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Expression of cytokine genes in human cardiac allografts: correlation of IL-6 and transforming growth factor-beta (TGF-β) with histological rejection

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SUMMARY

Cytokines may play critical roles in allograft rejection. Currently, a clear pattern of cytokine production that correlates with rejection has not emerged. Our preliminary studies suggested a trend toward increased IL-6 and TGF-β gene expression in cardiac allografts during rejection. We have extended these studies using reverse transcriptase/polymerase chain reaction (RT/PCR) to detect the expression of IL-6, TGF-β, and T cell receptor β chain constant region (TCR-β) genes in 21 additional consecutive myocardial biopsies obtained from six heart transplant patients and from five pre-transplant donor hearts. Cytokine gene expression was compared with histological diagnosis of rejection. There was strong correlation between IL-6 as well as TGF-β gene expression, and histological rejection (6/8 biopsies with *versus* 0/7 without rejection ($P=0.006$) and 7/9 biopsies with *versus* 0/7 without rejection ($P=0.003$) respectively). Neither IL-6 nor TGF-β transcripts were detected in any pre-transplant donor heart. TCR-β chain mRNA was found in all allograft biopsies regardless of the presence of rejection, but was absent in pre-transplant donor hearts. Our results indicate that expression of IL-6 and TGF-β is highly correlated with allograft rejection and thus may play an important role in regulation of cardiac allograft rejection. T cell infiltration of allografted myocardium is invariably detected by PCR regardless of histological rejection. The long-term functional significance of these cells in transplanted hearts needs further investigation.

Keywords IL-6 transforming growth factor-beta T cell receptor β chain constant region heart transplantation

INTRODUCTION

Cytokines play central roles in the regulation of immune responses. Their interactions in allograft rejection are not yet completely understood. Although a number of studies on cytokine expression in human and animal allografts have been reported, a consistent pattern of cytokine expression has not emerged. In part this may reflect methodological differences, as studies using polymerase chain reaction (PCR) failed to find any correlation between histological rejection and presence of IL-1, IL-6, and tumour necrosis factor-alpha (TNF-α) in renal allografts [1], while Caillat-Zucman *et al.* [2] using *in situ* hybridization found significantly elevated IL-6 expression in acute rejection. It is also possible, however, that the absence of a consistent pattern of cytokine expression thus far reflects true differences in the mechanisms underlying graft rejection in various tissues.

Our previous studies found that T cell receptor gene transcripts were present in all cardiac allograft biopsies regardless of rejection, but did not detect expression of IL-1, IL-4, IL-5, interferon-gamma (IFN-γ), or TNF-α. A trend toward increased expression of IL-6 and TGF-β during rejection was noted [3]. In the current study, we have continued analysis of IL-6, TGF-β and TCR-β gene expression in additional allograft myocardial biopsies and extended this analysis to pre-transplant donor hearts. The results confirmed that expression of mRNA for IL-6 and TGF-β is highly correlated with rejection, and is not found in normal myocardium before transplantation.

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PATIENTS AND METHODS

Isolation of RNA from myocardial biopsy specimens

Allograft myocardial biopsies were collected at the time of regular surveillance biopsy. Biopsy specimens were read for histopathology and classified by staff pathologists who were unaware of the study results. Myocardium from pre-transplant donor hearts was obtained immediately after organ excision. Individual biopsies for reverse transcriptase (RT)/PCR were placed in sterile freezer vials (Nunc, Roskilde, Denmark) and stored at −70°C until used for RNA preparation. Total cytoplasmic RNA was isolated from myocardial biopsy

Table 1. Preliminary study (a) and current study (b) on IL-6 and TGF-β gene expression

a

Gene product	Rejection *n*(*N*)	Non-rejection *n*(*N*)
IL-6	6 (10)*	1 (7)*
TGF-β	4 (9)†	0 (7)†

b

Gene product	Rejection *n*(*N*)	Non-rejection *n*(*N*)	Pre-transplantation heart *n*(*N*)
IL-6	6 (8)‡	0 (7)‡	0 (5)
TGF-β	7 (9)§	0 (7)§	0 (5)

n, Number of biopsies positive for gene expression; *N*, number of biopsies studied.
* $P=0{\cdot}07$.
† $P=0{\cdot}07$.
‡ $P=0{\cdot}006$.
§ $P=0{\cdot}003$.

specimens by the guanidinium thiocyanate-phenol chloroform method in 500 μl homogenization buffer [4]. The RNA was dissolved in 10 μl diethyl pyrocarbonate-treated distilled water for reverse transcription reaction. All patients participating in this study received standard immunosuppression (cyclosporine, azathioprine, and prednisone). The studies were approved by the Institutional Review Board.

Reverse transcription and polymerase chain reaction

RNA isolated from myocardial biopsy specimens was used for first strand cDNA synthesis with oligo dT and MMLV reverse transcriptase (BRL, Gaithersburg, MD) as suggested by the manufacturer. PCR amplification of cDNA was performed with primers for IL-6, TGF-β (Clontech, Palo Alto, CA), and TCR β chain constant region (sense primer 5′TTCCCACCCGAGGTCGCTGT 3′ and anti-sense primer 5′AGGCCTCGGCGCTGACGATC 3′). Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Stratagene, La Jolla, CA) primers were used as positive controls. PCR reactions were performed in 10 mM Tris/HCl, pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 0·2 mM dNTP, and 2 U of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). Primers were added at a final concentration of 0·5 μM for IL-6 and TGF-β primers, and 0·3 μM for T cell receptor β chain primers. Reactions were carried out in a DNA Thermal Cycler (Perkin Elmer Cetus) for 39 cycles, including denaturing at 94°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min for each cycle. PCR products were analysed on 2% agarose gels.

Cytokine gene expression was compared with histological diagnosis of rejection. Statistical significance was determined by Fisher's exact test.

RESULTS

Consecutive myocardial biopsies ($n=32$) from 12 heart transplant recipients were analysed. The results from the first 16 biopsies in six patients have been reported previously [3]. These

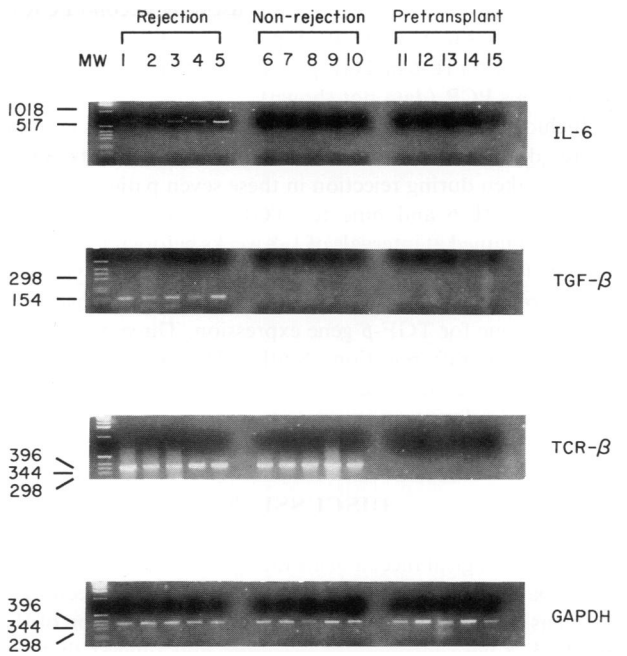

Fig. 1. Representative agarose gel electrophoresis of reverse transcriptase/polymerase chain reaction (RT/PCR) amplification products. Lane MW, DNA molecular weight markers; lanes 1–5, samples with histological rejection; lanes 6–10, samples without histological rejection; lanes 11–15, samples from pretransplant hearts. GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; TCR-β, T cell receptor β-chain constant region.

results demonstrated the presence of T cells in all allograft biopsies and suggested a trend toward increased expression of IL-6 and TGF-β during rejection (Table 1a). To investigate this further, RT/PCR for IL-6, TGF-β, and T cell receptor β was performed on 21 additional biopsies from six patients and five pre-transplant normal donor hearts. Expression of the GAPDH gene served as a control to ensure that RNA from myocardial biopsies was intact (Fig. 1). Histology of allograft myocardial biopsies was studied in parallel with PCR and classified according to International Society for Heart Transplantation (ISHT) criteria. For statistical analysis, the results of the experiments performed during the preliminary study and the current study are presented separately in Table 1.

As found previously [3], T cell receptor β chain transcripts were observed in all cardiac allograft biopsies regardless of rejection. No TCR-β transcript was detected in any pre-transplant heart (Fig. 1). As shown in Table 1b, IL-6 transcripts were detected in 6/15 allograft biopsies in the current study (Fig. 1) and were strongly correlated with histological rejection, 6/8 biopsies with rejection, and 0/7 without rejection ($P=0{\cdot}006$). TGF-β message was amplified in 7/9 biopsies with rejection and 0/7 without rejection (Fig. 1, Table 1b). Correlation between TGF-β gene expression and histological rejection was statistically significant ($P=0{\cdot}003$).

The concentration of RNA in each sample, and hence the amount of cDNA in each PCR, could not be quantified because of the small amount of tissue in myocardial biopsies. However, PCR for GAPDH was performed on serial dilutions of cDNA to determine the relative concentration of each sample. To equalize the concentration of cDNA in samples, a two- to four-fold

increase in the amount of cDNA was used in a second PCR for several samples (e.g. 8 and 10, Fig. 1). This did not result in a positive PCR for IL-6 or TGF-β in samples that were negative in the previous PCR (data not shown).

Included in these studies were sequential biopsies taken before, during and after rejection in seven patients. Of 15 biopsies taken during rejection in these seven patients, 10 were positive for IL-6 and nine for TGF-β gene expression. Six biopsies obtained at intervals of 1–8 weeks before rejection were negative for IL-6 and TGF-β transcripts. Of nine myocardial biopsies obtained 2–4 weeks after rejection, one was positive for IL-6 and none for TGF-β gene expression. Three patients had recurrent allograft rejection. Neither IL-6 nor TGF-β gene expression were detected in the interval between the two rejection episodes.

DISCUSSION

The immunological mechanisms that govern allograft rejection are probably a complex network of regulatory and effector cells as well as cytokines. Our data show a strong correlation of IL-6 and TGF-β with histological rejection, and suggest that they play important roles in regulation of allograft rejection. IL-6 is a multifunctional cytokine that regulates the immune system [5]. Along with TNF-α and IL-1, IL-6 belongs to the group of endogenous mediators of the host response to injury [6], infection [7], and transplantation [8,9]. IL-6 acts via a specific dimeric receptor [10] and plays an important role in the activation of human T lymphocytes and natural killer (NK) cells [11]. In normal individuals IL-6 synthesis becomes detectable after injury or infection [12]. The likely cellular sources of IL-6 in allografts include T cells [13], NK cells [13], monocytes [14], endothelial cells [15], and smooth muscle cells [16]. Specific up-regulation of IL-6 gene expression and its products in cardiac allografts and other organ allografts during rejection [2,17–20] is testimony to its central role in allograft rejection. It is not clear whether increased IL-6 expression is generated by non-immune components in response to rejection injury, or by T cells infiltrating the myocardium. IL-6 transcripts were absent in 30% of the rejecting biopsies in our study. This may reflect focal rejection and discordance of samples for histology and RT/PCR, or simply the sensitivity of the assay. It is also possible that variable immune mechanisms may dominate during different rejection episodes.

TGF-β is an important regulatory factor and exhibits a complex range of biological activities in a large number of cell types. *In vitro* studies have shown that TGF-β impairs the proliferation of lymphoid cells and inhibits certain differentiated functions, including antibody production by B cells [21] and cytolytic activity of cytotoxic cells [22–24]. TGF-β is a potent chemotaxic molecule for monocytes [25] and increases IL-1, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) gene transcription [25]. Both lymphocytes and monocytes have high-affinity TGF-β receptors [21,26]. *In vivo*, exogenous TGF-β can delay onset of rejection of ectopic cardiac transplants [27] and suppress autoimmune diseases [28,29]. TGF-β also appears fundamental to tissue repair. TGF-β has been shown to increase production of collagen, fibronectin and other matrix proteins [30], decrease synthesis of enzymes which degrade matrix proteins [31,32], and increase synthesis of proteolytic inhibitors [30]. The correlation of TGF-β with histological rejection in our studies suggests TGF-β may play a role in immunoregulation and repair processes during rejection.

Results of immunohistochemical studies on human cardiac allografts differ somewhat from our data. Ruan *et al.* [33] observed prominent expression of IL-2 and IFN-γ in allograft myocardial biopsies during severe cellular rejection. IL-6 expression was infrequently present. In contrast, we found IL-2 mRNA transcripts in only 2/13 rejecting biopsies, and no IFN-γ message. However, none of these biopsies was classified as severe rejection [3]. These disparities may also be due to inherent differences in kinetics between gene expression and protein synthesis.

Analysis of cytokine expression in other human solid organ transplants has revealed diverse results. Similar to our finding, Caillat-Zucman *et al.* [2] using *in situ* hybridization demonstrated up-regulation of IL-6 gene expression during renal allograft rejection. Bishop *et al.* [18] observed elevated IL-6 expression by immunohistology in chronic liver rejection, and occasionally in acute rejection. In contrast, Martinez *et al.* [34] using PCR found that IL-5 gene expression was associated with rejection in liver allografts, while IL-1, IL-6 and TNF-α were expressed variably and not correlated with rejection. Lipman *et al.* [1], however, found an association of cytotoxic T lymphocyte (CTL)-specific serine protease gene expression, but no correlation of IL-1, IL-6, or TNF-α with rejection in renal allografts. Further studies will be needed to clarify the divergent results obtained thus far. In contrast to data on human allograft rejection, multiple cytokines are detected in animal models of unmodified organ transplantation across MHC barriers [35–39] and in some syngeneic transplants [39]. In part this reflects the intensity of rejection occurring in the absence of immunosuppression, and may also be related somehow to different mechanisms underlying immune responses in disparate species.

The results of our current studies confirm the presence of TCR-β in all allografts and its absence in pre-transplant hearts. Thus, T cell infiltration occurs in all allografts regardless of rejection, and is not a feature of normal hearts. Similar findings have been reported in murine pancreatic islet grafts [38] and human renal allografts [40], with the latter study demonstrating that the T cells infiltrating the grafts are polyclonal. The small number of T cells may be insufficient to elicit rejection, but may nonetheless generate a variety of interactions contributing to chronic immune injury.

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REFERENCES

1 Lipman ML, Stevens AC, Bleackley RC *et al.* The strong correlation of cytotoxic T lymphocyte-specific serine protease gene transcripts with renal allograft rejection. Transplantation 1992; **53**:73–79.

2 Caillat-Zucman S, Vanden Broecke C, Legendre C *et al.* Differential *in situ* expression of cytokine genes in human renal rejection. Transplant Proc 1991; **23**:229–30.

3 Zhao XM, Yeoh TK, Hiebert M, Frist WH, Miller GG. Expression of acidic fibroblast growth factor and cytokine genes in human cardiac allografts and T cells. Transplantation 1993 (In press).

4 Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol chloroform extraction. Ann Biochem 1987; **162**:156–9.

5 Kishimoto T. The biology of interleukin 6. Blood 1989;**74**:1–10.

6 Shenkin A, Fraser WD, Series J *et al.* The serum interleukin-6 response to elective surgery. Lymphokine Res 1989; **8**:123–7.

7 Helfgott DC, Tatter SB, Santhanam U *et al.* Multiple forms of IFN-β2/IL-6 in serum and body fluids during acute bacterial infection. J Immunology 1989; **142**:948–53.

8 Van Oers MHJ, Van der Heyden AAPAM, Aarden LA. Interleukin-6 (IL-6) in serum and urine of renal transplant recipients. Clin Exp Immunol 1988; **71**:314–9.

9 Ray A, Sehgal PB. Cytokines and their receptors: molecular mechanism of interleukin-6 gene repression by glucocorticoid. J Am Soc Nephrol 1992; **2**(Suppl. 12):S214–21.

10 Akira S, Hirano T, Taga T, Kishimoto T. Biology of multifunctional cytokines: IL-6 and related molecules. FASEB J 1990; **4**:2860–7.

11 Luger TA, Schwarz T, Krutmann J *et al.* Interleukin-6 is produced by epidermal cells and plays an important role in the activation of human T-lymphocytes and natural killer cells: regulation of the acute phase and immune responses. Ann NY Acad Sci 1989; **557**:405–14.

12 Lindemann A, Ganser A, Hoelzer D, Mertelsmann R, Herrmann F. *In vivo* administration of recombinant human interleukin-3 elicits an acute phase response involving endogenous synthesis of interleukin-6. Eur Cytokine Netw 1991; **2**:173–6.

13 Bauer J, Herrmann F. Interleukin-6 in clinical medicine. Ann Hematol 1991; **62**:203–10.

14 Bauer J, Ganter U, Geiger T *et al.* Regulation of interleukin-6 expression in cultured human blood monocytes and monocyte-derived macrophages. Blood 1988; **72**:1134–40.

15 Sironi M, Breviario F, Proserpio P *et al.* IL-1 stimulates IL-6 production in endothelial cells. J Immunol 1989; **142**:549–53.

16 Loppnow H, Libby P. Proliferating or interleukin-1-activated human vascular smooth muscle cells secrete copious interleukin-6. J Clin Invest 1990; **85**:731–8.

17 Jordan SC, Kondo T, Prehn J, Marchevsky A, Waters P. Cytokine gene activation in rat lung allografts: analysis by northern blotting. Transplant Proc 1991; **23**:604–6.

18 Bishop GA, Matsumoto Y, McCaughan GW, Kenney JS. Identification of cytokine protein expression in human liver allograft rejection: methods development and expression of IL-1β, IL-6, IFN-γ, and basic fibroblast growth factor. Transplant Proc 1992; **24**:2310–2.

19 Ford HR, Hoffman RA, Wang SA, Simmons RL. Profile of cytokine production within the rejecting allograft. Transplant Proc 1991; **23**:251–2.

20 Ford HR, Hoffman RA, Tweardy J *et al.* Evidence that production of interleukin 6 within the rejecting allograft coincides with cytotoxic T lymphocyte development. Transplantation 1991; **51**:656–661.

21 Kehrl JH, Roberts AB, Wakefield LM *et al.* Transforming growth factor β is an important immunomodulatory protein for human B lymphocytes. J Immunol 1986; **137**:3855–60.

22 Wahl SM, Hunt DA, Song HL *et al.* Transforming growth factor-β ia a potent immunosuppressive agent that inhibits IL-1 dependent lymphocyte proliferation. J Immunol 1988; **140**:3026–32.

23 Rook AH, Kehrl JH, Wakefield LM *et al.* Effects of transforming growth factor-β on the functions of natural killer cells: depressed cytolytic activity and blunting of interferon responsiveness. J Immunol 1986; **136**:3916–20.

24 Ranges GE, Figari IS, Espevik T, Palladino MA. Inhibition of cytotoxic T cell development by transforming growth factor-β and reversal by recombinant tumor necrosis factor-α. J Exp Med 1987; **166**:991–8.

25 Wahl SM, Hunt DA, Wakefield LM *et al.* Transforming growth factor beta (TGF-β) induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci USA 1987; **84**:5788–92.

26 Kehrl JH, Wakefield LM, Roberts AB *et al.* The production of TGF-β by human T lymphocytes and its potential role in the regulation of cell growth. J Exp Med 1986; **163**:1037–50.

27 Ruscetti FW, Palladino MA. Transforming growth factor-β and the immune system. Prog Growth Factor Res 1991; **3**:159–75.

28 Brandes ME, Allen JB, Ogawa Y, Wahl SM. Transforming growth factor β1 suppresses acute and chronic arthritis in experimental animals. J Clin Invest 1991; **87**:1108–13.

29 Kuruvilla AP, Shah R, Hochwald GM *et al.* Protective effect of transforming growth factor β1 on experimental autoimmune disease. Proc Natl Acad Sci USA 1991; **88**:2918–21.

30 Ignotz RA, Endo T, Massague J. Regulation of fibronectin and type 1 collagen mRNA levels by transforming growth factor-β. J Biol Chem 1987; **262**:6443–6.

31 Chiang CP, Nilson H. Opposite and selective effects of epidermal growth factor and human platelet transforming growth factor-β on the production of secreted proteins by murine 3T3 cells and human fibroblasts. J Biol Chem 1986; **261**:10478–81.

32 Laiho M, Saksela O, Andreasen PA, Keski-Oja J. Enhanced production and extra-cellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-β. J Cell Biol 1986; **103**:2403–10.

33 Ruan XM, Qiao JH, Trento A *et al.* Cytokine expression and endothelial cell and lymphocyte activation in human cardiac allograft rejection: an immunohistochemical study of endomyocardial biopsy samples. J Heart Lung Transplant 1992; **11**:1110–6.

34 Martinez OM, Krams SM, Sterneck M *et al.* Intragraft cytokine profile during human liver allograft rejection. Transplantation 1992; **53**:449–56.

35 Wu CJ, Lovett M, Wong-Lee J et al. Cytokine gene expression in rejecting cardiac allografts. Transplantation 1992; **54**:326–32.

36 Hancock WW, Tanaka K, Salem HH *et al.* TNF as a mediator of cardiac transplant rejection, including effects on the intragraft protein C/protein S/Thrombomodulin pathway. Transplant Proc 1991; **23**:235–7.

37 Sun J, Matsumoto Y, McCaughan GW *et al.* Levels of cytokine expression in rat liver allograft rejection compared to allograft tolerance. Transplant Proc 1992; **24**:2309.

38 O'Connell PJ, Pacheco-Silva A, Nickerson PW *et al.* Unmodified pancreatic islet allograft rejection results in the preferential expression of certain T cell activation transcripts. J Immunol 1993; **150**:1093–104.

39 Dallman MJ, Larsen CP, Morris PJ. Cytokine gene transcription in vascularized organ grafts: analysis using semiquantitative polymerase chain reaction. J Exp Med 1991; **174**:493–6.

40 Krams SM, Falco DA, Villanueva JC *et al.* Cytokine and T cell receptor gene expression at the site of allograft rejection. Transplantation 1992; **53**:151–6.