Mechanisms of disease

Endothelial injury mediated by cytotoxic T lymphocytes and loss of microvessels in chronic graft versus host disease

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Summary

Background Vascular endothelial cells form the interface between recipient tissues and circulating alloreactive donor T cells after allogeneic stem cell transplantation. Vascular injury has been seen in patients with acute graft versus host disease (GVHD) in the skin. We aimed to see whether vascular injury mediated by cytotoxic T lymphocytes and microvessel loss arises in patients with chronic GVHD.

Methods We investigated eight patients with acute GVHD and ten with chronic GVHD for signs of endothelial injury and microvessel loss by measurement of von Willebrand factor (vWF) in plasma and blood vessel density in biopsy samples taken from lesional skin. Controls consisted of nine patients without GVHD who survived for longer than 100 days and nine healthy people. Inflammation and endothelial injury were assessed in selected samples by immunostaining for CD8 T cells, activated cytotoxic T lymphocytes, and vascular endothelial cells.

Findings We identified more extensive loss of microvessels in the skin of patients with GVHD (median 66 capillaries/mm²; IQR 16–98) than of healthy controls (205 capillaries/mm²; 157–226; p=0.005). Patients with GVHD had higher concentrations of vWF (238%; 168–288) than did those without GVHD (102%; 88–118; p=0.0005). Perivascular CD8 T cell infiltrates in skin correlated with vWF plasma concentrations in patients with GVHD (p=0.01), and activated cytotoxic T lymphocytes and endothelial injury were present in these same samples.

Interpretation Host endothelial cells are a target of alloreactive donor cytotoxic T lymphocytes. Substantial blood vessel loss may lead to impaired blood perfusion and tissue fibrosis, the hallmark lesion of chronic GVHD.

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Introduction

Allogeneic bone marrow or stem cell transplantation is a very effective treatment of malignant blood cell disorders. Up to 60–70% of patients who have had allogeneic stem cell transplantation develop chronic graft versus host disease (GVHD). Chronic GVHD is an important cause of morbidity and mortality in long-term survivors of allogeneic stem cell transplantation and GVHD is the major obstacle to broader use of the treatment.

Vascular endothelial cells have been recognised as an important target for alloreactive cytotoxic T lymphocytes in vascular rejection of solid organ transplants. Endothelitis or intimal arteritis, the subendothelial infiltration of the arterial intima by cytotoxic T lymphocytes, is a histopathological hallmark lesion of severe acute rejection of transplants. Chronic graft rejection, characterised by replacement fibrosis of the graft parenchyma, is frequently a consequence of tissue ischaemia resulting from progressive vascular occlusion of the graft. Although the mechanism of allograft vasculopathy is unknown, it has been associated with presence of cytotoxic T lymphocytes adjacent to injured endothelial cells. After allogeneic stem cell transplantation, vascular endothelial cells are the first alloimmune recipient cells encountered by circulating immunocompetent donor T cells. Vascular injury has been described in patients with acute GVHD, and arterial changes similar to allograft vasculopathy have been described in patients with chronic GVHD. We postulated that when left untreated, vascular injury arising in GVHD, mediated by cytotoxic T lymphocytes and manifested as persistent perivascular inflammation and endothelial cell death, would lead to progressive loss of microvessels. The replacement fibrosis of chronic GVHD, like that of chronic allograft rejection, could result from ischaemia, in this case secondary to microvascular loss. We thus assessed the extent of vascular injury by counting microvessels in skin biopsy samples and by measuring von Willebrand factor (vWF) in plasma. vWF is stored in the Weibel-Palade bodies of vascular endothelial cells and raised concentrations in plasma correlate with endothelial injury. We also assessed selected skin biopsy samples from patients with late acute or chronic GVHD for evidence of ongoing immune-mediated vascular injury.

Methods

Patients

We assessed 27 patients who underwent allogeneic stem cell transplantation and nine healthy controls. Healthy controls were age-matched volunteer donors (mostly scientists and physicians) working at our institution. All procedures were approved by the institutional ethical review board and written informed consent was obtained from every participant. Patients had undergone...
allogeneic stem cell transplantation at the Department of Hematology, University Hospital Basel, Switzerland, between February, 1988, and March, 2001. We included eight patients with acute GVHD and ten with chronic GVHD. Acute and chronic GVHD was diagnosed by the treating physicians (MG, AG) on the basis of standard clinical criteria. In all these patients, diagnosis of GVHD was confirmed histologically. The formalin-fixed skin biopsy samples used to confirm the diagnosis were obtained from the Department of Dermatology, University Hospital Basel, for further immunohistological analysis. A skin biopsy was not available in three patients with fatal acute GVHD, but the diagnosis was confirmed at autopsy. Nine recipients of stem cell transplantation who did not have chronic GVHD and nine healthy people were included as controls. We obtained 10 mL blood in EDTA, which was then centrifuged for 20 min at 2000 g, with plasma stored at −70°C.

**Procedures**

For quantification of vascular endothelial cells and for triple staining for perivascular cytotoxic T lymphocytes, we embedded formalin-fixed skin biopsy samples in paraffin and cut 5 μm-thick sections. Paraffin was removed by xylol and treatment with 100%, 96%, and 70% ethanol. After rinsing the slides in phosphate-buffered saline (pH 7·4), we incubated them in 10 mmol/L citrate buffer in a steam-presser chamber for 2 min. The slides were washed again with phosphate-buffered saline. For immunofluorescence stainings, non-specific binding sites were blocked with 3% fat-free dry milk in phosphate-buffered saline (pH 7·4), we incubated them in 3% hydrogen peroxide in 50% methanol. Vascular endothelial cells were detected with ULEX EUROPAEUS AGGLUTININ I (UEA-I) binding. We incubated slides with UEA-I (1 in 400, DAKO, Denmark) and incubated slides with UEA-I (1 in 400, DAKO, Denmark).
Glostrup, D) for 30 min at room temperature. UEA-I was detected by rabbit antiserum to UEA-1 (1 in 400, DAKO) and by a peroxidase conjugated goat antiserum to rabbit (DAKO Envision, rabbit). 3-amino-9-ethylande was added as a substrate (AEC-ready to use, DAKO). In the superficial dermis (a subepidermal region 150–200 µm deep), we counted vascular profiles and measured the length of blood vessels lined by endothelial cells with image analysis software (ImagePro, Media Cybernetics, Silver Spring, MD, USA). An average of 0·2 mm² of the superficial dermis was examined per patient. CD8 T cells were stained by a rat monoclonal antibody to human CD8 (Serotec Ltd, Oxford, UK) and detected by a peroxidase conjugated goat antiserum to rat (Multilink, Medite, Nunningen, Switzerland). 3,3’-diaminobenzidine was added as a substrate (DAB ready-to-use, DAKO). We quantified CD8 T cell infiltrates by counting CD8 positive cells per high-power visual field. Perivascular cytotoxic T lymphocytes were detected by incubating slides with rat monoclonal antibody to CD3 (1 in 200, Serotec), with a mouse monoclonal antibody to GRANULE MEMBRANE PROTEIN 17 (GMP-17) (1 in 100, Immunotech, France) and with UEA-I (1 in 400, DAKO) for 30 min at room temperature. Slides were rinsed with phosphate-buffered saline and incubated with rabbit antiserum to UEA-I (1 in 400, DAKO) for 30 min. After rinsing again with phosphate-buffered saline, Cyanin 3 (red fluorescent) conjugated donkey antirat (1 in 500), fluorescein isothiocyanate (green fluorescent) conjugated donkey antimouse (1 in 40) and Cyanin 5 (blue fluorescent) conjugated donkey antirabbit (1 in 200) (all from Jackson Immuno Research, West Grove, PA) were added for 30 min. We then rinsed the slides again with phosphate-buffered saline and mounted them under glass with hydrophilic embedding medium (Faramount, DAKO). Immunofluorescence triple staining was analysed with a confocal microscope (Zeiss, Jena, G). Cytotoxic T lymphocytes were identified as cells coexpressing CD3 and GMP-17.

Circulating vWF antigen was measured in EDTA plasma with a commercially available ELISA kit (Corgenics, Westminster, CO) in accordance with the manufacturer’s instructions. As a standard curve, serial dilutions of normal pool plasma were used and values expressed as a proportion of undiluted normal pool plasma.

**Statistical analysis**

Group comparisons were done with the Mann-Whitney U test. A p value of less than 0·05 was judged significant.
transplantation). On day 69, chronic GVHD was diagnosed histologically. At this time, we counted 100 blood vessels/mm² in the superficial dermis. Despite intensive immunosuppressive treatment, this number fell to 37/mm² on day 90. The patient's condition subsequently stabilised and on day 160, his subepidermal blood vessel count rose to 100/mm², suggesting that the process of vascular loss is reversible if GVHD can be stopped.

As an indirect sign of endothelial injury after allogeneic stem cell transplantation, we measured plasma vWF concentrations in patients with acute and chronic GVHD.
assessed for presence of activated cytotoxic T lymphocytes with acute and three with chronic GVHD) were further (p=0·01, figure 4). Five of these biopsy samples (two infiltrations correlated with plasma vWF concentrations patients, the amount of subepidermal CD8 T cell on the same day as we obtained blood samples. In these patients (four with acute and four with chronic GVHD) for vascular endothelial cells (blue), T lymphocytes (red), and a membrane marker of activated cytotoxic T lymphocytes (green). (A) normal skin contains very few T lymphocytes. (B) both mononuclear cell infiltrates and microvascular endothelial cell injury can be seen, consisting of cell swelling (arrowheads) and denudation (arrows). (C) perivascular infiltration by activated cytotoxic T lymphocytes coexpressing CD3 and GMP-17 (arrowheads) can be seen. (D) occasionally, activated cytotoxic T lymphocytes are attached to the luminal surface of microvascular endothelial cells (arrowheads).

and in patients without GVHD. vWF was higher in patients with acute GVHD (median 269%, IQR 214–295) than in controls (102%, 88–118) (p=0·0005, figure 3). Furthermore, vWF concentrations were also higher in patients with chronic GVHD (226%, 198–312) than in controls (p=0·0005; figure 3), or in long-term surviving patients without GVHD (131% [101–144], p=0·0005; figure 3).

Patients with acute GVHD had thrombocytopenia (34×10^9/L, 32–136), but those with chronic GVHD and those without GVHD had normal platelet counts (215×10^9/L [142–265] and 209×10^9/L [159–299], respectively).

Six patients with GVHD were not given ciclosporin at the time of investigation (table). These patients still had higher vWF concentrations (238%, 168–288) than did healthy controls (102%, 88–118, p=0·0005). Patients with GVHD given ciclosporin had the same median vWF concentrations (240%, 219–312) as patients without ciclosporin treatment.

We took biopsy samples from the lesional skin of eight patients (four with acute and four with chronic GVHD) on the same day as we obtained blood samples. In these patients, the amount of subepidermal CD8 T cell infiltration correlated with plasma vWF concentrations (p=0·01, figure 4). Five of these biopsy samples (two with acute and three with chronic GVHD) were further assessed for presence of activated cytotoxic T lymphocytes by the marker protein GMP-17. In four of five patients analysed, we identified activated cytotoxic T lymphocytes in the perivascular region (figure 5), and in some instances, these cells were attached to the luminal surface of microvascular endothelial cells (figure 5). In these patients, histological signs of vascular injury such as endothelial swelling or denudation were seen (figure 5). However, not all CD3 positive T lymphocytes coexpress GMP-17 and these cells may be CD4 T helper cells known to be present in cutaneous GVHD or incompletely activated CD8 cytotoxic T cells.

Discussion

The pathogenesis of chronic GVHD in human beings is not well understood. Clinically, this disorder resembles some autoimmune diseases such as progressive systemic sclerosis or Sjögren’s syndrome. However, the true molecular or cellular effector mechanisms leading to chronic GVHD are unknown. Our results show that chronic GVHD is characterised by extensive loss of microvessels in affected target tissues (eg, in skin), and suggest that vascular remodelling does not compensate for the extensive loss of capillaries seen in patients with chronic GVHD. The earliest significant drop of blood vessel counts in our series was recorded 69 days after stem cell transplantation, and because this time is fewer than 100 days, the disease would thus be generally classified as late acute GVHD rather than chronic GVHD. Our observations suggest that chronic GVHD is the progressive manifestation of a process of vessel loss, mediated by cytotoxic T lymphocytes, that begins during acute GVHD. We identified high concentrations of circulating vWF in patients with GVHD. By contrast with Tsakiris and colleagues’ findings, our results suggest that ciclosporin was not the reason for these raised concentrations of vWF. Platelets were also not the reason for these raised concentrations. We conclude that vWF is released from vascular endothelial cells injured by cytotoxic T lymphocytes. In some patients, the amount of vascular loss was substantial. The loss may cause tissue ischaemia, with resultant activation of hypoxia responsive genes such as vascular endothelial growth factor. The activation of angiogenic factors could account for the reversibility of the process, which was recorded in one patient. Angiogenesis can take place even in severely injured tissues and restore perfusion. This observation is important from a therapeutic standpoint: irreversible fibrosis might be avoided by specifically protecting regenerating endothelial cells against injury caused by cytotoxic T lymphocytes.

Such cytotoxic T-cell mediated injury has been implied in the pathogenesis of some autoimmune diseases. Specific nuclear autoantibodies recognising cell cycle antigens, which appear exclusively during apoptosis induced by cytotoxic T lymphocytes. Allergic T cells of fetal origin have been identified in lesional skin of female patients with progressive systemic sclerosis.
Our data suggest that activated cytotoxic T lymphocytes may cause endothelial injury leading to microvessel loss in chronic GVHD in human beings. This process takes place independently of epidermal injury, which is a histopathological characteristic of acute cutaneous GVHD. Endothelitis, the subendothelial accumulation of activated cytotoxic T lymphocytes, is the harbinger of severe rejection of solid organs, and is an important risk factor for chronic rejection of allografts.21

Our data suggest that endothelitis mediated by cytotoxic T lymphocytes in cutaneous GVHD might be the precursor lesion for blood vessel loss which evolves into chronic GVHD.

Our results have implications for management of patients after allogeneic stem cell transplantation. First, persistently high concentrations of vWF in plasma may be a useful test for early identification of patients at risk to develop chronic GVHD. Second, if our interpretation of the pathogenetic sequence is correct, then preventative or interventional treatment for chronic GVHD should be targeted at protecting endothelial cells from injury.

Contributors
B Biedermann designed the scientific project, analysed histopathological slides, interpreted results, and wrote the report. S Sahner did histopathological stainings. D Tsakiris measured vWF concentrations. C Jeanneret was involved in the morphometric analysis and gave advice on the report. J Pober contributed to the design of the study and gave advice on the report. M Gregor, D Tsakiris, and A Gratwohl were responsible for patient management and clinical care and gave advice on the report.

Conflict of interest statement
None declared.

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