Microangiopathy in Patients on Cyclosporine Prophylaxis Who Developed Acute Graft-Versus-Host Disease After HLA-Identical Bone Marrow Transplantation

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Severe microangiopathy has been reported as a rare complication of cyclosporine A (CsA) prophylaxis in allogeneic bone marrow transplantation (BMT). We found morphological and biochemical changes indicative of generalized endothelial damage in 49 of 66 allogeneic marrow graft recipients receiving cyclosporine, but none in 11 patients treated with methotrexate for prophylaxis of graft-*v*-host disease (GVHD). Changes occurred after engraftment of bone marrow and consisted of intravascular hemolysis with red cell fragmentation and de novo thrombocytopenia. They were preceded by a decrease in activated partial thromboplastin time and fibrinogen indicating activation of coagulation. Endothelial damage as the central lesion of microangiopathy was confirmed by a simultaneous increase of factor VIII related antigen. Severe microangiopathy was observed in ten patients and was fatal in seven. Risk factor analysis revealed a highly significant association of microangiopathy with severity of acute GVHD (aGVHD) ($P < .001$) and use of CsA prophylaxis ($P < .001$). Our data suggest endothelial damage as a result of cellular activation and subsequent release of cytokines in the course of aGVHD, which is not inhibited by CsA prophylaxis.

ACUTE graft-*v*-host-disease (aGVHD) is still a major risk in allogeneic bone marrow transplantation (BMT) and contributes to about 15% of transplant-related deaths in spite of the prophylactic use of immunosuppressive agents.[1] When cyclosporine A (CsA) was introduced for prophylaxis of aGVHD, several centers reported a beneficial effect of CsA on survival rates. Randomized trials comparing methotrexate (MTX) and CsA, however, were not able to confirm a significant reduction of the incidence of aGVHD or an improvement of survival.[2] Recently the combination of short-term MTX with long-term CsA has been shown to be superior to prophylaxis with CsA alone.[3]

In addition, the use of CsA has been accompanied by a series of novel side effects that were not seen in historical controls receiving MTX prophylaxis.[1,4] A high incidence of renal and vascular side effects has been observed in patients with bone marrow grafts, as well as in recipients of other organ transplants.[5-7] Chronic vascular CsA toxicity is associated with thrombotic microangiopathy[5]: A minority of patients with renal or bone marrow transplants developed severe and fatal syndromes resembling thrombotic thrombocytopenic purpura (TTP).[8-11] The pathogenesis of microangiopathic lesions, however, is still controversely discussed: Enhancement of CsA toxicity by immune mechanisms as vascular rejection in kidney transplants[5] and acute graft-*v*-host reactions in HLA mismatched BMT[12] has been postulated.

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CsA has been used in our bone marrow transplant unit since 1984 and was combined with a short course of MTX in most patients following allogeneic BMT. We repeatedly observed clinical and biochemical changes indicative of microangiopathy in patients receiving HLA identical bone marrow grafts. Clinical courses, risk factors, and indicators were, therefore, analyzed in 66 patients treated with CsA (53 patients in combination with short-term MTX), in 11 patients receiving MTX prophylaxis only, and in two recipients of syngeneic transplants. The data suggest a major pathophysiological role of aGVHD in the development of microangiopathy.

MATERIALS AND METHODS

Patients. Patients were admitted for a variety of hematological disorders or malignancies at various stages of their disease. Patient characteristics are summarized in Table 1. Patients with leukemia and refractory anemia with excess of blasts (RAEB) were conditioned with total body irradiation (single dose of 9.4 Gy or fractionated with 4 Gy on three consecutive days at a dose rate of 4.5 cGY/min) or busulphan 4 mg/kg on four consecutive days followed by cyclophosphamide between 120 mg/kg and 200 mg/kg over two to four days. Patients with severe aplastic anemia were treated with total lymphoid irradiation instead of total body irradiation. Seventy-seven patients received allogeneic HLA identical, and two syngeneic bone marrow grafts from sibling donors. All consecutive patients surviving at least 3 weeks after BMT and showing signs of engraftment were included in the analysis. Nineteen patients had major and another 17 patients minor ABO or Rh blood group incompatible grafts. Procedures and attendant risks were fully explained to patients, donors, and relatives. All patients and donors gave written informed consent.

Prophylaxis and treatment of GVHD. Prophylaxis of GVHD was performed by MTX in 11 patients according to the Seattle protocol,[13] and 66 patients received CsA. CsA was administered intravenously (IV) by continuous infusion from day −1 until day 28. It was started with 10 mg/kg on day −1, followed by 5 mg/kg/d from day 0 until day 4, and 3 mg/kg/d from day 5 until day 28. CsA was then administered orally (12 mg/kg/d in two divided doses). In 53 patients CsA was combined with a short course of IV MTX (15 mg/m^2 on day 1, 10 mg/m^2 on days 3 and 6). CsA dose was adjusted to plasma levels of 150 to 200 ng/mL in the first 2 to 3 months after BMT and then gradually reduced.

Acute GVHD was graded according to clinical and biochemical criteria[13,14] and confirmed by biopsy in a minority of patients.

Table 1. Characteristics of Patients and Frequency of Microangiopathic Changes

	Total No. of Patients	Patients with Microangiopathy		P
		N	(%)	
Sex				
Female	30	19	61	
Male	49	30	63	NS
Age				
<20 yr	16	8	50	
<35 yr	44	28	64	NS
35-50 yr	19	13	68	
Diagnosis				
Acute leukemias	43	28	65	
Chronic myeloid leukemia	25	15	60	NS
SAA	8	3	38	
RAEB	3	3	100	
Stage of disease				
Good risk	60	41	68	
Bad risk	19	8	42	NS
ABO/Rh incompatibility				
Absent	43	29	67	
Major or minor	36	20	56	NS
Pretransplant conditioning regimen				
STBI	10	2	20	
FTBI	56	40	71	NS*
TLI	7	3	43	
Busulphan	6	4	67	
Prophylactic immunosuppression				
None (syngeneic)	2	0	0	
MTX	11	0	0	
CsA	11	7	64	<.001
CsA (+MTX)	55	42	76	
Viral infections (HSV, CMV)				
Documented by cultures	29	18	62	NS
Absent	50	37	74	
Maximal overall aGVHD				
0-I	32	13	41	
>I-II	28	21	75	<.01
III, IV	19	15	79	
Maximal aGVHD of liver				
0-I	48	25	52	
>I-II	20	16	80	NS
III, IV	10	7	70	

Abbreviations: good risk, acute leukemias 1st or 2nd remission, chronic myeloid leukemia chronic phase, SAA and RAEB untransfused; bad risk, all other conditions. STBI, single dose; FTBI, fractionated total body irradiation; TLI, total lymphoid irradiation.

Statistical analysis was performed by Pearson chi-square statistics.

*STBI seems to be associated with less microangiopathic changes ($P < .05$), however eight of these patients received MTX-prophylaxis as well.

Patients with aGVHD grade II or more were treated with prednisolone (2 to 3 mg/kg/d). Corticosteroid treatment was reduced (10% of dosage every three days) as soon as progression of aGVHD stopped. Postmortem necroscopy sections and liver biopsies were performed in fatal courses.

Measurement of CsA levels. CsA plasma levels were assayed twice weekly by radioimmunoassay[15] using a polyclonal antibody (Sandoz, Basel) that recognizes both unmodified CsA and metabolites. EDTA was used as anticoagulant for samples, and before separation of plasma whole blood was equilibrated at 37°C for 60 minutes. During the IV period samples were drawn by peripheral vein puncture while CsA was continuously infused by a right atrial Hickman catheter. When CsA was switched to oral application, 12 hour trough samples were collected. For statistical analysis mean CsA levels were calculated for a 14-day period preceding occurrence of microangiopathy or maximal elevation of serum lactate dehydrogenase activity (LDH). Thirteen patients had to be excluded from this analysis because of incorrect drawing of samples.

Clinical and biochemical data. Clinical and biochemical data were retrospectively analyzed. Microangiopathy was defined by simultaneous occurrence of intravascular hemolysis characterized by appearance of schistocytes in peripheral blood smears and de novo thrombocytopenia. Intravascular hemolysis was diagnosed when a simultaneous rise of serum LDH activity (>250 U/L) and reticulocytes was associated with a decrease in hemoglobin levels. To exclude other causes of elevation of LDH, hemolysis was confirmed by LDH isoenzymes and/or determination of haptoglobin levels. Immune hemolysis could be excluded by a negative Coomb's test in 62 of 66 patients receiving CsA prophylaxis. Extent of de novo thrombocytopenia was calculated as percentual decrease of platelet counts from maximal counts observed after engraftment to minimal counts occurring at the time of hemolysis. Monocyte counts were determined by leukocyte counts and differentiation of peripheral blood smears. To characterize hemostaseological changes activated partial thromboplastin time (aPTT, reference interval 30 to 40 seconds), fibrinogen, prothrombin time, and antithrombin III levels were analyzed. Factor VIII related antigen (FVIIIR:Ag) was determined by a sensitive microELISA method in 30 patients receiving CsA prophylaxis.[16] Renal function was described by maximal serum creatinine.

Statistical analysis. The results are described by means and standard deviation of maxima or minima, for nonsymmetric variables medians and range are reported. For statistical evaluation groups were formed according to clinical criteria in CsA-treated patients. Differences between the groups were analyzed by Wilcoxon tests for metric or ordinal data and by Pearson chi-square analysis for nominal data. Correlations were calculated by Spearmans rank correlation coefficients. Multivariate risk factor analysis in CsA-treated patients included stratification for confounding variables and calculation of chi-square using Mantel-Haenszel statistics as well as multiple linear regression.

RESULTS

Clinical symptoms and relevance of microangiopathic changes in patients receiving CsA prophylaxis. After engraftment of bone marrow reproducible patterns of clinical, hematological, and biochemical changes indicating microangiopathy were found. Changes consisted of prolonged or de novo anemia in spite of high reticulocyte counts, occurrence of fragmented RBCs in peripheral blood smears, and a de novo drop of platelet counts. These patterns were frequently associated with deterioration of preexisting renal impairment and hypertension and detectable in a total of 49 patients (74%) receiving CsA prophylaxis. Eighteen patients required de novo transfusions of packed RBCs or platelets due to hemolytic anemia and thrombopenic purpura. Ten of these patients suffered from severe microangiopathy resembling TTP. Clinical symptoms included excessive hypertension, hemorrhagic diathesis, increase of body weight by interstitial edema, azotemia or renal failure, and mental changes like confusion. They were associated with signs of aGVHD. Seven patients finally died during the course of their disease with multi-organ failure acquiring fungal infections.

Hematological, biochemical, and hemostaseological changes indicating microangiopathy. For statistical analysis patients receiving CsA prophylaxis were grouped according to clinical severity of microangiopathy as indicated in Table 2. The most significant parameters associated with microangiopathy were elevation of LDH activity, schistocyte counts, thrombocytopenia, and elevation of FVIIIR:Ag. Maximal creatinine levels were not different in patients without and with slight to moderate microangiopathy. This reflects preexisting renal impairment that was worsened by microangiopathy in patients with TTP like changes. Hemostaseological changes included shortening of aPTT and a decrease of fibrinogen to subnormal levels. As prothrombine time and antithrombin III levels remained in a normal range in all patients our data indicate activation of coagulation in the course of microangiopathy, which is different from advanced disseminated intravascular coagulation.

Time course of microangiopathy. Microangiopathy always started after engraftment with maximal hemolysis occurring between 21 and 172 days (median, 61 days) and minimal platelet counts between 19 and 140 days (median, 63 days) after BMT. While aPTT and fibrinogen were in a normal range during bone marrow aplasia both parameters decreased after engraftment simultaneously with occurrence of first microangiopathic changes. This decrease was associated with an increase in monocyte counts, and there was a highly significant correlation of the time of maximal monocyte counts with the time of minimal aPTT and fibrinogen ($P < .001$, Fig 1).

Time course of microangiopathy is best reflected by elevation of FVIIIR:Ag levels (Fig 2): A significant rise could be observed from pretransplant normal levels (106 ± 18%) to a plateau of 172 ± 23% one day after starting CsA prophylaxis. This plateau remained stable until engraftment (median, day 19) and was followed by a subsequent rise in FVIIIR:Ag depending on severity of microangiopathy. In contrast, long-term survivors (more than 9 months after BMT) treated with comparable doses of CsA for chronic GVHD had FVIIIR:Ag levels in the range of patients during bone marrow aplasia (152 ± 38%, n = 15, data not shown). These patients never developed signs of more severe microangiopathy.

Risk factors of microangiopathy. Patient characteristics revealed no influence of age, sex, diagnosis, stage of disease, pretransplant conditioning regimens, frequency of major or minor blood group incompatibility, and incidence of viral infections (herpes- or cytomegaloviruses) on development of microangiopathy (Table 1). Use of CsA and aGVHD turned out to be the only significant risk factors in this univariate analysis.

Microangiopathy was not at all observed in patients receiving MTX prophylaxis or syngeneic transplants. Nine of 11 patients treated with MTX had elevated levels of LDH associated with a minor drop in hemoglobin, but fragmented RBC's were virtually absent in these patients as well as other indicators of microangiopathy (Table 3). Hemolysis might be explained by a higher incidence of blood group incompatibility in these patients (n = 6).

Absence of microangiopathy in patients receiving MTX prophylaxis allowed multivariate analysis of risk factors only in patients receiving CsA. Variance analysis of patients suffering from various degrees of microangiopathy showed a tendency to higher CsA levels in patients with severe microangiopathy ($P < .05$) but a strong correlation of microangiopathy with severity of aGVHD ($P < .001$). Correlation of microangiopathy with CsA levels beyond therapeutic range (>200 ng/mL), however, was not significant in chi-square analysis: Microangiopathy occurred in 18 of 25 patients with CsA levels below 200 ng/mL and in 24 of 28 patients with levels beyond this range (72% *v* 85%, not significant). In contrast 29 of 31 patients with aGVHD grade II or more suffered from microangiopathy while symptoms were absent in 15 of 35 patients without aGVHD or aGVHD grade I (43% *v* 94%, $P < .001$ in chi-square statistics). For multivariate analysis this correlation was stratified for confounding variables using a Mantel-Haenszel statistic: Age, sex, diagnosis, stage of disease, pretransplant conditioning regimens, major of minor blood group incompatibility, addition of

Table 2. Clinical Relevance of Microangiopathy and Associated Biochemical, Hematological, and Hemostaseological Changes in 66 Patients Receiving CsA Prophylaxis

Severity of Microangiopathy	Absent		Mild		Moderate		Severe
N	13		31		8		10
LDH (U/L)	211	***	372	NS	473	***	1,379
	(35)		(109)		(189)		(721)
Fragmented RBCs (%)	0.2	***	0.9	*	2.0	*	4.6
	(0.3)		(0.5)		(1.1)		(2.2)
Decrease of platelet counts (%)	20	***	45	*	64	NS	77
	(25)		(15)		(17)		(10)
Maximal creatinine (μmol/L)	107	NS	133	NS	109	***	277
	(19)		(40)		(13)		(145)
Minimal aPTT (sec)	30	NS	28	*	24	NS	26
	(5)		(4)		(2)		(4)
Minimal fibrinogen (g/L)	2.3	*	1.6	*	1.4	NS	1.1
	(0.9)		(0.8)		(0.3)		(0.3)
Prothrombine time (%)	96	NS	96	NS	98	NS	87
	(11)		(9)		(4)		(11)
Antithrombine III (%)	97	NS	105	NS	109	NS	102
	(22)		(16)		(18)		(21)
Maximal F VIII-related Ag (%)	207	*	269	***	466	*	642
	(77)		(60)		(63)		(143)
n	12		8		6		4

Microangiopathy was graded according to clinical symptoms: mild, biochemical and hematological signs; moderate, patients requiring transfusions due to hemolysis and thrombopenia; severe, TTP-like changes.

Mean and standard deviation of parameters are shown. FVIIIR:Ag was analyzed in 30 patients as indicated by n. Groups were compared by Wilcoxon tests, and statistical differences between the groups are indicated: *, $P < .05$; **, $P < .01$, ***, $P < .001$.

short-term MTX to CsA, CsA levels, and presence or absence of viral infections did not influence the strong association of microangiopathy with severity of aGVHD (P always <.01). This was also confirmed by multiple linear regression.

Attempts of prevention and treatment in patients suffering from microangiopathy. As revealed by our analysis, microangiopathy could not be prevented by addition of short-term MTX to CsA nor by increasing CsA doses to control aGVHD. In addition, treatment of patients with

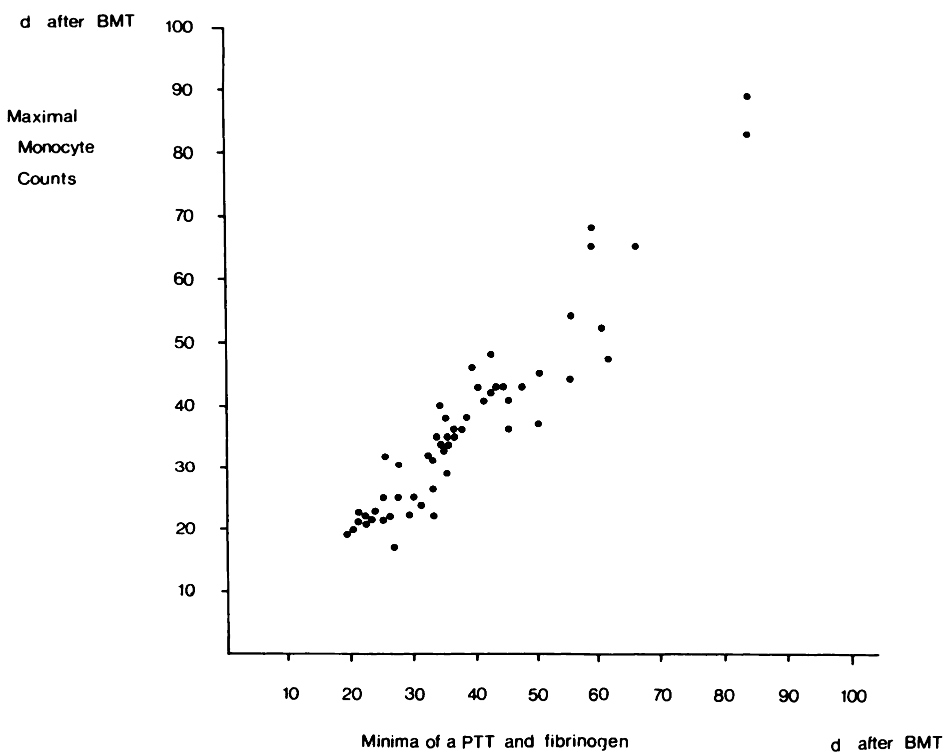

Fig 1. Association of maximal monocyte counts and minimal of aPTT and fibrinogen in CsA-treated patients: r = .93. Data of 55 patients were evaluable.

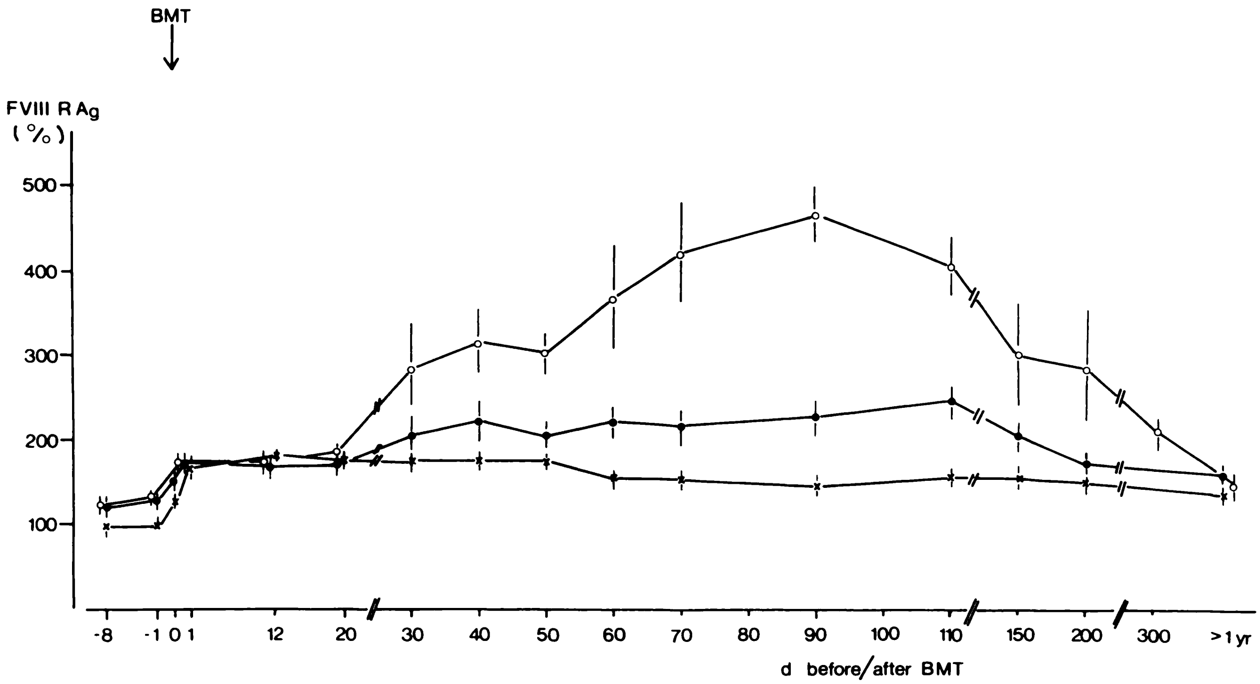

Fig 2. Time course of microangiopathy as indicated by FVIIIR:Ag levels. Thirty patients were analyzed. x, patients without microangiopathy (n = 12); ●, patients with slight microangiopathy (n = 8); O, patients with moderate and severe microangiopathy (n = 10). Mean and SE are shown. Statistical analysis was performed by *t* test for days 30, 60, 90, and 150 after BMT: Differences were significant for day 60 ($P < .05$) and for day 90 ($P < .001$).

corticosteroids had no influence on severity of microangiopathy as all patients with aGVHD grade II or more received prednisolone. In patients with severe microangiopathy treatment was attempted by reduction or discontinuation of CsA (n = 9), long-term oral dipyridamole (n = 4), infusions of fresh frozen plasma (n = 8), continuous infusion of prostaglandin E1 (n = 3), or plasmapheresis (n = 3). However, improvement of microangiopathy could not be related to any of these attempts.

DISCUSSION

Our data reveal a high incidence of microangiopathic lesions in patients receiving HLA identical bone marrow grafts and CsA prophylaxis or treatment. Microangiopathy could be graded from mild or asymptomatic lesions to severe and lethal courses, and its overall frequency is higher than reported by others.[7,9] Endothelial damage could be demonstrated as the central lesion by determination of FVIIIR:Ag levels.[17] Elevation of FVIIIR:Ag has been attributed to CsA nephrotoxicity in renal transplant patients.[18] This toxicity was shown to be mediated by inhibition of prostacyclin synthesis in endothelial cells,[19,20] which is a potent inhibitor of coagulation on endothelial surfaces. Thus CsA might cause vascular nephrotoxicity and further microangiopathic changes as also postulated for BMT recipients. Enhancement of microangiopathy by CsA and the exclusive occurrence of microangiopathy in CsA-treated patients in our study might therefore be explained by defective endothelial protection against further damage.

Table 3. Absence of Microangiopathic Changes in Patients Receiving MTX Prophylaxis as Compared With CsA-Treated Patients

	MTX	CsA	*P*
LDH (U/L)	472 (79)	591 (74)	NS
Decrease of platelets (%)	9.8 (4.6)	52.0 (3.1)	<.001
Maximal creatinine (μmol/L)	84 (9)	154 (13)	<.001
Fragmented RBCs (%)	Absent	2.0 (0.3)	—
Minimal fibrinogen (g/L)	2.4 (0.3)	1.6 (0.1)	<.05
Minimal aPTT (sec)	39.2 (2.4)	27.3 (0.6)	<.001

All patients showing elevation of LDH (>250 U/L) were compared (MTX n = 9, CsA n = 49). Mean ± SE are shown. Statistical analysis was performed by *t* tests.

Our data show generalized endothelial damage distinct from isolated nephrotoxicity and do not allow interpretation of microangiopathy as a consequence of CsA toxicity. Correlation of development and severity of microangiopathy with severity of aGVHD indicate pathophysiological mechanisms associated with aGVHD. This correlation could be explained by altered hepatic methobolism of CsA in the course of hepatic GVHD.[21] However severe microangiopathy with lethal outcome also occurred in four patients without any evidence of hepatic aGVHD. There are two other complications of BMT associated with endothelial damage: Veno-occlusive disease (VOD) and "endothelial leakage syndrome." FVIIIR:Ag levels have been reported to be elevated in patients with VOD.[22] Differential diagnosis of VOD by clinical criteria is difficult, especially with regard to aGVHD or drug toxicity of liver but usually this complication starts within the first 2 weeks after BMT and is not accompanied by intravascular hemolysis.[23] In addition postmortem liver biopsies failed to show VOD in patients suffering from severe microangiopathy. Endothelial leakage syndrome is a pulmonary complication reported in HLA mismatched BMT using CsA prophylaxis[12] and usually occurs during bone marrow aplasia. Though it might result from a similar pathophysiology it is different from microangiopathy observed in our study as our patients received HLA-matched bone marrow grafts and developed endothelial lesions after engraftment.

Endothelial damage might also be mediated by irradiation of cytotoxic drugs.[24] Risk factor analysis, however, revealed no difference in pretransplant conditioning regimens. Thus in our view aGVHD remains the most significant risk factor for microangiopathy. The biphasic increase in FVIIIR:Ag levels with a second rise following engraftment and the temporal coincidence of peak monocyte counts with activation of coagulation further suggest microangiopathy as a result of cellular activation in the course of aGVHD. Participation of mononuclear cells with macrophage activity would fit with present pathophysiological models: Endothelial cells synthesize thromboplastin after stimulation with a variety of agents including monocyte derived interleukin-1 (IL-1),[25,26] tumor necrosis factor (TNF),[27] and interferon gamma (IFNg) released by activated lymphocytes.[28] In addition, monocytes themselves secrete thromboplastin after stimulation with IFNg[29,30] and CsA has been reported to increase monocyte thromboplastin synthesis.[31] Preliminary results of experiments performed in our laboratory confirm thromboplastin release by activated monocytes. In a mixed lymphocyte culture system, allogeneic response could be monitored by determination of thromboplastin activity in supernatants. This response was completely abrogated by removal of monocytes from responder cells using immunorosetting.

Based on our observations the pathogenesis of microangiopathy might result from activation of lymphocytes in the course of aGVHD with subsequent stimulation of monocytes by IFNg. These in turn secrete cytokines such as TNF and IL-1 causing endothelial damage associated with activation of coagulation and development of microangiopathic lesions. Activation of these mediators is consistent with reported data on restoration of lymphokine production after BMT. While interleukin-2, (IL-2) production is suppressed in short-term patients[32] and CsA is known to inhibit lymphokine release[33] high IFNg levels have been observed early after BMT and in patients suffering from aGVHD under CsA prophylaxis.[34] In addition, monocyte functions and IL-1 production have been shown to be restored as soon as 30 days after BMT.[32]

A second pathway reflecting direct interaction of lymphocytes and endothelial cells seems possible as endothelial cells are able to express class II antigens after stimulation with IFNg and TNF[35,36] and thus might represent targets for aGVHD. However, this pathway should be observed in patients receiving MTX prophylaxis as well.

In our view exclusive occurrence of microangiopathy in patients with CsA prophylaxis calls for reevaluation of the role of CsA beyond defective endothelial repair. Our data suggest an altered pathophysiology of aGVHD in these patients as compared with patients receiving MTX prophylaxis. While MTX exerts its immunosuppressive action by a cytotoxic effect on graft-*v*-host reactive cells CsA is known to inhibit IL-2 production and responsiveness in in vitro systems. The proliferative response of activated cells, however, can easily be restored in the presence of CsA by addition of exogenous IL-2 in a mixed lymphocyte culture system.[37] In addition in vivo assays showed no effect of CsA on early activation of T cells by alloantigens.[38] As suggested by these observations and our analysis CsA might fail to inhibit activation of graft-*v*-host reactive cells in vivo and allow subsequent release of cytokines and development of microangiopathy. In our view more attention should be drawn to these interactions that might offer new diagnostic and therapeutic strategies in patients suffering from aGVHD.

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