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Connective Tissue Growth Factor Promotes Fibrosis Downstream of TGFβ and IL-6 in Chronic Cardiac Allograft Rejection

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Cardiac transplantation is an effective treatment for multiple types of heart failure refractive to therapy. Although immunosuppressive therapeutics have increased survival rates within the first year posttransplant, chronic rejection (CR) remains a significant barrier to long-term graft survival. Indicators of CR include patchy interstitial fibrosis, vascular occlusion and progressive loss of graft function. Multiple factors have been implicated in the onset and progression of CR, including TGFβ, IL-6 and connective tissue growth factor (CTGF). While associated with CR, the role of CTGF in CR and the factors necessary for CTGF induction *in vivo* are not understood. To this end, we utilized forced expression and neutralizing antibody approaches. Transduction of allografts with CTGF significantly increased fibrotic tissue development, though not to levels observed with TGFβ transduction. Further, intragraft CTGF expression was inhibited by IL-6 neutralization whereas TGFβ expression remained unchanged, indicating that IL-6 effects may potentiate TGFβ-mediated induction of CTGF. Finally, neutralizing CTGF significantly reduced graft fibrosis without reducing TGFβ and IL-6 expression levels. These findings indicate that CTGF functions as a downstream mediator of fibrosis in CR, and that CTGF neutralization may ameliorate fibrosis and hypertrophy associated with CR.

Key words: Chronic rejection, CTGF, fibrosis, IL-6, TGFβ

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Introduction

Chronic rejection (CR) is a significant barrier to long-term graft acceptance. Manifestations of CR include interstitial fibrosis, occlusion of luminal structures and progressive loss of graft function (1–7). The etiology of CR is not fully understood. However, multiple factors have been associated with its onset and progression, especially TGFβ. TGFβ overexpression is linked with CR (8,9), and may negatively impact graft survival through chemotactic and profibrotic effects (10). However, in addition to its deleterious fibrotic effects on the graft, TGFβ's immunosuppressive and antiproliferative functions may be indispensable for graft and host survival (11). For example, TGFβ plays a critical role in the induction and function of T regulatory cells (Treg), which are believed to contribute to graft acceptance (12–14). Further, TGFβ inhibits T- and B-cell proliferation (10) and represses cancers of epithelial cell origin (15). These opposing effects make TGFβ a suboptimal target for CR treatments and have prompted investigation into the downstream mediators of TGFβ in CR pathology. Identifying downstream mediators of CR may facilitate the development of therapeutics that negate the fibrosis-inducing activity of TGFβ while sparing its antiinflammatory and antiproliferative effects.

One such downstream mediator, known to be induced by TGFβ in multiple cell types (16), including cardiac myocytes and fibroblasts (17), is connective tissue growth factor (CTGF). CTGF plays an important role in the development of connective tissue as well as the formation of scar tissue (18,19), and is upregulated in multiple fibrotic disorders, including CR of cardiac and kidney grafts (8,20–22). CTGF mediates multiple profibrotic effects ascribed to TGFβ including increased extracellular matrix production, fibroblast proliferation and enhancement of adhesive responses (22). Thus, as CTGF is induced by TGFβ and because CTGF mediates profibrotic effects, CTGF has been proposed as a therapeutic target for limiting the deleterious fibrotic effects of TGFβ while sparing its immunemodulatory functions (8,23,24).

CTGF induction by TGFβ has been observed in settings of cardiac fibrosis (22). However, we have previously reported that transduction of syngeneic grafts with TGFβ is insufficient to induce CTGF or CR (8). Hence, TGFβ-mediated

induction of CTGF *in vivo* is contextually dependent. One such contextual difference between allogeneic and syngeneic grafts is the development of alloimmune responses which may provide factors that crosstalk with TGFβ signaling (25). This prompted further investigation into immune parameters that potentiated TGFβ-induced fibrosis and led to the identification of a critical role in the initiation and progression of CR for IL-6 (26), a cytokine that modulates the effects of TGFβ in multiple cell types (27–29).

Because TGFβ, CTGF and IL-6 have established associations with CR (8,26), we investigated the relationships between these cytokines utilizing overexpression and neutralization approaches. These findings support the role of CTGF as a promoter of cardiac graft fibrosis and indicate that it functions downstream of TGFβ and IL-6. Further, these findings indicate that CTGF neutralization holds promise as a therapeutic approach for limiting the fibrosis associated with CR.

Materials and Methods

Mice

Female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained from Charles River Laboratories (Raleigh, NC) and were kept under microisolator conditions. The use of mice for these studies was reviewed and approved by the University of Michigan's Committee on the Use and Care of Animals.

Vascularized cardiac transplantation

Heterotopic cardiac transplantation was performed as described (30). Briefly, the aorta and pulmonary artery of the donor heart were anastomosed end-to-side to the recipient's abdominal aorta and inferior vena cava, respectively. On perfusion with the recipient's blood, the transplanted heart resumes contraction. Graft function is monitored by abdominal palpation.

In vivo *mAb therapy*

Anti-CD4 (hybridoma GK1.5, obtained from American Type Culture Collection, Manassas, VA), anti-CD40L (hybridoma MR1, kindly provided by Dr. Randy Noelle, Dartmouth College) and anti-IL-6 (hybridoma MP5–20F3, obtained from American Type Culture Collection, Manassas VA, with permission of DNAX) mAbs were prepared by Bio X Cell (West Lebanon, NH). Allograft recipients were transiently depleted of CD4+ cells by i.p. injection of 1 mg of anti-CD4 mAb on days −1, 0 and 7 posttransplant (8,26). For inductive anti-CD40L therapy, allograft recipients were injected i.p. with 1 mg of anti-CD40L on days 0, 1 and 2 posttransplant (8,26). Anti-IL-6 mAb or control rat IgG (Sigma, St. Louis, MO) was administered by i.p. injection of 1 mg on days −1, 1 and 3 and weekly thereafter (26,31). Allograft recipients treated with anti-CTGF mAb (FG-3019, kindly provided by FibroGen Inc., San Francisco, CA (32,33)) or control human IgG (Sigma) received 0.5 mg i.p. twice weekly beginning on day 7 posttransplant.

Adenoviral-mediated transduction of cardiac grafts

Transduction was performed as previously described (8,34,35). Briefly, cardiac grafts were perfused via the aorta with 5×10^8 pfu of E1/E3 deleted adenoviral vectors encoding the active form of human TGFβ1 (AdTGFβ) (8,34), human CTGF (AdCTGF) (36) or beta-galactosidase (Adβgal) (8,34,35). Following perfusion, donor grafts were placed in iced Ringer's solution for 1 h prior to transplantation. Previous studies with Adβgal have revealed a patchy distribution of transgene expression by both cardiac and vascular cells that persists for at least 8 weeks posttransplant (35).

Morphometric analysis of cardiac graft fibrosis and hypertrophy

Graft fibrosis was quantified by morphometric analysis of Masson's trichrome-stained sections using iPLab software (Scanalytics Inc., Fairfax, VA). Mean fibrotic area was calculated from 10 to 12 areas per heart section analyzed at 200× magnification (26,37). To quantify cardiomyocyte area as a measure of hypertrophy, digital outlines were drawn around at least 80 cardiomyocytes from views of H&E-stained sections at 200× magnification. Areas within outlines were quantified using SCION IMAGE Beta 4.0.2 software (Scion Corporation, Frederick, MD) to measure cardiomyocyte cell size (38). A minimum of eight hearts were analyzed per group for both analysis techniques.

Quantitative real-time PCR

Graft RNA was isolated by homogenizing tissues in TRIzol reagent (Invitrogen, Carlsbad, CA) as per manufacturer's protocol. Five micrograms of total RNA were reverse transcribed using Oligo dT, dNTPs, MMLV-RT (Invitrogen), RNAsin (Promega, Madison, WI) in PCR buffer (Roche, Indianapolis, IN). Resulting cDNA was purified by a 1:1 extraction with phenol/chloroform/isoamyl (25:24:1) then precipitated in one volume 3 M NaOAc and two volumes absolute ethanol. Levels of atrial natriuretic peptide (ANP), CTGF, IL-6, TGFβ, IL-17 and T-cell receptor β constant region (TCRβ) message were determined by quantitative real-time PCR using iQ SYBR master mix (Bio-Rad, Hercules, CA) in a Rotor-Gene 3000 thermocycler (Corbett Life Science, San Francisco, CA). Expression levels were determined relative to GAPDH using the Rotor-Gene comparative concentration utility.

Primer sequences were as follows:

ANP (*Nppa*) forward 5′-GGAGGTCAACCCACCTCTG-3′

ANP (*Nppa*) reverse 5′-GCTCCAATCCTGTCAATCCTAC-3′

CTGF (*Ctgf*) forward 5′-GGAAAACATTAAGAAGGGCAAAA-3′

CTGF (*Ctgf*) reverse 5′-CCGCAGAACTTAGCCCTGTA-3′

GAPDH (*Gapdh*) forward 5′-CTGGTGCTGAGTATGTCGTG-3′

GAPDH (*Gapdh*) reverse 5′-CAGTCTTCTGAGTGGCAGTG-3′

IL-6 (*Il6*) forward 5′-CGTGGAAATGAGAAAAGAGTTGT-3′

IL-6 (*Il6*) reverse 5′-TCCAGTTTGGTAGCATCCATC-3′

TGFβ (*Tgfb 1*) forward 5′-CCTGAGTGGCTGTCTTTTGAC-3′

TGFβ (*Tgfb 1*) reverse 5′-CCTGTATTCCGTCTCCTTGGT-3′

IL-17 (*Il17a*) forward 5′-GGACTCTCCACCGCAATGA-3′

IL-17 (*Il17a*) reverse 5′-GACCAGGATCTCTTGCTGGA-3′

TCRβ (*Tcrb-C*) forward 5′-CTGCCAAGTGCAGTTCCAT-3′

TCRβ (*Tcrb-C*) reverse 5′-GGCCTCTGCACTGATGTTCT-3′

Flow cytometry

Splenocytes were labeled with FITC-conjugated anti-CD3, PE-conjugated anti-CD4 and CY5-conjugated anti-CD8 (PharMingen, San Jose, CA). Cell

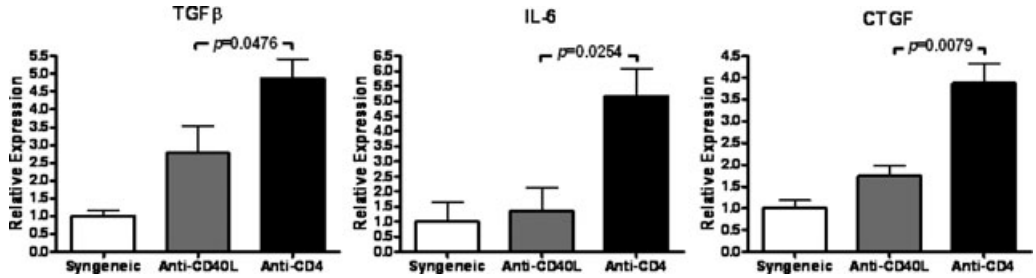

Figure 1: Elevated intragraft expression of TGFβ, IL-6 and connective tissue growth factor (CTGF) in cardiac allografts undergoing chronic rejection (CR). TGFβ, IL-6 and CTGF message levels were determined at day 30 posttransplant using quantitative real-time PCR in syngeneic cardiac grafts, cardiac allografts from recipients treated with anti-CD40L mAb therapy (Anti-CD40L) or cardiac allografts whose recipients were transiently depleted of CD4+ cells (Anti-CD4). Bars represent mean + S.E.M. of four to nine grafts with expression relative to GAPDH normalized to the syngeneic group.

analyses were performed on lymphocytes gated using forward vs. side scatter using a Becton Dickinson FACSCalibur (San Jose, CA).

Statistical analysis

Statistical significance was calculated using an unpaired *t*-test with Welch's correction. p-values ≤ 0.05 were considered statistically significant.

Results

Experimental system

BALB/c cardiac allografts in C57BL/6 recipients receiving anti-CD40L mAb continue to function for >60 days and do not develop CR, unless transduced with TGFβ (8). In contrast, allografts in recipients transiently depleted of CD4+ cells develop CR as CD4+ cells begin to repopulate the periphery between 3 and 4 weeks following initial depletion (8,39–41). Echocardiographic and histologic analysis revealed that day 30 posttransplant represents a critical point in this CR model as extensive graft hypertrophy and fibrosis are present at this time and are followed by degradation of cardiac contractility (26). Therefore, grafts were assessed at day 30 posttransplant in these studies. We have used these models to better understand the roles of TGFβ, IL-6 and CTGF in CR.

Elevated intragraft TGFβ, IL-6 and CTGF expression correlate with CR

Transduction of allografts, but not syngeneic grafts, with TGFβ is sufficient to induce CTGF and CR (8), indicating the involvement of an immune component in TGFβ-mediated fibrosis. This is further supported by our recent identification of IL-6 as a critical inducer of CR (26). Hence, the *in vivo* interactions of TGFβ, CTGF and IL-6 in CR were the focus of this study. TGFβ, CTGF and IL-6 transcripts were measured in grafts whose recipients were transiently depleted of CD4+ cells, which develop CR, and compared to allografts whose recipients were treated with anti-CD40L, which do not develop CR, or untreated syngeneic grafts. Intragraft levels of TGFβ, IL-6 and CTGF were significantly increased ($p = 0.0476$, 0.0254 and 0.0079, respectively) in cardiac allografts whose recipients were transiently depleted of CD4+ cells than in grafts whose recipients were treated with anti-CD40L or syngeneic controls (Figure 1). Thus, the upregulation of all three cytokines was observed in grafts undergoing CR.

Forced expression of CTGF or TGFβ promotes allograft fibrosis

To determine whether exogenous expression of CTGF promotes cardiac fibrosis, allografts and syngeneic grafts were transduced with AdCTGF. AdCTGF transduction of allografts in recipients treated with anti-CD40L caused a significant increase in fibrotic area by day 30 posttransplant compared to allografts transduced with control virus (Figure 2A). In contrast, syngeneic grafts transduced with AdCTGF had similar levels of fibrosis to controls. It should be noted that the mean fibrotic area for AdCTGF-transduced allografts was less than in hearts transduced with AdTGFβ, consistent with previous descriptions in lung transductions (42). This difference could not be accounted for by differences in transgene expression levels, as AdTGFβ and AdCTGF expression were comparable in these studies as determined by real-time PCR (data not shown). Thus, while forced expression of either TGFβ or CTGF promoted cardiac allograft fibrosis, they did so to different extents (Figure 2). This could in part be due to TGFβ induction of endogenous CTGF expression (8,17,43), thereby producing an additive effect.

It has been observed that TGFβ and CTGF are potently fibrotic in tandem while less fibrotic individually (44,45). Therefore, we asked whether cotransduction of both TGFβ and CTGF vectors would induce fibrosis and CR in syngeneic grafts. No increases in fibrosis were observed upon cotransduction of syngeneic grafts compared to single virus transduction (data not shown). Thus, while injection of TGFβ and CTGF synergize to cause fibrotic responses in the skin (45), forced expression of both was insufficient to induce fibrosis or CR in syngeneic cardiac grafts, further supporting the requirement of an immune component.

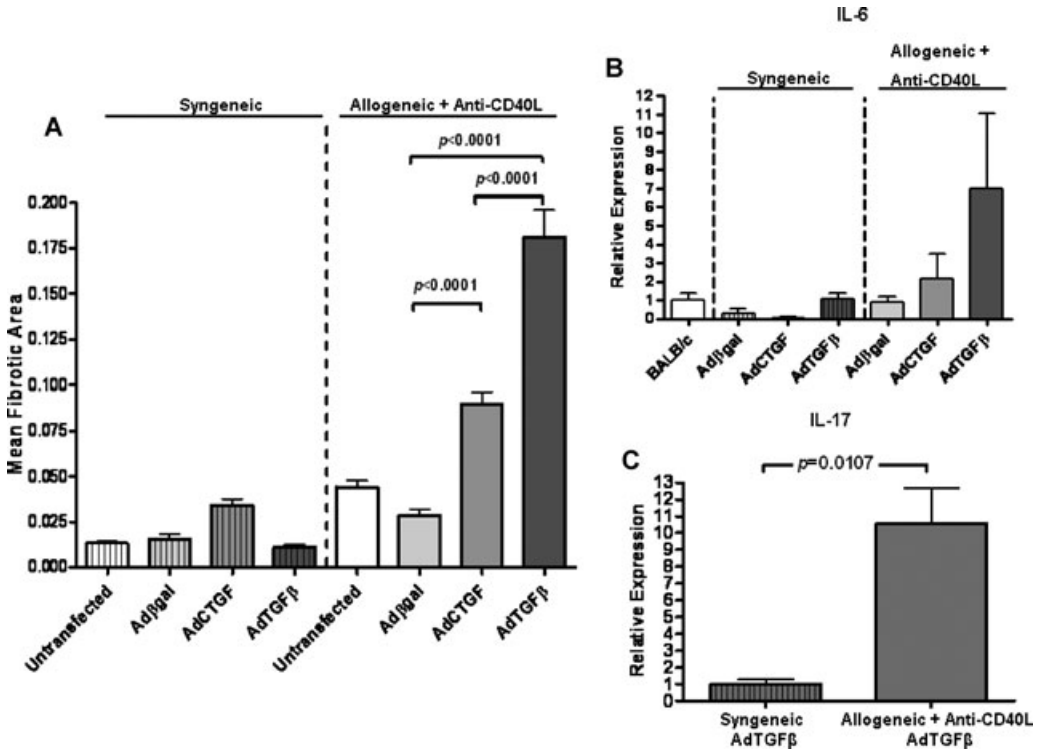

Figure 2: Forced expression of TGFβ or connective tissue growth factor (CTGF) promotes allograft fibrosis. (A) Morphometric analysis of Masson's trichrome staining at day 30 posttransplant in cardiac grafts that were left untransduced or transduced with adenoviral vectors encoding βgal (Adβgal), CTGF (AdCTGF) or TGFβ (AdTGFβ) before grafting into syngeneic recipients or allogeneic recipients treated with anti-CD40L. Bars represent the combined mean + S.E.M. of fibrotic (blue) area of 10–12 frames of view per heart taken from 5 to 12 different cardiac grafts per group. (B) Intragraft IL-6 message levels were determined at day 30 posttransplant using quantitative real-time PCR in groups from (A). Bars represent mean + S.E.M. of at least four hearts per group with expression relative to GAPDH normalized to naïve, untransplanted BALB/c hearts. (C) Intragraft IL-17 message levels were determined using quantitative real-time PCR in syngeneic grafts transduced with AdTGFβ or allogeneic grafts transduced with AdTGFβ whose recipients received anti-CD40L treatment. Bars represent mean + S.E.M. of at least five independent hearts per group with expression relative to GAPDH normalized to the naïve BALB/c group.

We next considered whether the greater fibrotic activity of AdTGFβ relative to AdCTGF could be due to immunologic effects. TGFβ is chemotactic for multiple immune cell types (10) that are able to produce IL-6, which we have recently reported to play a critical role in CR (26). Therefore, we asked whether differences in intragraft IL-6 expression might account for these disparate outcomes. IL-6 transcript levels exhibited a suggested increase in AdTGFβ, but not AdCTGF transduced allografts whose recipients received anti-CD40L therapy. No increases in IL-6 expression were observed in AdTGFβ or AdCTGF-transduced syngeneic grafts (Figure 2B).

TGFβ and IL-6 have been implicated in the development of Th17 responses (27), which have recently been linked to CR (46,47). Hence, we assessed the expression of IL-17 in allogeneic and syngeneic grafts transduced with AdTGFβ. IL-17 expression was significantly greater ($p = 0.0107$) in allografts than in syngeneic grafts (Figure 2C), whereas IL-17 expression was similar in allogeneic and syngeneic grafts transduced with AdCTGF (data not shown). Thus, increased IL-17 and CTGF transcript levels may promote fibrosis associated with AdTGFβ-transduced allografts, but not AdTGFβ-transduced syngeneic grafts that do not develop fibrosis.

IL-6 neutralization reduces intragraft CTGF and IL-17 transcripts

The association between TGFβ, IL-6 and CTGF (Figure 1) may be strengthened by previous reports that IL-6 enhances TGFβ signaling by altering receptor localization in the cell membrane (29) and that IL-6 can alter the outcome of TGFβ signaling (27,28). Indeed, we have previously reported that IL-6 neutralization prevents CR of cardiac allografts (26). We therefore asked whether IL-6 neutralization would inhibit CTGF or IL-17 expression (Figure 3). In allografts whose recipients were transiently depleted of CD4+ cells, treatment with anti-IL-6 mAb significantly reduced intragraft IL-6, IL-17 and CTGF expression ($p = 0.0216$, 0.0044 and 0.0180, respectively) compared to control antibody treatment. In contrast, TGFβ expression levels remained

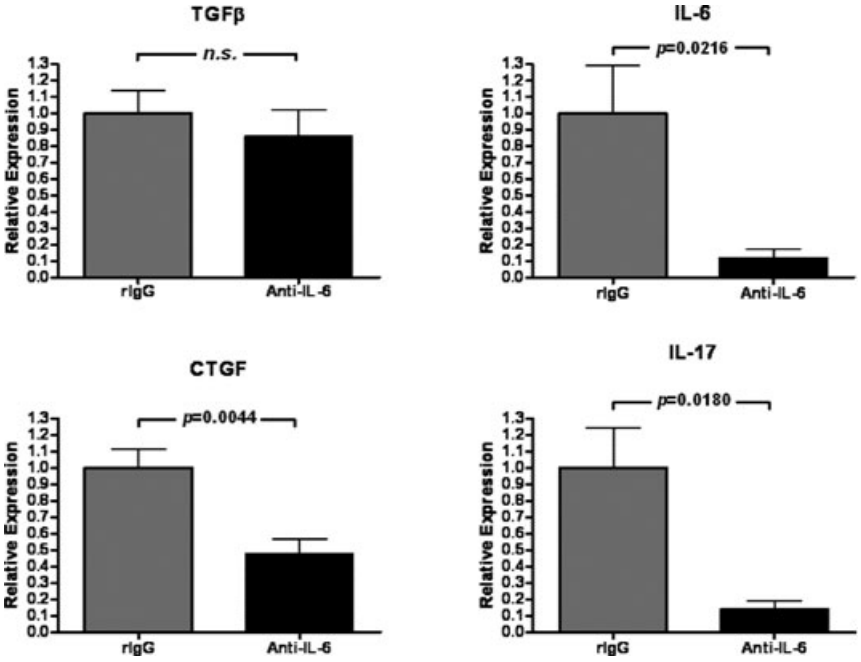

Figure 3: IL-6 neutralization reduces expression of IL-6, IL-17 and connective tissue growth factor (CTGF) but not TGFβ in cardiac allografts undergoing chronic rejection (CR). Intragraft IL-6, IL-17, CTGF and TGFβ message levels were determined at day 30 posttransplant using quantitative real-time PCR in cardiac allograft recipients that were transiently depleted of CD4+ cells and received either neutralizing anti-IL-6 (Anti-IL-6) or control rat IgG (rIgG). Bars represent mean + S.E.M. of six to eight grafts per group with expression relative to GAPDH normalized against rIgG-treated controls.

unchanged (Figure 3). Thus, IL-6 promotes intragraft IL-6, IL-17 and CTGF expression.

CTGF neutralization ameliorates allograft fibrosis

We next asked whether CTGF neutralization would inhibit the fibrosis associated with CR. To this end, we treated allograft recipients that were transiently depleted of CD4+ cells with neutralizing anti-CTGF mAb or control antibody. Treatment with anti-CTGF mAb resulted in significant reduction of fibrotic area ($p < 0.0001$, Figure 4A, B), but was not accompanied by reduction of intragraft TGFβ, CTGF or IL-6 transcripts (Figure 4C). These observations support a role for CTGF as a downstream mediator of fibrosis associated with CR.

CTGF neutralization decreases cardiomyocyte hypertrophy associated with CR

CTGF can induce cardiomyocyte hypertrophy (48,49), a function it shares with IL-6 (26). Because IL-6 neutralization inhibited CTGF expression (Figure 3), we assessed the effect of neutralizing CTGF on cardiomyocyte hypertrophy. Anti-CTGF treatment resulted in a significant decrease ($p < 0.0001$) in cardiomyocyte hypertrophy (Figure 5A) and significantly reduced ($p = 0.0102$) the intragraft expression of ANP (Figure 5B), a molecular marker of cardiac hypertrophy (50,51). For reference, cardiomyocyte area and ANP expression levels for naïve, untransplanted BALB/c hearts and allografts transplanted into recipients receiving anti-CD40L therapy are depicted.

CTGF neutralization inhibits T-cell infiltration of grafts

CTGF promotes integrin-mediated adhesive responses in multiple cell types (52–61) and induces the production of chemokines (62). We therefore asked whether CTGF neutralization might also alter the infiltration of immune cells into grafts undergoing CR. Histologic analysis indicated reduced cellular infiltrate in grafts receiving anti-CTGF (Figure 6A). Indeed, a significant decrease ($p = 0.0238$) in TCRβ constant region expression, a marker of graft-infiltrating T cells (63), was observed (Figure 6B). To verify that this difference was not due to CTGF neutralization preventing peripheral repopulation of CD4+ cells, we compared the percentage of CD4+ cells in anti-CTGF and control treated graft recipients. No significant differences were observed between these groups (Figure 6C).

Discussion

CR has been associated with multiple factors, perhaps most frequently with TGFβ (9). However, the role of TGFβ in CR is complicated by its pleiotropic activity encompassing immunosuppressive and antiproliferative effects in immune (10,64–66) and nonimmune (15,67) cells as well as the induction of Treg (68–70), which are associated with graft acceptance (12,13,24). Thus, TGFβ may promote graft survival and global immune tolerance while suppressing malignancy, making it ill-suited as a therapeutic target in the treatment of CR. This has prompted investigation into the downstream mediators of fibrotic TGFβ function (8,23).

Multiple reports indicate that TGFβ requires additional factors to drive fibrosis (8,44,45). Indeed, syngeneic grafts do not develop fibrosis in response to TGFβ, while allografts whose recipients receive anti-CD40L mAb develop marked fibrosis in response to TGFβ (Figure 2; (8)). Hence, alloimmune responses potentiate the profibrotic effects of TGFβ.

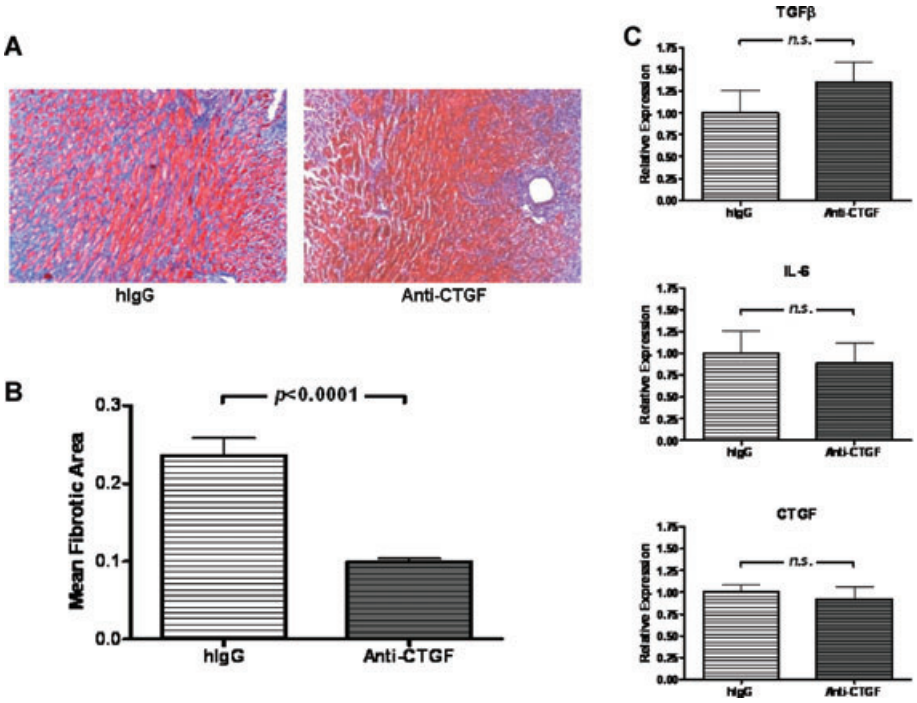

Figure 4: Connective tissue growth factor (CTGF) neutralization ameliorates fibrosis. (A) Representative sections of Masson's trichrome stains, in which fibrotic tissue stains blue, of cardiac allografts from recipients transiently depleted of CD4+ cells (Anti-CD4) at day 30 posttransplant in recipients treated with control IgG or neutralizing anti-CTGF mAb (200× magnification). (B) Morphometric analysis of trichrome staining of groups in (A). Bars represent mean + S.E.M. of 10–12 frames of view from each of six to nine hearts. (C) TGFβ, IL-6 and CTGF message levels were determined at day 30 posttransplant using quantitative real-time PCR in cardiac allografts described in (A). Bars represent mean + S.E.M. of samples taken from 8 to 12 different cardiac grafts with expression relative to GAPDH normalized against hIgG-treated controls.

We have reported a critical role for IL-6 in CR (26), whose elevated expression correlated with TGFβ and CTGF (Figure 1). Correlations of TGFβ with CTGF (8) and IL-6 (26) have previously been described. Further, we have previously observed CTGF expression associated with areas of graft-infiltrating mononuclear cells (8), whose recruitment during inflammatory responses has been linked to IL-6 (71,72). Therefore, we considered that there may be connectivity between all three cytokines.

To ascertain the sufficiency of TGFβ and CTGF to induce CR, allogeneic and syngeneic cardiac grafts were transduced with AdTGFβ or AdCTGF and transplanted into recipients receiving anti-CD40L mAb or syngeneic recipients. AdTGFβ and AdCTGF significantly increased mean fibrotic area compared to untransduced or control vector-treated allografts (Figure 2A). Consistent with a previous report of adenoviral transduction of lungs (42), the fibrotic response to TGFβ transduction in the heart was significantly greater than the response to CTGF transduction (Figure 2A). Greater fibrotic responses to AdTGFβ could be from synergy of TGFβ-induced immune factors and CTGF in cardiac allografts, an effect which is not observed in syngeneic grafts (8). Further, in cardiac allografts, TGFβ induction of endogenous CTGF may synergize with TGFβ-mediated chemotactic effects on multiple immune lineage cells (10), which may explain the suggested upregulation of IL-6 and significant upregulation of IL-17 (Figure 2).

Given the differences in AdTGFβ responses between allografts and syngeneic grafts and the correlation of TGFβ and CTGF with IL-6 in CR (Figure 1), we asked whether the presence of IL-6 was required for CTGF upregulation. In cardiac allograft recipients transiently depleted of CD4+ cells, IL-6 neutralization reduced the expression of IL-6 and CTGF without altering TGFβ transcript levels (Figure 3). This suggests that TGFβ transcript regulation lies upstream of IL-6 and CTGF in CR. It should be noted that IL-6 neutralization does not prevent repopulation of CD4+ cells in the periphery (26). This indicates that the ability of IL-6 neutralization to prevent CR (26) could function in part through reduction of intragraft CTGF. Further, IL-6 neutralization significantly inhibited IL-17 expression (Figure 3), indicating that IL-17 might play a role in CTGF induction, as IL-17 has been reported to induce collagen production in cardiac fibroblasts (73). Another explanation for this effect might be decreased recruitment of graft-infiltrating cells which may express or induce local cells to express CTGF (8), IL-6 and IL-17. Indeed, IL-6 induces chemotaxis and migration of immune cells (74,75).

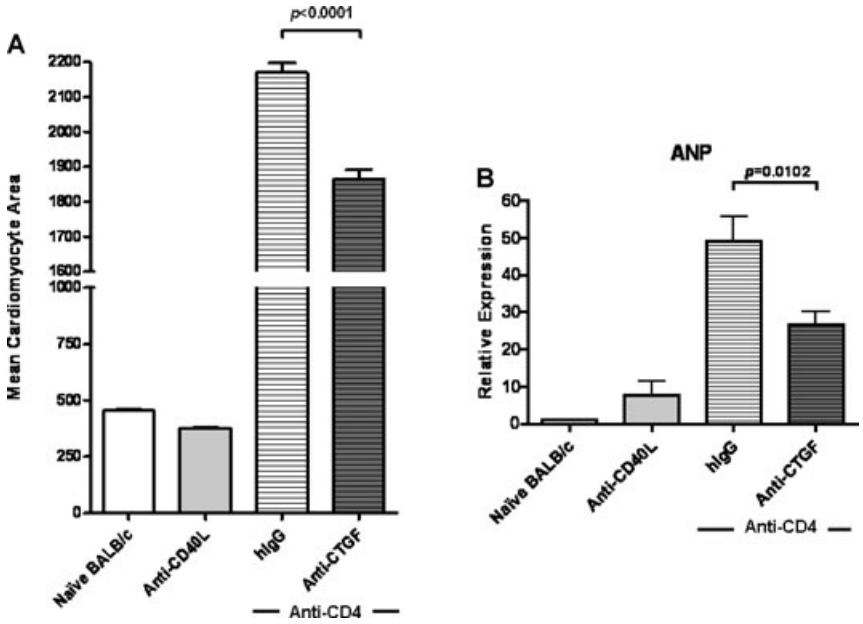

Figure 5: Connective tissue growth factor (CTGF) neutralization ameliorates cardiac hypertrophy in chronic rejection (CR) grafts. (A) Cardiomyocyte area was quantified from H&E stains of day 30 posttransplant cardiac allografts taken from recipients transiently depleted of CD4+ cells (Anti-CD4) and receiving CTGF-neutralizing mAb (Anti-CTGF) or control antibodies (hIgG), recipients treated with Anti-CD40L mAb, or naïve, untransplanted BALB/c hearts. Bars represent mean + S.E.M. of area measurements taken from ≥100 cardiomyocytes per heart from 5 (naïve BALB/c and Anti-CD40L), 8 (Anti-CD4 + hIgG) or 10 (Anti-CD4 + Anti-CTGF) different hearts per group. (B) Intragraft message levels of atrial natriuretic peptide (ANP), a marker of cardiac hypertrophy, were quantified with real-time PCR in cardiac grafts from groups in (A) at day 30 posttransplant. Bars represent mean + S.E.M. of 8–12 grafts per experimental group (Anti-CD4 + hIgG or Anti-CTGF) and four grafts per control group (Anti-CD40L and naïve BALB/c) with expression relative to GAPDH normalized against the naïve BALB/c hearts.

As IL-6 neutralization ameliorated CR (26) and decreased intragraft CTGF expression (Figure 3), we treated cardiac allograft recipients with neutralizing CTGF mAb. CTGF neutralization significantly reduced allograft fibrosis (Figure 4A, B) without significantly reducing intragraft TGFβ, IL-6 or CTGF expression (Figure 4C). These findings are consistent with CTGF being a downstream mediator of fibrosis in CR (16,23,42,76).

The significant but incomplete reduction in fibrotic area in response to CTGF neutralization may be explained by multiple factors. Our neutralization protocol, though effective, may not be optimal. Another possibility is the presence of CTGF-independent profibrotic effects of TGFβ and/or IL-6 (77). A further consideration is whether the mAb FG-3019, which recognizes CTGF module 2 in humans and rodents (33), might inhibit some but not all profibrotic effects of CTGF. However, this possibility seems unlikely in light of a recent report evaluating the antifibrotic efficacy of anti-CTGF antibodies directed against each of the four CTGF modules. In this report, only mAb directed against the von Willebrand factor type C domain (module 2) was able to inhibit TGFβ-induced fibrosis (78). Indeed, this is the same domain that the anti-CTGF mAb utilized in our study binds (32,33).

Beyond its roles in fibrosis, CTGF can exert other effects relevant to CR. Recent studies have described a concomitance of cardiomyocyte hypertrophy with CR (26,79,80). CTGF is produced by hypertrophic chondrocytes during development (81), and is produced by cardiac myocytes in response to hypertrophic stimuli (49). In addition, CTGF itself can induce cardiomyocyte hypertrophy (48). Treatment with neutralizing anti-CTGF mAb significantly reduced mean cardiomyocyte area (Figure 5A) and intragraft levels of ANP (Figure 5B), a marker of cardiac hypertrophy in multiple settings (26,50,51). However, it should be noted that anti-CTGF mAb did not inhibit cardiac hypertrophy to the extent previously observed with anti-IL-6 (26). This finding indicates that in addition to driving cardiac fibrosis, CTGF may augment cardiomyocyte hypertrophy associated with CR. Interestingly, hypertrophy is associated with downregulation of two recently discovered CTGF-inhibiting micro RNAs in cardiac myocytes (82). Thus, CTGF may be linked to cardiac hypertrophy on multiple levels.

Finally, as CTGF is known to play an important role in fibroblast adhesion in response to TGFβ (55,76), we asked whether CTGF might similarly influence recruitment of lymphocytes to the graft. Histologic assessment of infiltrating cells was indicative of reduced numbers of

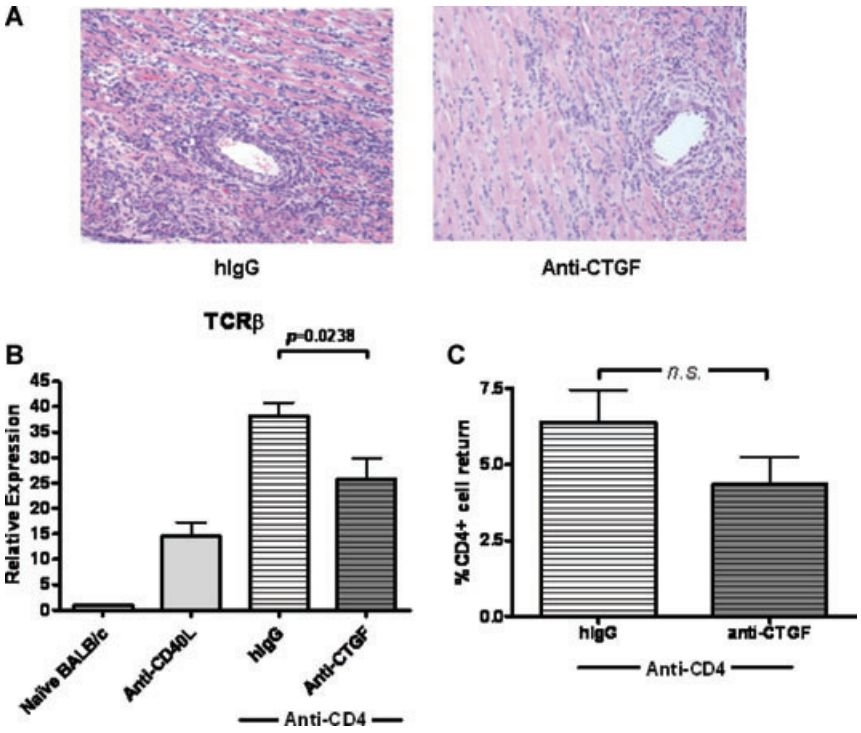

Figure 6: Connective tissue growth factor (CTGF) neutralization limits graft infiltration by T cells in chronic rejection (CR) grafts. (A) Representative H&E stains of day 30 posttransplant cardiac allografts taken from recipients transiently depleted of CD4+ cells (Anti-CD4) and receiving CTGF neutralizing mAb (Anti-CTGF) or control antibodies (hIgG). Stains suggest a reduction in perivascular infiltrate density in grafts treated with neutralizing Anti-CTGF. (B) Intragraft message levels of T-cell receptor β constant region (TCRβ) were quantified at day 30 posttransplant with real-time PCR as a measure of T-cell infiltration of allografts in recipients transiently depleted of CD4+ cells (Anti-CD4) and receiving anti-CTGF mAb or control hIgG antibodies, recipients treated with Anti-CD40L mAb, or naïve BALB/c hearts. Bars represent mean + S.E.M. of 8–12 grafts per group with expression relative to GAPDH normalized against the hIgG group. (C) Repopulation of CD4+ cells in the periphery at day 30 posttransplant was determined by flow cytometric analysis of splenocytes isolated from graft recipients. Bars represent mean + S.E.M. of the percentage CD4+ cells of the gated cell population in five to seven recipients tested.

graft-infiltrating lymphocytes (Figure 6A). This observation was further supported by significant reduction of intragraft TCRβ constant region expression (Figure 6B) in response to CTGF neutralization.

On the basis of these observations and others in the literature, we propose a model representing the interactions of TGFβ, IL-6 and CTGF and their induction of hypertrophy and fibrosis associated with CR

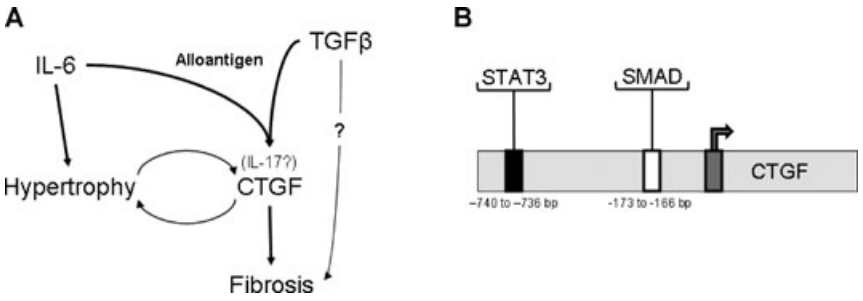

Figure 7: Proposed model of cytokine interactions in chronic rejection. (A) In cardiac allografts, TGFβ and IL-6 contribute to CTGF production. IL-6 and CTGF are both known to promote hypertrophy in cardiac myocytes, which in turn can produce CTGF. CTGF functions as a downstream mediator of fibrosis. (B) Induction of CTGF downstream of TGFβ and IL-6 could be explained by the respective presence of a consensus SMAD-binding element and a STAT3 response element in 5′ region upstream of the CTGF promoter. For expanded explanations, please see text.

(Figure 7A). In cardiac allografts that undergo CR, TGFβ (Figure 1 (8)) and IL-6 (Figures 1 and 2B (26)) are induced. In syngeneic grafts, forced expression of TGFβ is insufficient to upregulate CTGF and fibrosis (8), and IL-6 remains at basal levels (Figure 2B). IL-6 neutralization inhibits hypertrophy and fibrosis associated with CR (26), which may be in part through inhibition of CTGF and IL-17 expression whereas TGFβ expression remains unchanged (Figure 3). Thus, TGFβ and IL-6 appear to be cooperative upstream factors promoting CTGF expression and CR. CTGF neutralization limits fibrosis (Figure 4A, B) and cardiomyocyte hypertrophy (Figure 5), without altering intragraft TGFβ, IL-6 or CTGF transcripts (Figure 4C). These effects of CTGF neutralization coincide with reduction in graft-infiltrating T cells (Figure 6). Together, these observations support a downstream role for CTGF in fibrosis and hypertrophy.

Contexts in which TGFβ and IL-6 are present coincide with intragraft IL-17 expression, which has been implicated in promoting cardiac remodeling (83), fibrosis (73), bronchiolitis obliterans syndrome in lung transplant patients (46) and cardiac allograft vasculopathy (47). However, the effects of IL-17 on hypertrophy and CTGF expression are unclear and merit further investigation. Our proposed model of CTGF induction downstream of IL-6 and TGFβ (and perhaps IL-17) might be explained by previous identification of both a STAT3 responsive element (−740 to −736 bp) (84) and a consensus SMAD-binding element (−173 and −166) (85) upstream of the CTGF promoter (Figure 7B). Hence, optimal induction of CTGF in CR may require that CTGF producing cells receive both SMAD and STAT3 signals, likely provided by TGFβ (10) and IL-6 (86), respectively.

This study supports a role for CTGF as a downstream mediator of fibrosis and highlights the essential contributions of immune elements to CR and fibrosis of cardiac grafts while elucidating relationships between TGFβ, IL-6 and CTGF. Further, these studies indicate for the first time that mAb neutralizing CTGF can ameliorate fibrosis and hypertrophy associated with CR. These findings further implicate IL-6 as a critical immune factor in CR that may potentiate TGFβ-mediated CTGF induction. Finally, TGFβ-mediated induction of fibrosis in allogeneic but not syngeneic grafts was associated with a suggested increase in intragraft IL-6 expression and a significant increase in IL-17 expression, supporting the notion that TGFβ induction of fibrosis and CR requires interaction with immune parameters.

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