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Differential intragraft cytokine messenger RNA profiles during rejection and repair of clinical heart transplants. A longitudinal study

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Abstract After clinical heart transplantation, ischemia, acute rejection, and repair mechanisms can trigger the up-regulation of cytokines. To investigate the cytokine profile early after transplantation, we monitored messenger RNA (mRNA) expression levels of tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), transforming growth factor-b (TGF-β), platelet-derived growth factor-A (PDGF-A), and basic fibroblast growth factor (bFGF) by reverse transcriptase-polymerase chain reaction (RT-PCR) in serial endomyocardial biopsies ($n = 123$) from 16 cardiac allograft recipients during the first 3 post-operative months. In the first month, mRNA expression levels of MCP-1, TNF-α, TGF-β, and bFGF were significantly higher than in the period thereafter (acute rejection episodes excluded). Acute rejection (International Society for Heart and Lung Transplantation (ISHLT) rejection grade > 2) was strongly associated with the level of TNF-α mRNA. After acute rejection episodes, rising mRNA expression levels of PDGF-A and bFGF were found. The association between TNF-α mRNA and acute rejection reflects the importance of this cytokine in allogeneic responses. Elevated growth factor expression levels indicate repair responses after tissue damage due to either the transplantation procedure (surgery, ischemia, reperfusion) or acute allograft rejection.

Keywords Heart transplantation · Cytokines · Acute rejection · Endomyocardial biopsies

Introduction

Cytokines are regulatory proteins that play a central role in the anti-donor immune response [1, 3]. Their release by vascular endothelial cells, cardiac myocytes, and/or inflammatory cells can be triggered by various alloantigen-(in)dependent factors such as brain death, ischemia, reperfusion, and acute rejection episodes [15, 24]. It has been suggested that early cytokine responses after transplantation can influence later graft outcome. For example, elevated levels of interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) early after clinical heart transplantation corresponded to reduced survival [8], and elevated growth factor expression [e.g., transforming growth factor-b (TGF-β), platelet-derived growth factor-A (PDGF-A), and basic fibroblast growth factor (bFGF)] has been linked to unrestricted repair responses and development of chronic allograft rejection both in experimental and in clinical studies [3, 7]. Furthermore, increased pro-inflammatory cytokine and chemokine messenger RNA (mRNA) and protein levels have been found in association with acute allograft rejection [3, 13]. Thus, information regarding the kinetics of cytokine expression after clinical transplantation can lead to a

better understanding in events of graft adaptation and rejection. However, serial intragraft cytokine measurements in the recent clinical heart transplant setting are few [11, 17].

The aim of the present study was to identify changes in cytokine profiles in time and during acute allograft rejection early after clinical heart transplantation. Therefore, we analyzed serial patterns of intragraft cytokine mRNA expression during the first 3 post-operative months in cardiac allograft recipients. We selected a broad range of cytokines that can be released by activated endothelial cells, cardiac myocytes, and infiltrated mononuclear cells: TNF-α, monocyte chemoattractant protein-1 (MCP-1), TGF-β, PDGF-A, and bFGF. The pro-inflammatory cytokines TNF-α and MCP-1 play a significant role in the regulation and recruitment of cells that participate in inflammatory responses [12, 18]. The growth factors TGF-β, PDGF-A, and bFGF are polypeptides with potent mitogenic activity for fibroblasts, smooth muscle cells, and endothelial cells and are important in tissue repair processes [15, 19].

Patients and methods

Patients

We studied 16 consecutive cardiac allograft recipients who were given transplants between November 1997 and October 1998. Maintenance immunosuppressive therapy consisted of cyclosporin A and low-dose steroids. Serial endomyocardial biopsies (EMBs) obtained from the right ventricle were studied during the first 3 months after transplantation. Timing of surveillance biopsies in this period was weekly during the first 6 weeks and bi-weekly during the following 8 weeks. During routine biopsy sampling, an additional biopsy was harvested for cytokine studies after informed consent from the patients. In total, 123 biopsies (on average eight time points per patient) were available for cytokine analysis. In addition, "time-zero" biopsies were sampled from ten patients during the transplantation procedure. Acute rejection was diagnosed by histological assessment of endomyocardial biopsies and graded according to the guidelines of the International Society for Heart and Lung Transplantation (ISHLT) [5]. Patients with an ISHLT rejection grade of over 2 were considered to have an acute rejection and received additional immunosuppressive treatment.

Cytokine mRNA detection by quantitative reverse transcriptase-polymerase chain reaction

Competitive reverse transcriptase-polymerase chain reaction (RT-PCR) was used for quantitative measurement of TNF-α, MCP-1, TGF-β, PDGF-A, bFGF, and the constitutively expressed housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Total RNA was extracted from snap-frozen EMBs, and complementary DNA (cDNA) was synthesized with random primers as described previously in detail [4]. Aliquots of cDNA were directly used for PCR amplification, using sequence-specific primers for TNF-α (sense: 5′-GAG-TGA-CAA-GCC-TGT-AGC-CCA-TGT-TGT-AGC-A-3′, antisense: 5′-GCA-ATG-ATC-CCA-AAG-TAG-ACC-TGC-CCA-GAC-T-3′), MCP-1 (sense: 5′-TAG-CAG-CCA-CCT-TCA-TTC-C-3′, anti-sense: 5′-TTC-CCC-AAG-TCT-CTG-TAT-CT-3′), TGF-β (sense: 5′-GCC-CTG-GAC-ACC-AAC-TAT-TGC-3′, anti-sense: 5′-GCT-GCA-CTT-GCA-GGA-GCG-CAC-3′), PDGF-A (sense: 5′-AGA-AGT-CCA-GGT-GAG-GTT-AGA-GGA-GCA-T-3′, anti-sense: 5′-CTG-CTT-CAC-CGA-GTG-CTA-CAA-TAC-TTG-CT-3′), bFGF (sense: 5′-GGC-TTC-TTC-CTG-CGC-ATC-CA-3′, anti-sense: 5′-GCT-CTT-AGC-AGA-CAT-TGG-AAG-A-3′), and GAPDH (sense: 5′-GGT-GAA-GGT-CGG-AGT-CAA-CG-3′, anti-sense: 5′-CAA-AGT-TGT-CAT-GGA-TGA-CC-3′). PCR conditions were 10-min denaturation at 94 °C, followed by 40 cycles of 1-min denaturation at 94 °C, 2-min annealing at optimal temperatures for TNF-α (60 °C), MCP-1 (56 °C), TGF-β (60 °C), PDGF-A (60 °C), bFGF (58 °C), or GAPDH (60 °C), and 3-min extension at 72 °C, prolonged for 7 min during the last cycle. Positive control samples were produced by mRNA extraction and cDNA synthesis from 10^6 human spleen cells stimulated with 1% phytohemagglutinin-M (PHA; Difco, Detroit, Mich.) for 24 h at 37 °C. Negative control samples consisted of diethyl pyrocarbonate-treated H_2O as no-template reaction.

To quantify the initial amount of functional cytokine mRNA in EMBs, we used a competitive template RT-PCR assay in which known amounts of specific internal control fragment in different dilutions were added to constant amounts of sample cDNA for competitive co-amplification. We designed the internal controls for each cytokine to generate a smaller PCR product to allow differentiation between the amplified target and the internal control. The intensity of internal control and target products on gel was measured by luminescence with a DC-40 camera in combination with analysis software (Kodak, Rochester, N.Y.). The relative concentration of cytokine mRNA was divided by the relative concentration of GAPDH to indicate the initial cytokine mRNA expression level in EMBs. EMBs negative for GAPDH mRNA expression were excluded from further analysis.

Statistics

Differences in median cytokine mRNA expression levels between EMBs sampled during the first month and during the second/third month after transplantation, or EMBs with and without histologically proven acute rejection, were analyzed by non-parametric Mann-Whitney *U*-test. Correlations of ischemia time with cytokine mRNA levels were calculated by Pearson's correlation coefficient (r) after log-transformation of the cytokine mRNA ratios. Associations with P values of under 0.05 were considered statistically significant.

Results

To determine the relationship between cytokine mRNA expression levels and time, without the influence of acute rejection on cytokine mRNA expression, we excluded the biopsies sampled during and 3 weeks following an acute rejection episode ($n = 30$). Figure 1 shows the median (and 25th and 75th percentiles) of the cytokine mRNA expression levels in the remaining serial biopsies. Peak cytokine mRNA expression was measured in "time-zero" biopsies for MCP-1, bFGF, and PDGF; in the first week for TNF-α, and bFGF; and in the third week for TNF-α, MCP-1, TGF-β, and bFGF (Fig. 1). Grouping of the time points revealed that the expression levels of TNF-α, MCP-1, TGF-β, and bFGF mRNA were significantly higher in the first post-operative month (including "time-zero" biopsies) than in the

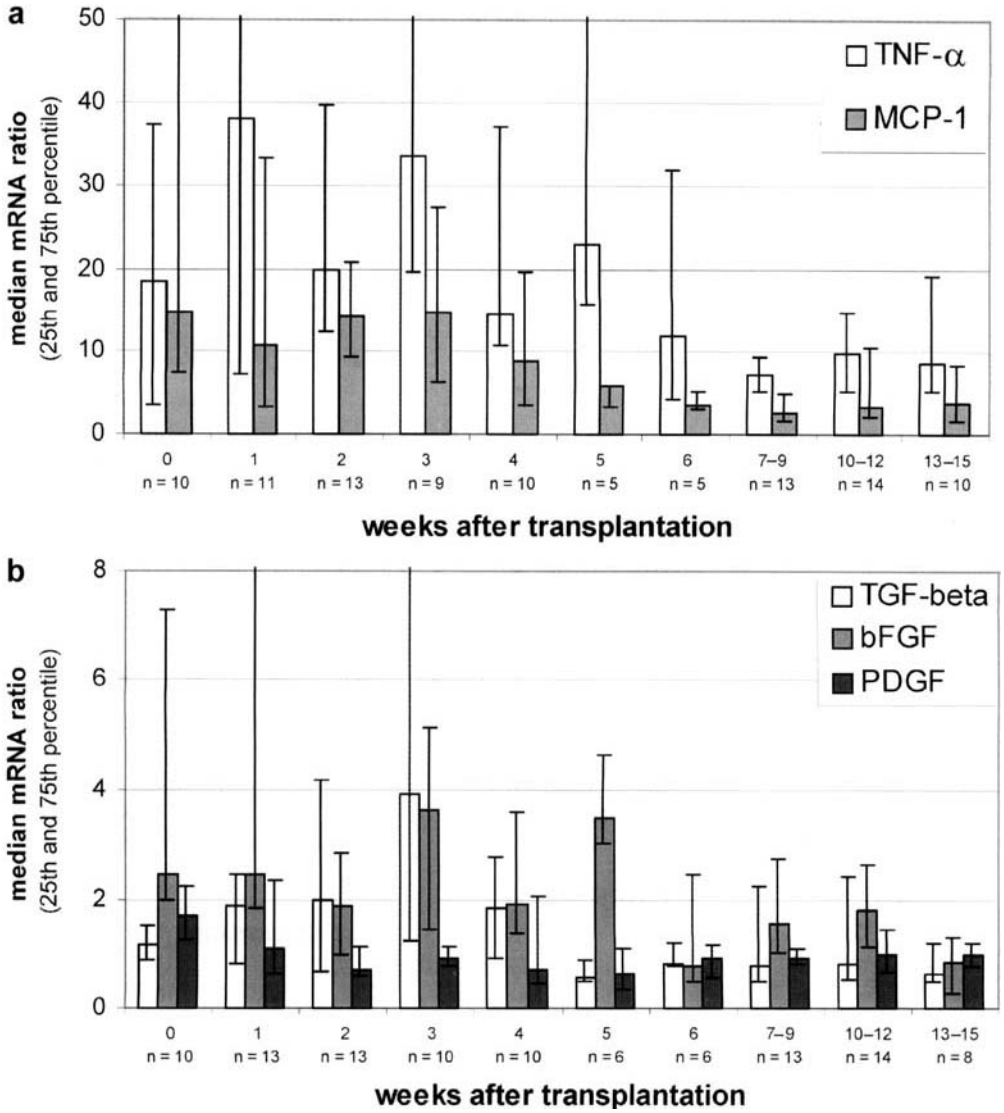

Fig. 1 Median cytokine/GAPDH mRNA expression levels of pro-inflammatory cytokines (**a**) and growth factors (**b**) during follow up after heart transplantation (*HTx*). Acute rejection episodes are excluded

period thereafter (Fig. 2). The median cytokine/GAPDH mRNA ratio in the first month vs 2–3 months after transplantation were 13.8 vs 3.4 for MCP-1 ($P < 0.0001$), 23.0 vs 8.6 for TNF-α ($P = 0.007$), 1.6 vs 0.8 for TGF-β ($P = 0.002$), and 2.5 vs 1.5 for bFGF ($P = 0.006$). The expression level of PDGF-A was higher in "time-zero" biopsies, but remained at a constant expression level during follow-up.

The length of duration of cold ischemia ranged from 112 to 272 min and was not correlated with cytokine mRNA expression levels, neither in the first EMBs (TNF-α: $r = -0.16$, $P = 0.61$; MCP-1: $r = -0.20$, $P = 0.53$; TGF-β: $r = -0.19$, $P = 0.49$; PDGF-A: $r = -0.02$, $P = 0.95$; bFGF: $r = -0.09$, $P = 0.76$) nor with median ratios during the first month after transplantation (TNF-α: $r = -0.15$, $P = 0.59$; MCP-1: $r = -0.23$, $P = 0.39$; TGF-β: $r = -0.01$, $P = 0.98$; PDGF-A: $r = 0.16$, $P = 0.56$; bFGF: $r = 0.01$, $P = 0.98$).

In four of 16 patients no histological signs of acute rejection were observed during the first 3 months after transplantation. The remaining 12 patients had one or more biopsies with histological signs of acute rejection. Overall, histological signs of acute rejection were found in 20 of 123 biopsies. Most episodes of acute rejection occurred between the fourth and the sixth post-operative week. We compared the cytokine mRNA expression levels in all biopsies with and without acute rejection. This revealed that acute rejection was not associated with the mRNA expression levels of MCP-1, TGF-β, PDGF-A, or bFGF. In contrast, Fig. 3 shows that the expression level of TNF-α mRNA was significantly higher in the biopsies sampled during rejection than in non-rejection biopsies ($P = 0.008$).

Next, we analyzed median cytokine mRNA expression levels before, during, and after individual rejection episodes in the 12 rejecting patients, to identify cytokine

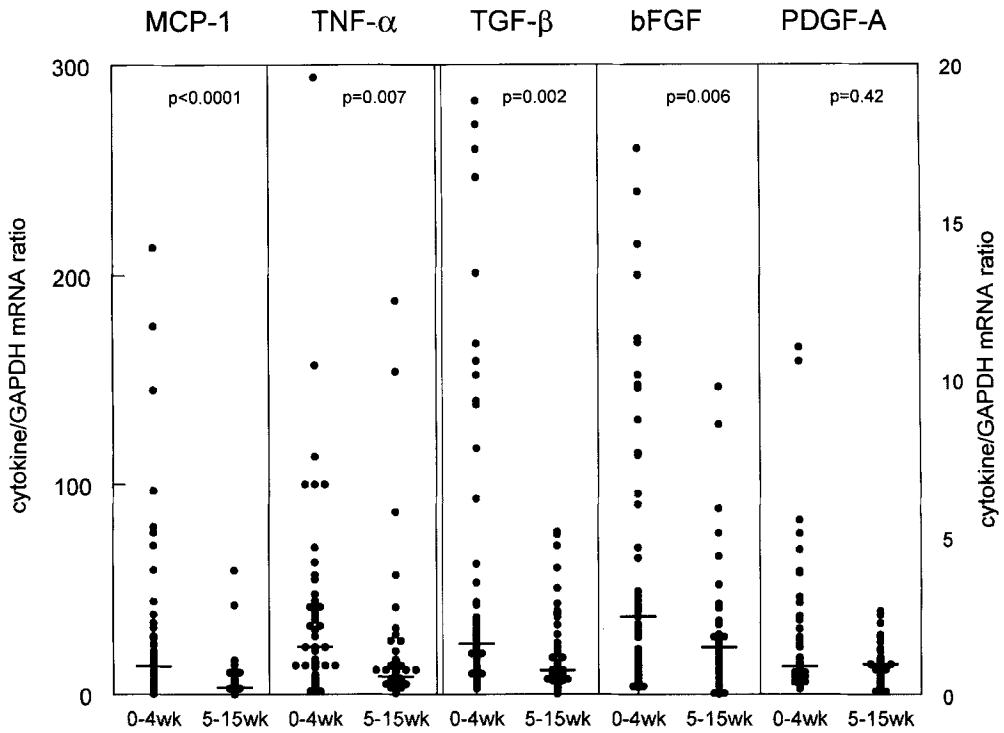

Fig. 2 Cytokine/GAPDH mRNA expression levels in the first month (0–4 weeks) after transplantation vs the second/third month (5–15 weeks) after transplantation

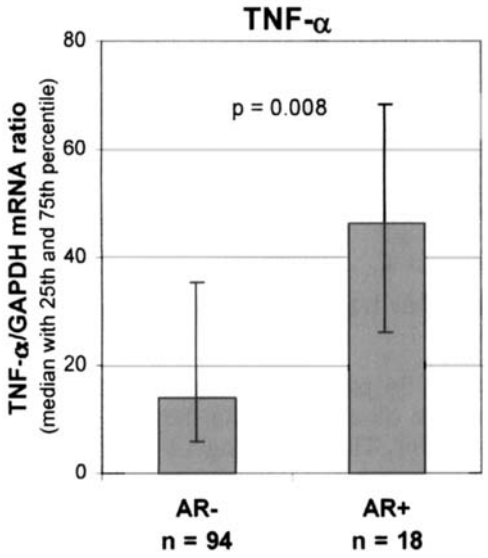

Fig. 3 Median TNF-ŏ/GAPDH mRNA expression levels in biopsies without (*AR–*) and with (*AR+*) histological signs of acute rejection after heart transplantation

patterns around individual rejection episodes. We observed a trend towards higher median TNF-α mRNA expression during individual rejection episodes. Median MCP-1 mRNA expression was higher before and during rejection than in the biopsy after rejection. In the first biopsy after treatment of acute rejection, decreased median TGF-β mRNA expression levels, as well as increased PDGF-A and bFGF mRNA expression levels, were found. However, these associations were not statistically significant.

Discussion

In the present study we found a relatively high mRNA expression level of TNF-α, MCP-1, TGF-β, and bFGF early after transplantation. These findings are in line with previous reports of elevated cytokine levels in serum (TNF-α, IL-6, IL-8) early after clinical heart transplantation [9, 10]. The increased intragraft cytokine expression in the first post-operative month most likely reflects the inflammatory response triggered by surgery, ischemia, and reperfusion, leading to cell activation and/or tissue injury [15, 16, 18]. However, we could not confirm a direct relationship between ischemia time and cytokine mRNA expression levels in the graft, suggesting that ischemia is not the major factor leading to a cytokine response early after cardiac transplantation. The fact that ischemia times after heart transplantation are relatively short compared with those after other transplanted organs (generally within 2–4 h) might be another explanation for the lack of association between ischemia times and cytokine mRNA expression levels in our study.

When we excluded acute rejection episodes, we showed a significant decrease in the measured cytokine mRNA expression levels after the first post-operative month (Figs. 1 and 2). This is probably due to down-regulation

of the early non-specific inflammatory response. The transcription factor NF-χB might play a central role in this process because it enhances the gene expression of many pro-inflammatory mediators including cytokines [21, 23]. It is likely that early after transplantation the production of NF-χB is up-regulated by ischemia and reperfusion [23]. Because it is known that cyclosporine interferes with the activation of NF-χB [21], the decreased cytokine mRNA levels in the graft after the first month after transplantation might also be the effect of ongoing immunosuppressive therapy.

During acute rejection we found higher expression levels of TNF-α mRNA within the graft. This is in agreement with previous observations that TNF-α mRNA and protein expression, as well as TNF-α gene polymorphism, was associated with acute allograft rejection [15, 18, 22]. Furthermore, TNF-α mRNA and protein expression in "time-zero" biopsies have been associated with right ventricular failure after clinical heart transplantation [6]. TNF-α in the heart is produced by cardiac myocytes and macrophages and is able to stimulate vascular endothelial cells to express adhesion molecules (e.g., VCAM-1) and HLA. This triggers increased adherence of monocytes and T cells to the endothelium, followed by infiltration into the cardiac tissue [15, 18]. Our data underline the important role of this pro-inflammatory cytokine in the regulation of the alloimmune response after clinical heart transplantation.

Previous serial measurements of cytokines in serum or in the graft could not predict acute allograft rejection [9, 10, 13, 17]. Our intragraft measurements also failed to identify an individual predictive parameter for development of acute rejection. We cannot entirely exclude the influence of bacterial or viral infection episodes such as cytomegalovirus on cytokine mRNA expression within the graft [14], although intragraft cytokine measurements do not automatically reflect peripheral events. After individual acute rejection episodes we found up-regulation of PDGF-A and bFGF mRNA expression. Other investigators have previously shown increased expression of PDGF-A and bFGF mRNA and protein in transplanted heart tissue compared with control hearts that was not associated with histological signs of acute rejection [2, 20, 25]. This may suggest that these growth factors play an important role in alloantigen-independent repair of tissue injury rather than in the alloantigen-dependent immune response.

In conclusion, the association between intragraft TNF-α mRNA expression and acute rejection episodes emphasizes the importance of this cytokine in allogeneic responses. Furthermore, the increased growth factor expression levels in allografts early after transplantation and after acute rejection indicate the activation of a repair response to restore tissue injury caused by the transplantation procedure or by acute rejection episodes.

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