Cerebral Endothelial Expression of Adhesion Molecules in Mice with Chronic Graft-Versus-Host Disease

Petra Sostak, MD; Petra Reich, MD; Claudio S. Padovan, MD; Armin Gerbitz, MD; Ernst Holler, MD; Andreas Straube, MD

Background and Purpose—Graft-versus-host disease (GvHD) is a major complication after allogeneic bone marrow transplantation (BMT). The theory that the central nervous system (in addition to the peripheral nervous system) participates in GvHD has been supported by detection of cerebral lymphomononuclear infiltrates in experimental GvHD and the observation of cerebral angiitis-like disease in patients with chronic GvHD.

Methods—In a murine BMT model, we investigated the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on cerebral endothelium after allogeneic and syngeneic transplantation. Through the use of ICAM-1–knockout mice, the effect of ICAM-1 deficiency on cellular infiltration was evaluated. As an indicator of enhanced apoptotic cell death, we examined the cerebral expression of Fas antigen (Fas), the occurrence of the poly-ADP ribose polymerase p85 fragment, and the distribution of TUNEL positive–stained cells.

Results—In close correlation with earlier findings of cerebral infiltration in the same animals, we found cerebral endothelial upregulation of ICAM-1 and especially of VCAM-1 in allogeneic recipients compared with syngeneic animals without GvHD and unmanipulated controls. In ICAM-1–knockout mice, leukocytic infiltration did not differ from that in wild-type animals. Neither cerebral histopathologic changes nor an apoptotic effect of cellular infiltrates on brain parenchyma could be detected.

Conclusions—In this model of experimental GvHD, VCAM-1 may play a critical role in leukocyte recruitment into the central nervous system of animals with chronic GvHD. (Stroke. 2004;35:1158-1163.)

Key Words: immunohistochemistry ■ cerebrovascular disorders ■ endothelium, vascular ■ cell adhesion molecules

Neurologic complications can occur in up to 70% of allogeneic bone marrow recipients,1 and involvement of the central nervous system (CNS) has been reported because of the neurotoxicity of the conditioning regimen, neurovascular complications, and side effects of immunosuppression. Chronic graft-versus-host disease (GvHD), a systemic complication after allogeneic bone marrow transplantation (BMT), is known to cause peripheral neuromuscular disorders,2,3 and there is growing evidence that it can also affect the CNS. In animal models of chronic GvHD, cerebral perivascular and parenchymal lymphomononuclear infiltrations and upregulation of major histocompatibility (MHC) class I and II antigens have been reported.4 Chronic GvHD has also been considered a possible cause of cerebral ischemic and hemorrhagic complications in patients after allogeneic transplantation.5

Because cellular adhesion molecules (CAMs) play a major role in the recruitment of leukocytes into the brain, we investigated the endothelial expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in the brains of mice with chronic GvHD. The same animals were described earlier to have cerebral cellular infiltrations6; we therefore sought to correlate these earlier findings with our results on vascular CAM expression. Through the use of ICAM-1–knockout mice, the influence of ICAM-1 deficiency on leukocyte influx was investigated. To determine whether the infiltrating leukocytes led to cerebral tissue damage in mice with chronic GvHD, we examined the expression of Fas antigen (Fas) and the occurrence of the poly-ADP ribose polymerase (PARP) p85 fragment and terminal deoxynucleotidyl transferase–mediated biotinylated UTP nick end-labeling (TUNEL) positive–stained cells in murine brain parenchyma.

Materials and Methods

Bone Marrow Transplantation

Transplantation was performed according to a previously described protocol.7 All aspects of the following studies were carried out in...
according to the Principles of Laboratory Animal Care and German law on the protection of animals.

After conditioning with total-body irradiation (800 cGy split into 2 doses), C57/B6 (H-2b) mice received 10^6 bone marrow cells and 10^5 spleen cells from LP (H-2b) mice for allogeneic BMT (n=14) or from C57/B6 (H-2b) mice for syngeneic BMT (n=6). The degree of systemic GvHD was assessed by a clinical score that included weight loss, posture, activity, fur texture, and skin integrity. Each parameter was graded from 0 to 2, and a clinical index was generated by summation of the 5 criteria scores (maximum index=10). Five unmanipulated mice served as controls.

Detection Methods (Immunohistology and Immunofluorescence)

On day 42 (maximum of the clinical GvHD index), mice were deeply anesthetized and the brains removed. Each brain was divided into 2 pieces at the level of the diencephalon; the rostral part was snap-frozen and stored at −80°C in isopentane; the caudal part was fixed in 10% buffered formalin and embedded in paraffin. Coronal cryostat sections (6 μm) at the level of the basal ganglia and paraffin sections (3 μm) at the levels of the basal ganglia, hippocampus, and brain stem/cerebellum were obtained. Cryostat sections were fixed with 4% buffered paraformaldehyde. Paraffin sections were dewaxed, rehydrated with xylene and ethanol, and stripped of proteins by incubation in phosphate-buffered saline (PBS) containing 1% Triton X (Sigma) and protease K (Sigma). Brain morphology was evaluated by hematoxylin/eosin staining.

For detection of activated blood vessels, double-immunofluorescence staining was performed on cryostat sections. Fixed sections were incubated in PBS containing 10% normal goat serum (DAKO) and 0.3% Triton X (Sigma) for blocking and permeabilization. The following primary antibodies were serially incubated for 2 hours: polyclonal rabbit anti-mouse laminin (DAKO, 1:400; labels the basement membrane of blood vessels) and either rat anti-mouse CD54 (IgG2a, Serotec, 1:100; detects ICAM-1) or rat anti-mouse CD106 (IgG, Pharmingen, 1:50; marks VCAM-1). Secondary antibodies from Jackson ImmunoResearch were used and incubated for 30 minutes: fluorescein-isothiocyanate–conjugated goat anti-rabbit IgG (1:200, to detect laminin) and Cy3-conjugated goat anti-rat IgG (1:200, to detect ICAM-1 or VCAM-1).

Various pathways that could indicate apoptotic changes as effector mechanisms of cellular infiltration were studied. DNA fragmentation was determined by the TUNEL technique on paraffin sections and the in situ cell death detection kit (fluorescein, Roche Diagnostics GmbH). Immunofluorescent staining against the PARP p85 fragment, which results from caspase cleavage of the 116-kDa intact molecule, was performed on cryostat sections. Fixed sections were incubated in PBS containing 10% normal swine serum (DAKO) and 0.3% Triton X (Sigma) for blocking and permeabilization. The polyclonal antibody against the PARP p85 fragment (Promega) was incubated in a 1:100 dilution overnight. Cy3–conjugated goat anti-rabbit secondary antibody (Jackson, 1:200) was used to detect labeled cells by immunofluorescence. Expression of Fas was assessed by immunohistochemistry on cryostat sections with use of the polyclonal antibody M20 sc-716 (Santa Cruz Biotechnology, 1:200), as well as a peroxidase detection system (biotinylated swine anti-rabbit antibody, 1:200, and streptavidin–horseradish peroxidase, 1:1000; DAKO), with diaminobenzidine as the chromogen.

As positive controls, a tissue section was pretreated with DNase I (Sigma) for 10 minutes before the TUNEL reaction mix was applied; murine spleen tissue was used as a positive control for PARP and Fas detection. All fluorescently labeled sections were covered in fluorescent mounting medium; chromogen-stained sections were mounted in ethanol/xylene and embedded in eukitt (FLUKA).

Stained sections were analyzed by light or fluorescence microscopy on an Olympus BX 60 microscope equipped with an AxioCam camera and KS 300 as the image-analyzing software (Zeiss). Examination was performed by 2 blinded observers who were unaware of the identity of the investigated brain sections with regard to the 3 animal groups. Five hundred randomly selected laminin-positive vessels within the brain parenchyma were examined to determine simultaneous CAM positivity. Vessels were differentiated by size and vessel wall anatomy and were classified as arteriolar/venous or capillary vessels by light microscopy.

The extent of cellular CD45 infiltration into the brain parenchyma has already been determined earlier in the same animals. In short, CD45+ cells within 6 brain parenchymal areas were counted in 10 high-power fields each (magnification, 400×), and the mean number of CD45+ cells (parenchymal CD45+ score per field) was determined. On the basis of these results, parenchymal cellular infiltration was correlated with the extent of brain vessel activation.

Chronic GvHD Model in ICAM-1–Knockout Mice

Transplantation and preparation of brain sections were performed as described earlier. C57/B6 (H-2b) wild-type and ICAM-1–knockout mice received bone marrow from either LP (H-2b) mice for allogeneic transplantation (wild-type mice, n=6; ICAM-1–knockout mice, n=5) or C57/B6 (H-2b) mice for syngeneic transplantation (wild-type mice, n=3; ICAM-1–knockout mice, n=2). Three unmanipulated animals served as controls. Whole brains were snap-frozen, and coronal cryostat sections were obtained that encompassed the diencephalon and the brain stem/cerebellum. Cellular infiltration was detected by immunohistochemistry as described earlier, and the mean CD45+ score per field in 4 brain areas (cortex, hippocampus, brain stem, and cerebellum) was calculated, including both parenchymal and perivascular CD45+ cells.

Statistical Analysis

Values are presented as mean±SD. Animal groups were compared with the Mann–Whitney U test. To describe cellular infiltration as a function of vascular CAM expression, the Pearson correlation coefficient was calculated.

Results

Systemic GvHD

During the first week after transplantation, both syngeneic and allogeneic animals initially showed an increased GvHD score because of weight loss due to irradiation (day 7: syngeneic, 1.5±0.5; allogeneic, 2.0±0.5; P<NS). In the syngeneic group, the GvHD score normalized during the following weeks (day 14, 0.2±0.1; day 42, 0.2±0.3), whereas allogeneic animals subsequently developed elevated GvHD indices (day 14, 1.2±0.3; day 42, 3.3±2.1; both P<0.01 vs syngeneic animal values). In controls, no GvHD signs were detected. Neither controls nor transplanted animals showed neurologic deficits.

Histological Examination

The histologic appearance of the brain parenchyma and vasculature on hematoxylin/eosin-stained slices was normal in all animals.

Vascular Expression of Adhesion Molecules

Figure 1 shows examples of double-immunofluorescent staining of cerebral blood vessels for both groups of transplanted animals. We found constitutive expression of VCAM-1 and constitutive expression of ICAM-1, with ICAM-1–knockout mice having less expression of ICAM-1. In contrast, ICAM-1–knockout mice showed less expression of ICAM-1 compared to wild-type mice.
and activated capillaries (VCAM-1, 4.9±1.7%; ICAM-1, 17±5.9%) was elevated, but compared with controls, a statistically significant difference in vessel activation was assessed for ICAM-1 only on arterioles and venules. In allogeneic mice, a significantly higher proportion of arterioles/venules showed expression of adhesion molecules (VCAM-1, 85.2±2.5%; ICAM-1, 81.1±2.8%) compared with controls and syngeneic animals. Especially for capillaries, there was an elevated CAM expression on vessels of allogeneically transplanted mice (VCAM-1, 21±2.1%; ICAM-1, 39.8±3.9%) versus controls and versus syngeneic BMT recipients. Two of 14 allogeneic animals were excluded from ICAM-1 evaluation because the signal of ICAM-1 immunopositivity could not be differentiated with certainty from the nonspecific background staining.

Cellular Infiltration and Correlation with Vessel Activation
Immunohistologic staining showed diffuse brain parenchymal infiltration in allogeneic animals (2.8±0.6 cells per 400× field), but CD45+ cells were absent after syngeneic BMT (0.1±0.1 cells per 400× field) and in unmanipulated controls (0.2±0.1 cells per 400× field). Parenchymal cellular infiltration after allogeneic BMT consisted of lymphocytes (41%), activated microglial cells (44%), and monocytes (7%); 8% of CD45+ cells could not be classified. In the allogeneic animals, perivascular infiltration (intravascular cells not counted) was detectable in 27% of arterioles and in 9% of capillaries, whereas in controls and syngeneic animals, <2% of vessels were infiltrated. In the brain sections of 3 of 14 animals stained for VCAM-1 and of 1 of 12 animals stained for ICAM-1, CD45+ infiltration could not be determined because of poor tissue quality and reduced delimitation of cellular elements.

Parenchymal CD45+ scores6 were correlated with endothelial expression of CAMs on brain vascular endothelium (ICAM-1, K=0.6, P<0.005; VCAM-1, K=0.8, P<0.001). In most cases, low vascular CAM expression in controls or syngeneic animals was related to minor CD45+ parenchymal scores, whereas a combination of higher values was found in allogeneic animals (Figure 3). The delineation of allogeneic mice from controls or syngeneic animals was more obvious for VCAM-1 than for ICAM-1.

Apoptosis
In none of the transplanted animals was there evidence to suggest apoptotic cell death, in either the TUNEL or the PARP detection system; Fas was also not detected. In the positive control for TUNEL staining, all nuclei stained positively. Murine spleen tissue used as the positive control for Fas and PARP showed positively stained cells.
ICAM-1–Knockout Mice
The CD45−/H11001 score of the allogeneic wild-type mice (3.6+/−1.8 cells per 400×/H11003 field) was slightly higher compared with the values of the allogeneic ICAM-1–knockout mice (2.4+/−0.4 cells per 400×/H11003 field), but no statistically significant difference could be found (Figures 4 and 5). In syngeneic animals (wild type, 0.4+/−0.3 cells per 400×/H11003 field; ICAM-1 knockout, 0.5+/−0.2 cells per 400×/H11003 field) and in the control animals (0.2 cells per 400×/H11003 field), CD45−/H11001 cells were rare. We observed large CD45−/H11001 cells resembling mononuclear phagocytic cells and smaller cells noted as lymphocytes or activated microglia.

Discussion
Chronic GvHD is a systemic complication of allogeneic BMT, and it has long been a matter of debate whether chronic GvHD manifests in the CNS. Although a large autopsy study has not provided evidence for the involvement of the brain in chronic GvHD,10 cerebral lymphomononuclear infiltrates and activation of microglia were detected in single patients with chronic GvHD.11,12 Long-term survivors with chronic GvHD were suspected to have cerebral angiitis because of the clinical course and treatment response in 4 patients, and the diagnosis was confirmed by biopsy in 1 patient.5 In experimental GvHD soon after transplantation, T lymphocytes were rarely found in addition to the parenchymal and vascular expression of MHC class I and II antigens,4 whereas in chronic disease, cerebral lymphomononuclear infiltrations were observed more frequently.6

Leukocyte recruitment into the brain is thought to be controlled by adhesion molecules in various CNS pathologies, eg, in multiple sclerosis13 or ischemic cerebrovascular disease.14 In the present study, we showed that an increased expression of ICAM-1 and VCAM-1 on the endothelium of cerebral vasculature, irrespective of vessel size, also occurred in mice with chronic GvHD. The influence of conditioning was investigated in syngeneic animals, and an effect on the expression of adhesion molecules could be observed only for ICAM-1 on arterioles/venules. The induction of endothelial ICAM-1 expression by irradiation has been reported in the oral mucosa of humans, but, as in our study, no upregulation of VCAM-1 was observed.15 As expected, in healthy controls, basal endothelial expression of ICAM-1 and VCAM-1 was found on leptomeningeal vessels and the choroid plexus,16 whereas constitutive expression on larger intracerebral vessels has been mentioned inconsistently in the literature and could be due to the divergent detection methods used or a reliance on strain-specific variations. Despite infiltrating cells, no histomorphological changes or apoptotic cell death could be detected. Apoptotic mechanisms have so far been reported for liver or spleen GvHD,17 but TUNEL-positive cells in the CNS of rats with GvHD were very rare.18 Otherwise, signs of tissue damage might occur in a later phase in our GvHD model if the inflammatory reaction were to become more severe. No alternative indicators of brain pathology, such as myelin and axonal damage, were assessed in this study.
Our results are in line with the widely accepted pathophysiological concept of chronic GVHD. In GVHD, a complex signaling cascade is thought to promote migration of leukocytes into tissue. Conditioning as the first step in this cascade may activate host cells, induce cytokine release, and increase expression of adhesion molecules like ICAM-1, as found in our study. Donor T-cell activation and cytokine release constitute the second step in GVHD pathophysiology. In chronic GVHD, the interleukins IL-4 and IL-10, produced by the T-helper cell subset Th-2, are supposed to constitute the major cytokine mediators. In accordance with our findings, it has been reported that IL-4 induces VCAM-1 and ICAM-1 expression on endothelial cells. Endothelial VCAM-1 or ICAM-1 upregulation, together with tissue infiltration, has already been reported in experimental GVHD for noncerebral tissues, such as the skin and liver. Treatment studies with anti-ICAM-1 either decreased vessel inflammation and tissue damage or had no effect. A study on idiopathic pneumonia syndrome and GVHD, both caused by donor-cell alloreactivity after BMT, has shown amelioration of pathologic changes in the lung in ICAM-1–knockout mice, whereas GVHD-induced lesions in the liver and colon were not affected. Antibodies against VCAM-1 or against its receptor, very late antigen (VLA-4), were found to reduce the incidence, severity, and GVHD-related mortality or to inhibit GVHD-related hepatic lesions in experimental murine BMT. There are no studies on VCAM-1–knockout mice because this strain is not viable.

In most of the investigated animals in our study, endothelial activation was correlated positively with the extent of cerebral inflammatory infiltration. The observation of higher values for both variables in animals after allogeneic BMT may support the functional importance of VCAM-1 and ICAM-1 in leukocyte recruitment into the brain during chronic GVHD. Low or no cellular infiltration, despite elevated expression of adhesion molecules in some animals, has been reported earlier. A higher expression of VLA-4 on peripheral T lymphocytes has been observed only in patients with GVHD but not in patients without GVHD. This finding probably explains the lower infiltration rate in syngeneic compared with allogeneic animals. We failed to demonstrate a significant effect of ICAM-1 deficiency on cellular entry into the brain after allogeneic transplantation. This result, together with our observation of a more significant upregulation of VCAM-1 compared with ICAM-1 in allogeneic recipients, provides evidence of an important role for VCAM-1 in leukocytic recruitment in our animal model. The central importance of this pathway in cerebral leukocyte adhesion has already been suggested in an animal model of systemic lupus erythematosus using an anti–VCAM-1 antibody.

In conclusion, our findings show enhanced expression of ICAM-1 and VCAM-1 on cerebral vessels in experimental chronic GVHD and might therefore contribute to the understanding of encephalitis and cerebral vasculitis in patients with chronic GVHD.

Acknowledgments

This study was supported by a grant to C.S.P. from the Deutsche Krebshilfe (PA 702655) and to P.R. from the Friedrich-Baur Stiftung (AZ 0063/2001 8766026). We wish to thank J. Benson for editing the manuscript.

References


Cerebral Endothelial Expression of Adhesion Molecules in Mice with Chronic Graft-Versus-Host Disease
Petra Sostak, Petra Reich, Claudio S. Padovan, Armin Gerbitz, Ernst Holler and Andreas Straube

*Stroke*. 2004;35:1158-1163; originally published online April 8, 2004;
doi: 10.1161/01.STR.0000125865.01546.bb
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/35/5/1158

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/