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Donor-specific CTL frequencies in peripheral blood in relation to graft vascular disease after clinical heart transplantation

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Abstract Cellular mechanisms may play a role in the development of graft vascular disease (GVD). We previously demonstrated that GVD correlated with an increase of donor-specific T-helper 1 cytokine production by graft-infiltrating lymphocytes but not by peripheral blood mononuclear cells (PBMC). These T-helper 1 cytokines aid the generation of cytotoxic T-lymphocytes (CTL). In the present report, we investigated whether there is a relationship between the frequency of donor-specific CTL precursors (pCTL) in PBMC and the development of GVD. We tested PBMC samples of five patients with GVD and five patients without GVD in the periods 3–6 months, 1 year, and 3 years after heart transplantation. At all time points, GVD was not related to the number of pCTL. In conclusion, donor-specific cellular tests in peripheral blood could not be related to GVD. Apparently, donor-specific reactions associated with the induction of GVD can only be monitored in the graft.

Key words Peripheral blood · Chronic rejection · CTL frequencies

Introduction

While the morphology of GVD (graft vascular disease) is well recognized, the mechanisms leading to GVD are still largely unknown. Both immunological and non-immunological factors may play a role in the development of GVD. We focused on donor-specific cellular test systems both intragraft and in peripheral blood in an attempt to find associations with GVD.

In a previous study, we demonstrated that increased T-helper 1 cytokine [interleukin (IL)-2 and interferon-γ] production by donor-stimulated graft-infiltrating lymphocyte cultures derived from endomycocardial biopsies taken within the first postoperative year is associated with GVD diagnosed at 1 year after heart transplantation (HTx) [5]. These T-helper 1 cytokines aid the differentiation and maturation of cytotoxic T-lymphocytes (CTL). Therefore, we determined the possible role of CTL in GVD development. Because functional tests in the graft, necessitating culturing graft-infiltrating lymphocytes, are invasive and time consuming, we performed experiments in peripheral blood. Several studies have demonstrated that a reduction in circulating CTL is associated with stable engraftment after kidney, liver, and heart transplantation [2, 3, 9]. Therefore, we wondered whether the frequency of CTL in PBMC (peripheral blood mononuclear cells) might also reflect an unstable situation, as in GVD. We stimulated the PBMC with donor cells to determine the total pool of CTL in peripheral blood. Because circulating CTL are predominantly CTL precursors (pCTL) and not the mature, committed CTL [4], we considered the measured number of CTL as pCTL and related their frequency to the development of GVD.

Materials and methods

Patients

We tested a total number of 15 PBMC samples from five patients with GVD and five patients without GVD in the period 3–6 months, 1 year, and 3 years after HTx. All samples were taken

Table 1 Frequencies of cytotoxic T-lymphocyte precursors *(pCTL)* of peripheral blood mononuclear cell *(PBMC)* samples taken at different time points after heart transplantation *(HTx)* from five patients with graft vascular disease *(GVD)* and five patients without GVD (*pCTLf* frequency of pCTL per million PBMC, *CI* confidence interval)

Patient	Three to 6 months after HTx		One year after HTx		Three years after HTx	
	pCTLf	95% CI	pCTLf	95% CI	pCTLf	95% CI
Patients without GVD						
BU	145	100–190	144	103–185	199	146–261
MU	13	6–20	65	40–90	31	19–43
HE	57	27–87	142	91–192	14	7–22
FI	302	230–375	192	145–239	41	28–54
SC	11	2–20	0	0–0	0	0–0
Patients with GVD						
ZE	1150	675–1625	320	235–404	126	90–163
JO	224	152–296	74	47–101	227	156–299
KA	0	0–0	–[a]	–	0	0–0
PO	6	2–9	11	5–18	3	0–6
OL	1080	807–1353	1442	1027–1857	3079	2034–4125

[a] PBMC were inviable according to the phytohemagglutinin M stimulation

at the day an endomyocardial biopsy was performed. To avoid bias of acute rejection, only those blood samples were analyzed that had no histological signs of myocytolysis in their accompanying biopsy.

All patients received standard immunosuppressive therapy, consisting of cyclosporin A and low-dose prednisolone. One year after HTx, GVD was visually assessed on coronary angiograms by one of us (A.H.M.M.B.) and defined as all vascular wall changes including minimal wall irregularities [1].

Frequency of cytotoxic T-lymphocytes

Responder PBMC were isolated from heparinized blood by density gradient centrifugation using Ficoll-Isopaque. PBMC were stimulated with phytohemagglutinin M (1:100 final dilution; Difco Laboratories, Detroit, Mich., USA) to control the viability of the cells.

Limiting dilution cultures were set up in 96-well U-bottomed tissue culture plates (Costar, Cambridge, Mass., USA). Twenty-four replicates of a graded number of PBMC responders were titrated in seven-step double dilutions from 2.5×10^4 to 391 PBMC/well and were stimulated with an irradiated and washed Epstein-Barr-transformed B-cell line of donor origin (5×10^3 cells/well) in 200 µl culture medium (RPMI-1640 DM; Life Technology, Paisley, UK; supplemented with 10% pooled human heat-inactivated serum, 4 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). After 3 days of incubation, 100 µl of the supernatant was replaced by 100 µl culture medium containing 40 U/ml recombinant IL-2 (final concentration 20 U/ml IL-2; Biotest, Dreieich, Germany). After another 7 days of culture, we performed a cell-mediated lympholysis. Each cell was tested for cytolytic activity against 2.5×10^3 ^{51}Cr-labelled T-cell blasts of donor origin. These T-cell blasts can only be used as a HLA class I target, and not as a HLA class II target [8]. After 4 h of incubation at 37°C in 5% CO_2, the supernatants were harvested (Skatron harvesting system; Skatron, Lierse, Norway) and the release of ^{51}Cr was determined in a γ-counter (Packard Instruments, Downers Grove, Ill., USA). Maximum and spontaneous release were determined in quadruplicate by incubation of target cells with culture medium in the presence and absence of Triton X-100 (5% v/v fourfold in TRIS buffer), respectively.

The percentage of lysis was calculated according to the formula:

$$\% \text{ lysis} = 100\% \times \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}}$$

The microcultures were considered cytolytic when the percentage lysis exceeded 10%.

Results

Donor-specific pCTL for HLA class I antigens were detected in PBMC of both patients with and without GVD. Their number fluctuated per patient with time after HTx. We found no differences in the frequency of donor-specific pCTL between PBMC derived from patients with GVD and patients without GVD at 3–6 months, 1 year, and 3 years after HTx (Table 1).

Analyzing the individual increase or decrease in frequencies also did not result in differences between the patient groups.

Discussion

To elucidate whether a quantitative change of alloreactive pCTL in PBMC is one of the mechanisms accounting for the development of GVD, we determined the frequency of pCTL specific for donor HLA class I antigens of PBMC samples from patients with and without GVD.

Several studies have demonstrated that a reduction in circulating CTL is associated with stable engraftment [2, 3, 9]. Interestingly, our study suggests that in patients with stable engraftment who remained free from GVD, no consistent reduction in circulating pCTL numbers was observed over time. We also found that in an unsta-

ble situation, such as GVD development, both the number of pCTL and the variability in time of pCTL appearance were not different from the results of the patients without GVD. Apparently, the presence of a pool of pCTL specific for donor HLA class I antigens in PBMC does not reflect GVD development. In line with this, we previously reported that we were also not able to find a relationship between the induction of GVD and T-helper 1 cytokines produced by PBMC, which are suggested to be essential for CTL generation [6]. So, it seems that the parameters of an activated immune system in peripheral blood do not parallel the pathogenesis of GVD.

What about the immune reactivity in the graft? We have investigated the possible role of CTL in lymphocytes that infiltrate the transplanted heart. The frequency of in vivo primed or committed CTL with specificity for donor HLA class I in these graft-infiltrating cell cultures in our hands again did not discriminate between patients with and without GVD [7]. However, we found that a high frequency of CTL with specificity for donor HLA class II in the cultures may protect against the development of GVD [7]. We hypothesized that these particular CTL act by killing HLA class II-bearing donor cells (endothelial cells and smooth muscle cells) responsible for GVD induction. In the case of GVD development, the frequency of donor-directed HLA class II specific CTL is too low to lyse the donor cells. Activated donor endothelial cells secrete growth factors which promote donor smooth muscle cell proliferation, leading to intima thickening. In other words, we think that GVD is an autoreactive phenomenon of the donor tissue induced by acceptor-derived cytokines. We have not yet measured the number of pCTL specific for donor HLA class II antigens in PBMC. Further studies on this hypothesis of donor autoreactivity could be important for understanding the pathogenesis of GVD.

In summary, the frequency of circulating pCTL with donor HLA class I specificity does not correlate with the development of GVD. Only donor-specific T-helper 1 production and the frequency of CTL specific for donor HLA class II antigens in the graft are associated with the induction of GVD.

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