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Cytokine gene expression profiles in human endomyocardial biopsy (EMB) derived lymphocyte cultures and in EMB tissue

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Abstract The reverse transcriptase polymerase chain reaction (RT-PCR) technique was used to analyse cytokine gene expression in relation to acute cardiac rejection. Expression of interleukins, IL-2, IL-4, IL-6 and IL-10 mRNA was studied in sequential endomyocardial biopsies (EMB) and in graft-infiltrating lymphocyte (GIL) cultures propagated from EMB taken after heart transplantation. The cytokine gene expression of GIL propagated from EMB taken during an episode of rejection and of immunological quiescence was comparable. In contrast, posttransplant EMB showed selective IL-2 gene expression when rejection was diagnosed. IL-4 mRNA was absent in pretransplant EMB but present in posttransplant EMB taken during periods of rejection and of immunological quiescence. Both IL-6 and IL-10 transcripts were found in pre- and posttransplant EMB. These findings confirmed that IL-2 is specifically involved in cardiac rejection, while IL-4 may play a role in immune responses leading to graft rejection or graft tolerance.

Key words Human cardiac transplantation · Cytokine Graft-infiltrating lymphocyte Acute rejection · RT-PCR Endomyocardial biopsy

Introduction

The process of acute graft rejection is mediated, to a large extent, by cytokines (reviewed by Halloran et al. [5]). Analysis of intragraft cytokine gene expression has demonstrated the appearance or upregulation of various cytokine gene transcripts interleukin (IL-2 [4], IL-4 [3], IL-5 [7], IL-6 [11]) during allograft rejection. Besides, data obtained by experimental allograft models suggest that cytokines such as IL-4 and IL-10 mediate specific unresponsiveness in organ transplantation (reviewed by Lowry [6]). To unravel immunological processes leading to graft rejection or tolerance, it is, therefore, necessary to systematically investigate the presence of several cytokines after transplantation. Accordingly, we used reverse transcriptase polymerase chain reaction (RT-PCR) to study cytokine gene transcription in human cardiac allografts during episodes of rejection and of immunological quiescence. Expression of IL-2, IL-4, IL-6 and IL-10 mRNA was analysed in graft-infiltrating lymphocytes (GIL) propagated from posttransplant endomyocardial biopsies (EMB), in pretransplant EMB (control heart tissue) as well as in sequentially taken posttransplant EMB.

Materials and methods

Patients

Cardiac transplantation patients received cyclosporin A and low-dose steroids as maintenance immunosuppressive therapy. Rejection was diagnosed by ISHT criteria [1].

EMB tissue

41 EMB taken from ten cardiac allograft recipients (8–346 days after transplantation) were divided into three groups: those obtained before transplantation (control heart tissue; $n = 6$), those without myocyte damage indicative of an episode of immunological quiescence ($n = 18$; ISHT grade 0 and 1) and those with myocyte damage indicative of an episode of rejection ($n = 17$; ISHT grade 2 and 3). All EMB were directly snap-frozen and stored at −80 °C.

Propagation of GIL from EMB

EMB taken routinely after transplantation (19–563 days posttransplantation) were cocultured with 10^5 irradiated (40 Gy) autologous PBMC in RPMI 1640 containing 10% (vol/vol) lectin-free Lymphocult-T-LF as exogenous source of IL-2 and 10% (vol/vol) human serum. When necessary, GIL cultures were restimulated with third-party Epstein Barr virus immortalized B cells. To study the cytokine gene expression profile, 5×10^4 GIL were cultured for 24 h in IL-2 free medium and subsequently stimulated for 4 or 20 h by addition of 5×10^4 irradiated donor derived cells. After stimulation, cell pellets were washed twice with cold PBS and stored at −80 °C.

RNA preparation and polymerase chain reaction (PCR)

Snap-frozen EMB were homogenized using disposable pistols (Merck/Eppendorf), after which 10^4 mouse 3T3 cells (ATCC, Rockville, Md.) were added to improve yield. Total RNA was extracted from snap-frozen EMB or EMB-derived cultures using a modified version of the guanidinium method described by Chomczynski and Sacchi [2]. After extraction, RNA was precipitated and dissolved in 50 µl diethylpolycarbonate-treated H_2O (DEPC-H_2O). First-strand cDNA synthesis was performed at 42 °C for 90 min using 25 µl extracted RNA, 2.5 µl Moloney murine leukaemia virus (MMLV) reverse transcriptase (200 U/µl; Gibco-BRL, Gaithersburg, Md.) and 0.25 µg hexanucleotides (0.5 µg/µl; Promega Corporation, Madison, Wis.) in a total reaction volume of 50 µl. Subsequently, 5 µl cDNA was amplified in a thermal cycler using 2 U TAQ DNA polymerase (Promega) and 50 pmol of 3′ and 5′ sequence specific primers. The amplification temperatures and times were: 95 °C for 5 min, followed by 40 cycles with a 1-min 95 °C denaturation, a 2-min 60 °C annealing of primers and a 3-min 72 °C extension of primers. The last step was extended for 7 min. PCR products were electrophoresed on a 2% agarose gel in TBE, transferred to Hybond N^+ and detected by hybridisation of γ^{32} P endlabelled internal probes. Expression of keratin (EMB) or β-actin (EMB-derived cultures) mRNA was used as an internal control to establish the efficiency of RNA yield.

Results and discussion

The cytokine gene expression of GIL cultures is summarized in Table 1. GIL cultures grown in medium supplemented with IL-2 occasionally expressed IL-2, IL-4, IL-6 or IL-10 mRNA. Incubation of these lymphocytes for 24 h in IL-2-free medium, however, abrogated the expression of those cytokines. After 4 h of stimulation by donor cells, IL-2, IL-4, IL-6 and IL-10 transcripts were observed in the majority of cultures propagated from EMB with histological signs of myocyte damage and also in those without. A reduction in the number of EMB showing IL-2 and/or IL-6 cytokine gene expression was found after 20 h of stimulation. This was not the case for IL-4 and IL-10 mRNA expression. These results demonstrated that IL-2 and IL-6 transcripts reached maximal expression within 20 h after donor stimulation whereas IL-4 and IL-10 mRNA expression continued to increase at least until 20 h after stimulation. Similar kinetics of IL-2 and IL-10 mRNA expression have been found by Yssel et al [12]. More importantly, the ubiquitous expression of IL-2, IL-4, IL-6 and IL-10 mRNA in donor-stimulated GIL cultures allowed no discrimination between EMB with and without signs of rejection. We need to develop a quantitative PCR technique to reveal eventual differences in the level of cytokine gene expression during periods of rejection and of immunological quiescence.

In contrast, EMB sequentially taken after heart transplantation showed different cytokine expression profiles during a period of immunological quiescence compared to a period of rejection (Fig. 1). The presence of IL-2

Table 1 The cytokine gene expression of graft-infiltrating lymphocyte (GIL) cultures derived from endomyocardial biopsies (EMB) with or without histological signs of myocyte damage (*ND* not done, *IL* interleukin)

	EMB with myocyte damage				EMB without myocyte damage			
	IL-2	IL-4	IL-6	IL-10	IL-2	IL-4	IL-6	IL-10
Unstimulated								
IL-2[a] present	0/5	1/4	1/4	2/5	2/7	4/7	2/7	3/7
IL-2[b] absent	0/3	ND	ND	0/3	0/4	0/2	0/2	0/4
Donor stimulated								
For 4 h	5/6	3/3	3/3	6/6	9/9	6/7	6/7	8/9
For 20 h	1/6	3/3	1/3	6/6	5/9	6/7	4/7	7/9

[a] GIL cultured in IL-2-containing medium
[b] GIL cultured in IL-2-containing medium were maintained in IL-2-free medium for 24 h

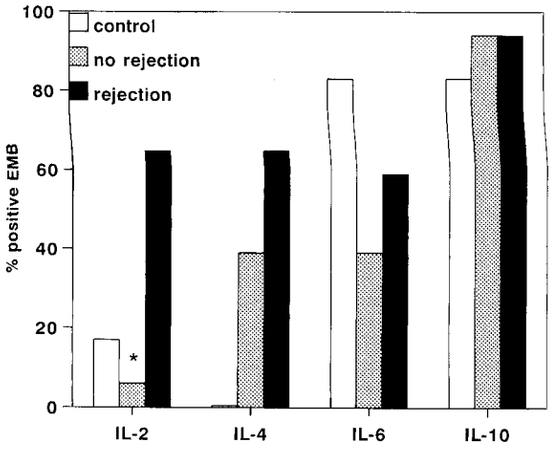

Fig. 1 Intragraft cytokine gene expression in cardiac allograft recipients. Control heart tissue ($n = 6$) and posttransplant endomyocardial biopsies (EMB) with ($n = 17$) or without ($n = 18$) histological signs of rejection (myocyte damage) were analysed for IL-2, IL-4, IL-6 and IL-10 mRNA expression. The percentage of cytokine gene expressing EMB is shown. * Correlation between IL-2 gene expression and histological rejection was statistically significant ($P < 0.001$: X^2-test)

mRNA was specifically correlated with acute cardiac rejection as 11 of 17 EMB with histological signs of myocyte damage expressed the IL- 2 gene compared to 1 of 18 EMB without myocyte damage. Expression of IL-2 mRNA during acute rejection was often found to be accompanied by the expression of IL-4 (9/11), IL-6 (9/11) and IL-10 (10/10) mRNA. Nevertheless, none of these latter cytokine transcripts appeared to be characteristic of rejection.

The majority of pretransplant EMB showed IL-6 and IL-10 mRNA expression (5/6), whereas IL-4 and IL-2 mRNA were absent (0/6 and 1/6; Fig. 1). The absence of IL-2 and IL-4 mRNA in control heart tissue and their presence in posttransplant EMB indicated that both cytokines are involved in immunological processes occurring at the graft site after transplantation. Similar findings have been reported for murine cardiac allografts [3, 8]. Elevated IL-6 levels found in serum samples of human lung transplant recipients in the immediate postoperative period (4 h) were thought to be the outcome of preservation injury [9]. IL-6 gene transcription in pretransplant EMB might, therefore, be induced following the non-specific insult of the transplant procedure itself. Likewise, cells of the monocyte-macrophage lineage may have expressed the IL-10 gene in pretransplant EMB as a response to ischaemic injury during surgery.

Taken together, IL-2, IL-4, IL-6 and IL-10 transcripts were present in donor-stimulated GIL cultures irrespective of the histological status of the EMB they have been propagated from. This was also the case for IL-4, IL-6 and IL-10 transcripts in posttransplant EMB tissue. Nevertheless, the increased frequency of IL-4 and IL-6 mRNA expression in solid EMB taken during rejection demonstrated that their involvement in the rejection process cannot be ignored. Takeuchi and coworkers [10] have reported the presence of IL-4 and IL-10 mRNA in mouse heart allografts during acute rejection as well as in heart allografts of mice rendered tolerant. The ubiquitous expression of IL-4 and IL-10 mRNA found in the present study may, therefore, result in cardiac allograft rejection, on the one hand, and tolerance on the other hand. IL-2 gene expression is exclusive for posttransplant EMB with signs of rejection, a finding that confirms the importance of this cytokine during cardiac rejection. Monitoring of IL-2 mRNA expression in snap-frozen EMB may, therefore, be of particular interest in the diagnosis of clinical transplant rejection.

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