Mechanisms of disease

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

Barbara C Biedermann, Silvia Sahner, Michael Gregor, Dimitrios A Tsakiris, Christina Jeanneret, Jordan S Pober, Alois Gratwohl

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 capilllaries/mm^2; IQR 16–98) than of healthy controls (205 capilllaries/mm^2; 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.[1] Up to 60–70% of patients who have had allogeneic stem cell transplantation develop chronic graft versus host disease (GVHD).[2] Chronic GVHD is an important cause of morbidity and mortality in long-term survivors of allogeneic stem cell transplantation[3] 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.[4] Endothelialitis 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.[5] 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.[6] Although the mechanism of allograft vasculopathy is unknown, it has been associated with presence of cytotoxic T lymphocytes adjacent to injured endothelial cells.[7,8] After allogeneic stem cell transplantation, vascular endothelial cells are the first allogeneic recipient cells encountered by circulating immunocompetent donor T cells. Vascular injury has been described in patients with acute GVHD,[9] and arterial changes similar to allograft vasculopathy have been described in patients with chronic GVHD.[10] 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.[11] 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

GLOSSARY

CYTOTOXIC T LYMPHOCYTES
Cytotoxic T lymphocytes are usually CD8 positive T lymphocytes that recognise major histocompatability complex class-I bound antigens or foreign major histocompatability complex class-I molecules. They are capable of killing antigen-bearing target cells within minutes to hours. Their major cytolytic effector molecules are perforin, granzymes, fas ligands, and tumour necrosis factor.

GRANULE MEMBRANE PROTEIN 17 (GMP-17)
Granule membrane protein 17 is a membrane-bound protein of activated cytotoxic T lymphocytes. It is also expressed on macrophages, neutrophils, and natural killer cells and it is not specific for cytotoxic T cells. However, if this protein is coexpressed with CD3, it discriminates activated effector cytotoxic T cells from naive CD8 T lymphocytes.

ULEX EUROPAEUS AGGLUTININ I
Ulex europaeus agglutinin I is a lectin derived from the furze plant that recognises blood group H. It is a specific and useful marker for cutaneous vascular endothelial cells.

WEIBEL PALADE BODIES
Weibel palade bodies are endothelial intracellular storage granules containing von Willebrand factor (vWF) and P selectin. After exposure to histamine or thrombin, Weibel Palade bodies degranulate and release vWF into the plasma and vessel wall, while P selectin is displayed on the cell surface.

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.[12] 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 (pH 6) 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 for 30 min. For immunohistochemistry, slides were preincubated with 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,

Sex	Donor (sex, relationship)	Age at SCT (years)	Underlying disease	Conditioning	Time of investigation (days after SCT)		Treatment at investigation
					Biopsy	Blood plasma	
Acute GVHD (n=8)							
F	M, sibling	39	ALL	Etoposide, cyclophosphamide, TBI	48	26	Prednisone, ciclosporin
M	M, sibling	22	ALL	Etoposide, cyclophosphamide, TBI	ND	98	Prednisone, ciclosporin
F	M, unrelated	26	CML	Etoposide, cyclophosphamide, TBI	ND	82	Prednisone, ciclosporin
F	M, sibling*	40	MM	Etoposide, cyclophosphamide, TBI	ND	22	Prednisone, ciclosporin
F	M, sibling	57	NHL	Fludarabine, TBI	83	83	None
M	F, unrelated	31	CML	Etoposide, cyclophosphamide, TBI	7	7	Prednisone, ciclosporin
M	M, sibling†	54	CLL	Fludarabine, TBI	89	89	None
F	F, sibling	63	MM	Fludarabine, TBI	94	94	None
	Median (IQR)	40 (30–55)			83 (48–89)	82 (25–90)	
Chronic GVHD (n=10)							
M	F, sibling	15	AML	Etoposide, cyclophosphamide, TBI	1931	4606	Prednisone, ciclosporin
M	M, sibling	29	CML	Etoposide, cyclophosphamide, TBI	3479	3106	Prednisone
M	F, sibling	41	CML	Etoposide, cyclophosphamide, TBI	1917	1699	Prednisone, ciclosporin
W	M, unrelated	19	ALL	Etoposide, cyclophosphamide, TBI	826	826	None
M	M, sibling	53	MPS	Etoposide, cyclophosphamide, TBI	488	488	Prednisone
M	M, sibling	40	CML	Cyclophosphamide, TBI	160	518	Prednisone, ciclosporin, mycophenolate mofetil
M	F, sibling	59	MDS	Cyclophosphamide, ATG	188	370	Prednisone, ciclosporin
M	F, sibling	36	ALL	Cyclophosphamide, TBI	111	111	Prednisone, ciclosporin
M	F, sibling*†	42	CML	Cyclophosphamide, ATG	196	331	Prednisone, ciclosporin
F	M, unrelated	61	MDS	Fludarabine, TBI	206	206	Ciclosporin
	Median (IQR)	40 (31–50)			347 (190–1644)	503 (341–1481)	
Long-term survivors without GVHD (n=9)							
F	F, sibling	30	AA	Cyclophosphamide	ND	3192	None
M	F, sibling	45	AML	Etoposide, cyclophosphamide, TBI	ND	2058	None
M	M, unrelated	30	ALL	Etoposide, cyclophosphamide, TBI	ND	1561	None
M	M, unrelated	42	CML	Etoposide, cyclophosphamide, TBI	ND	1490	None
M	M, sibling	37	CML	Etoposide, cyclophosphamide, TBI	ND	1435	None
F	F, sibling	55	AML	Etoposide, cyclophosphamide, TBI	ND	1407	None
M	F, sibling	30	NHL	Etoposide, cyclophosphamide, TBI	ND	819	None
M	M, sibling	43	AML	Etoposide, cyclophosphamide, TBI	ND	375	None
M	M, sibling	34	MM	Etoposide, cyclophosphamide, TBI	ND	371	None
	Median (IQR)	37 (30–43)				1435 (819–1561)	

SCT=stem cell transplantation. AA=aplastic anemia. AML=acute myeloid leukemia. ALL=acute lymphoblastic leukemia. CML=chronic myeloid leukemia. CLL=chronic lymphatic leukemia. MM=multiple myeloma. NHL=non-Hodgkin lymphoma. MPS=myeloproliferative syndrome. MDS=myelodysplastic syndrome. TBI=total body irradiation. ATG=antithymocyte globulin. ND=not done. All patients had 100% donor chimerism except where indicated. *One HLA mismatch. †Donor chimerism >95%.

Patients' characteristics

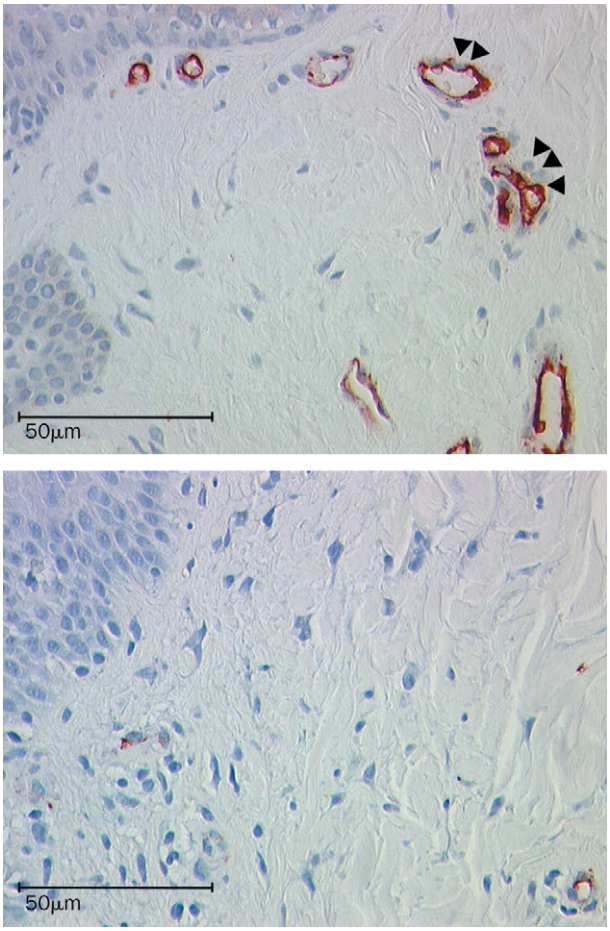

Figure 1: **Visualisation of cutaneous blood vessels by UEA-I binding**
(Upper) skin biopsy sample from a healthy participant showing capillary loops (arrows). (Lower) skin biopsy sample from a patient with chronic GVHD showing extensive loss of blood vessels.

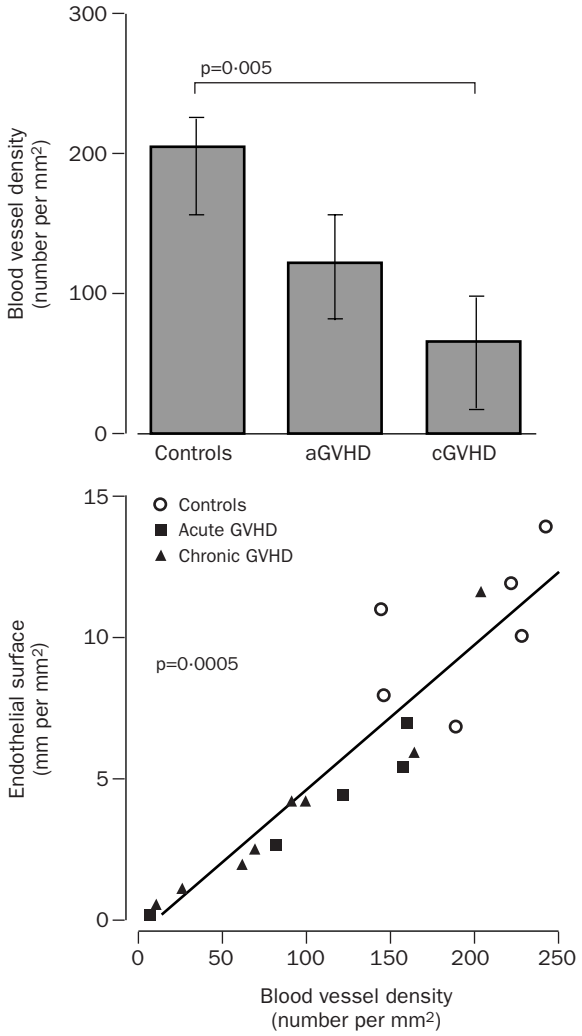

Figure 2: **Microvessel density in skin biopsy samples**
(Upper) blood vessel density in healthy controls (n=6) is compared with patients with acute GVHD (aGVHD, n=5) and those with chronic GVHD (cGVHD, n=10). Values are median (IQR). (Lower) true loss of the vessels is confirmed by the strong correlation of vessel number with endothelial surfaces for all groups.

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-ethylcarbazole 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). On average, 0·2 mm^2 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.

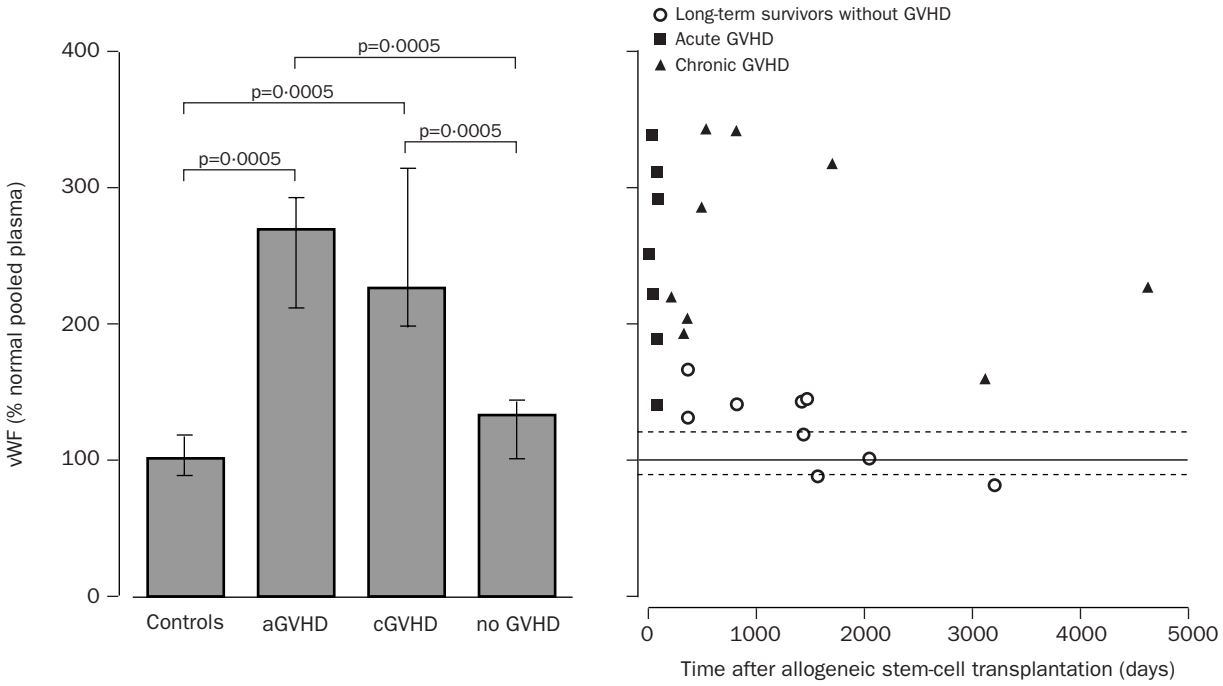

Figure 3: **Circulating concentrations of von Willebrand factor (vWF)**
(Left) plasma concentrations of vWF from patients with acute (n=8) and chronic GVHD (n=10) were compared with those of healthy controls (n=9) or long-term survivors without GVHD (n=9). Values are median (IQR). (Right) sustained elevation of plasma vWF concentrations is seen in patients with GVHD. Long-term survivors without GVHD have normal concentrations of vWF. Solid line=median vWF concentration of nine healthy controls; dashed line=IQR.

Role of the funding source
The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or in the writing of the report.

Results

Patient characteristics are shown in the table. The median age of controls was 39 (IQR 33–47). In our analysis of tissue biopsy samples from patients with chronic GVHD, we noted profound loss of blood vessels in the superficial dermis (figure 1). Compared with skin from healthy controls, few blood vessels were detected in patients with chronic GVHD. To quantify the amount of vascular loss, we counted blood vessels in cross-sections of the superficial dermis and normalised the counts to the surface analysed (figure 2). Healthy controls had a median of 205 capillaries per mm^2 (IQR 157–226). In patients with chronic GVHD, we counted only 66 capillaries per mm^2 (16–98) (p=0·005). Since the reduced number of capillaries could be compensated for by dilatation of the remaining vessels, we also measured the length of luminal vascular surface lined by vascular endothelial cells. For every mm^2 of superficial dermis, healthy controls had a median of 10·4 mm (8·4–11·7) luminal vascular surface lined by endothelial cells and patients with chronic GVHD had 2·2 mm (0·7–4·2) (p=0·005). The number of capillaries correlated well with the luminal endothelial surface (figure 2). We also recorded microvessel loss to 123 capillaries per mm^2 (IQR 83–158) in patients with acute GVHD, and the pattern of blood vessel loss was similar to that seen in those with chronic GVHD (p=0·1 figure 2). In these patients, the earliest time by which significant loss of the vessels was documented was 69 days after stem cell transplantation. Three sequential skin biopsy samples were available from one patient with early-onset chronic GVHD (arising <100 days after stem cell transplantation). On day 69, chronic GVHD was diagnosed histologically. At this time, we counted 100 blood vessels/mm^2 in the superficial dermis. Despite intensive immunosuppressive treatment, this number fell to 37/mm^2 on day 90. The patient's condition subsequently stabilised and on day 160, his subepidermal blood vessel count rose to 100/mm^2, 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

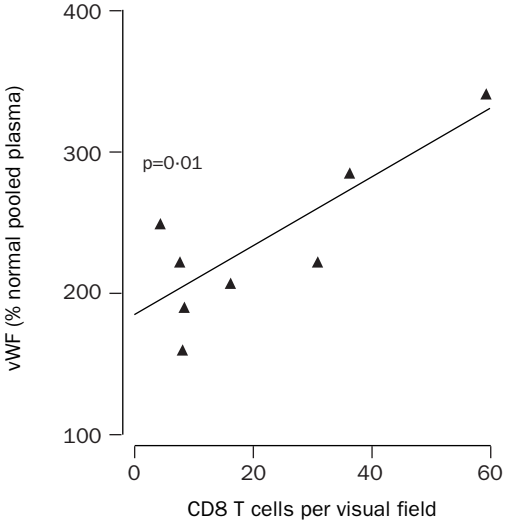

Figure 4: **Plasma concentration of von Willebrand factor (vWF) correlates with T lymphocyte infiltrate**
Concentrations of vWF in plasma are plotted against the number of infiltrating, dermal CD8 T lymphocytes.

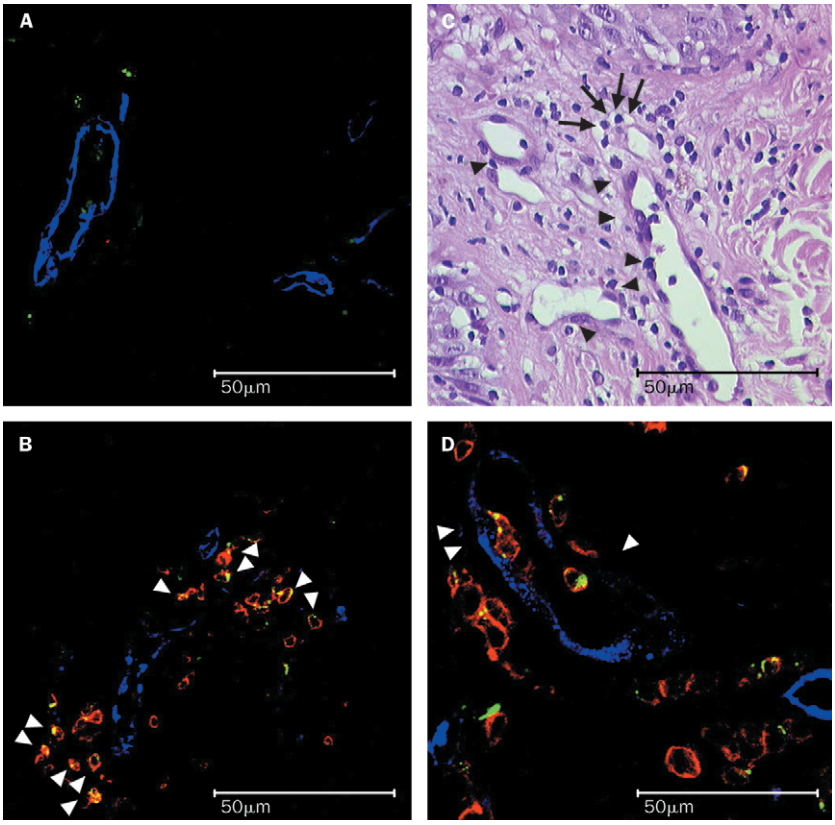

Figure 5: **Immune-mediated vascular injury in GVHD**
Skin biopsy samples from a healthy individual (A) and a patient with chronic GVHD (B–D) are stained for vascular endothelial cells (blue), T lymphocytes (red), and a membrane marker of activated cytotoxic T lymphocytes (green). (A) normal skin contains 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 significantly 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[13] 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 progessive systemic sclerosis or Sjögren's syndrome.[14] 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.[16] 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,[15] 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.[17] 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 recognise nuclear antigens, which appear exclusively during apoptosis induced by cytotoxic T lymphocytes.[18] Alloreactive T cells of fetal origin have been identified in lesional skin of female patients with progressive systemic sclerosis.[19,20]

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. Endothelialitis, the subendothelial accumulation of activated cytotoxic T lymphocytes, is the harbinger lesion of severe rejection of solid organs,[5] and is an important risk factor for chronic rejection of allografts.[21] Our data suggest that endothelialitis 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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