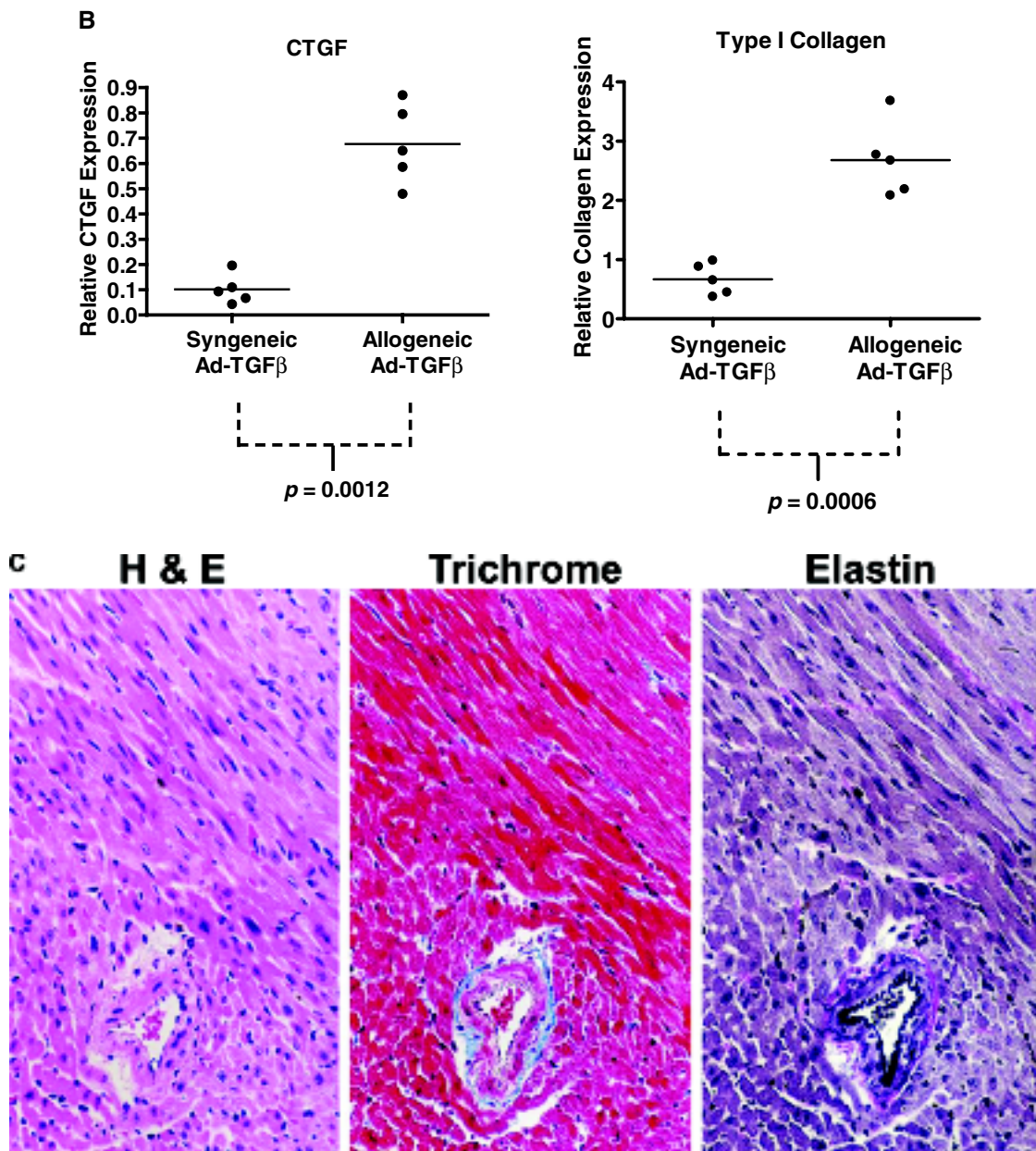
B

CTGF

Type I Collagen

p = 0.0012

p = 0.0006

C   **H & E**   **Trichrome**   **Elastin**

Figure 3. Continued.

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