Journal of Cerebral Blood Flow and Metabolism
14:348–352 © 1994 The International Society of Cerebral Blood Flow and Metabolism
Published by Raven Press, Ltd., New York

Short Communication

Quantitation of Perivascular Monocytes and Macrophages Around Cerebral Blood Vessels of Hypertensive and Aged Rats

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Summary: The numbers of monocytes and macrophages in the walls of cerebral blood vessels were counted on perfusion-fixed frozen brain sections (16 μm) of spontaneously hypertensive rats (SHR), stroke-prone SHR (SHR-SP), normotensive Wistar–Kyoto (WKY) rats, and young (16-week-old) and old (2-year-old) normotensive Sprague–Dawley rats (SD-16w and SD-2y, respectively) using monoclonal antibodies against rat macrophages (ED2). The staining was visualized with fluorescein-labeled second antibodies. The ED2-specific staining in brain sections was restricted to macrophages in a perivascular location. The number of perivascular cells per square millimeter of high-power field was significantly greater in SHR-SP (8.6 ± 2.1; n = 4) and SHR (6.7 ± 0.9; n = 6) than in normotensive WKY (4.0 ± 0.5; n = 6; $p < 0.01$). The number of perivascular macrophages was also greater in SD-2y (7.5 ± 2.7; n = 9) than in SD-16w (2.9 ± 1.8; n = 8; $p < 0.01$). No ED2 staining was found in the resident microglia or in the endothelial cells, which were identified by double staining with rhodamine-labeled anti-factor VIII–related antigen antibodies. The results suggest that the stroke risk factors hypertension and advanced age are associated with increased subendothelial accumulation of monocytes and macrophages. This accumulation could increase the tendency for the endothelium to convert from an anticoagulant to a procoagulant surface in response to mediators released from these subendothelial cells. **Key Words:** Immunofluorescence—ED2—Von Willebrand factor—Rats—Brain.

We have been interested in the possibility that, in individuals with risk factors for stroke, chronic activation of monocytes and macrophages and/or endothelium promotes a perivascular accumulation of monocytes and macrophages and an intensified cytokine-mediated interaction between monocytes and macrophages and endothelial cells that prepares local segments of extracranial and intracranial vessels for subsequent thrombosis or hemorrhage. Routine histological assessment by morphologic criteria has demonstrated increased numbers of macrophages around large arteries in old rats with hypertension (Chobanian, 1990) as well as in human hyperlipidemia (Ross, 1986) and diabetes mellitus (Bierman, 1992). RNA messages for monocyte-derived cytokines, monocyte chemoattractant protein-1, tumor necrosis factor-α (TNF-α), and immunoreactive TNF-α have been detected in atherosclerotic lesions (Barath et al., 1990; Ylä-Herttuala et al., 1991). Previous studies from this laboratory have demonstrated that hypertension and advanced age, along with other risk factors for stroke, "prepared" brainstem vasculature of rats for ischemia

Received April 8, 1993; final revision received July 29, 1993; accepted September 1, 1993.

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Abbreviations used: ABC, avidin–biotin–peroxidase complex; ANOVA, analysis of variance; ED2, monoclonal antibodies against rat macrophages; FITC, fluorescein isothiocyanate; FVIII-RA, factor VIII–related antigen; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; SD-2y, 2-year-old Sprague–Dawley rats; SD-16w, 16-week-old Sprague–Dawley rats; SHR, spontaneously hypertensive rats; SHR-SP, stroke-prone spontaneously hypertensive rats; TNF-α, tumor necrosis factor-α; WKY, Wistar-Kyoto.

and hemorrhage (Hallenbeck et al., 1988). The output of TNF-α into CSF in vivo in response to the standardized cytokine stimulus, lipopolysaccharide, was also greater in hypertensive and aged rats than in normotensive or young controls (Sirén et al., 1992, 1993).

In this study we continued to test the predictions of our working hypothesis. Therefore, the number of monocytes and macrophages in the walls of cerebral blood vessels was quantified in hypertensive rats and in aged and young normotensive rats. The serial counts of monocytes and macrophages were done on perfusion-fixed frozen brain sections of spontaneously hypertensive rats (SHR), stroke-prone SHR (SHR-SP), normotensive Wistar-Kyoto (WKY) rats, and 2-year-old and 16-week-old Sprague-Dawley (SD-2y and SD-16w, respectively) using immunofluorescence with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies specific to rat macrophages (ED2).

METHODS

Male 16–20-week-old SHR, male WKY rats (Taconic Farms, Germantown, NY, U.S.A.), and male SHR-SP (Laboratory of Animal Sciences, SmithKline Beecham, King of Prussia, PA, U.S.A.) were used. Male 16-week-old and 2-year-old Sprague-Dawley rats (Zivic-Miller, Zelienople, PA, U.S.A.) were also used. The rats were housed at 22°C with a 12-h–12-h light–dark cycle, with food and tap water ad libitum.

The animals were anesthetized with pentobarbital sodium (50 mg/kg i.p.), and the tissues were perfusion-fixed through cardiac perfusion with 10% formalin and phosphate-buffered saline (PBS), cryoprotected with 20% sucrose immersion for 2 days, and frozen in isopentane over dry ice. Coronal sections of the forebrain and hindbrain were cut in a cryostat; one 16-μm section was mounted on a gelatin-subbed slide and the next 25 sections were discarded, then a 16-μm section was mounted and the next 25 sections were discarded, then a third 16-μm section was mounted. In this way, each histoslide contained 3 sections at 400-μm intervals from each other.

For staining of the endothelial cells in each section, the sections were first incubated with polyclonal rabbit antibodies against human factor VIII–related antigen (FVIII-RA; Dako, Carpinteria, CA, U.S.A.) at 4°C for 24 h, washed three times (10 min each wash) in 0.2% Triton-PBS, and exposed to rhodamine-labeled goat antirabbit antibodies (Jackson Laboratories, Bar Harbor, ME, U.S.A.) for 30 min at room temperature. After two washes (10 min each), the sections were incubated for 48 h at 4°C with monoclonal antibodies against rat macrophages (mouse antirat ED2; Accurate Chemical and Scientific Corporation, Westbury, NY, U.S.A.), which react with a membrane epitope in rat macrophages (Dijkstra et al., 1985; Barbé et al., 1990) and are expressed in the brain solely in the perivascular cells (Graeber et al., 1989). Thereafter, the sections were washed again three times (10 min each), and exposed to FITC-labeled goat antimouse antibodies (Jackson Laboratories) for 30 min at room temperature.

After washing, the sections were coverslipped and examined under fluorescence microscope. Incubations with the primary antibodies were done in 0.3% Triton-PBS with 1% normal goat serum. All washes were done with 0.2% Triton-PBS. The primary antibodies were diluted 1:1,000; rhodamine-labeled second antibodies were diluted 1:100, and FITC-labeled antibodies were diluted 1:300.

Serial counts of ED2-positive cells represent counts of 90 high-power fields of brain sections. The counting of ED2-positive cells was done by an investigator unaware of the experimental source of the material. Some sections were processed for immunohistochemistry for bright-field microscopy using the avidin–biotin–peroxidase complex (ABC) method (Vectastin ABC-Kit, Vector Laboratories, Burlingame, CA, U.S.A.) with 3,3′-diaminobenzidine as peroxidase substrate (Fig. 1A). Sections of spleen and lymph nodes stained with ED2 antibodies served as positive controls; for assessment of nonspecific staining, the primary antibodies were either omitted or replaced by normal mouse IgG_{2a}.

Statistical analysis

The data in the text and figures are presented as means ± SD for the indicated number of individual values. Since initial analysis of the data revealed that they were not normally distributed, the nonparametric Kruskal–Wallis analysis of variance (ANOVA) using the CSS/pc statistical package for microcomputers (Complete Statistical System, StatSoft, Tulsa, OK, U.S.A.) was used. The Mann–Whitney *U* test was used to analyze the differences between individual treatment groups.

RESULTS

The ED2-specific staining in brain parenchyma was restricted to macrophages in a perivascular location (Fig. 1A and C). Macrophages in the ependyma and choroid plexus also stained positive with ED2 antibodies; however, for the quantification of perivascular macrophages, only cells closely apposed to the FVIII-RA–stained endothelium were counted. No ED2 staining was observed in the endothelial cells visualized with FVIII-RA antibodies (Fig. 1D) or in the resident microglia, which stained positive with antibodies for the rat CR3 (OX-42; Fig. 1B). The number of perivascular cells per square millimeter of high-power field was significantly greater in SHR-SP and SHR than in WKY rats (Fig. 2). The number of perivascular macrophages was also greater in 2-year-old SD rats than in 16-week-old SD rats (Fig. 2 and Table 1).

DISCUSSION

It has become clear that the immune privilege of the CNS is not absolute. Traffic of inflammatory cells through the blood–brain barrier has been demonstrated to take place via a receptor-mediated endothelial adhesion and penetration across the en-

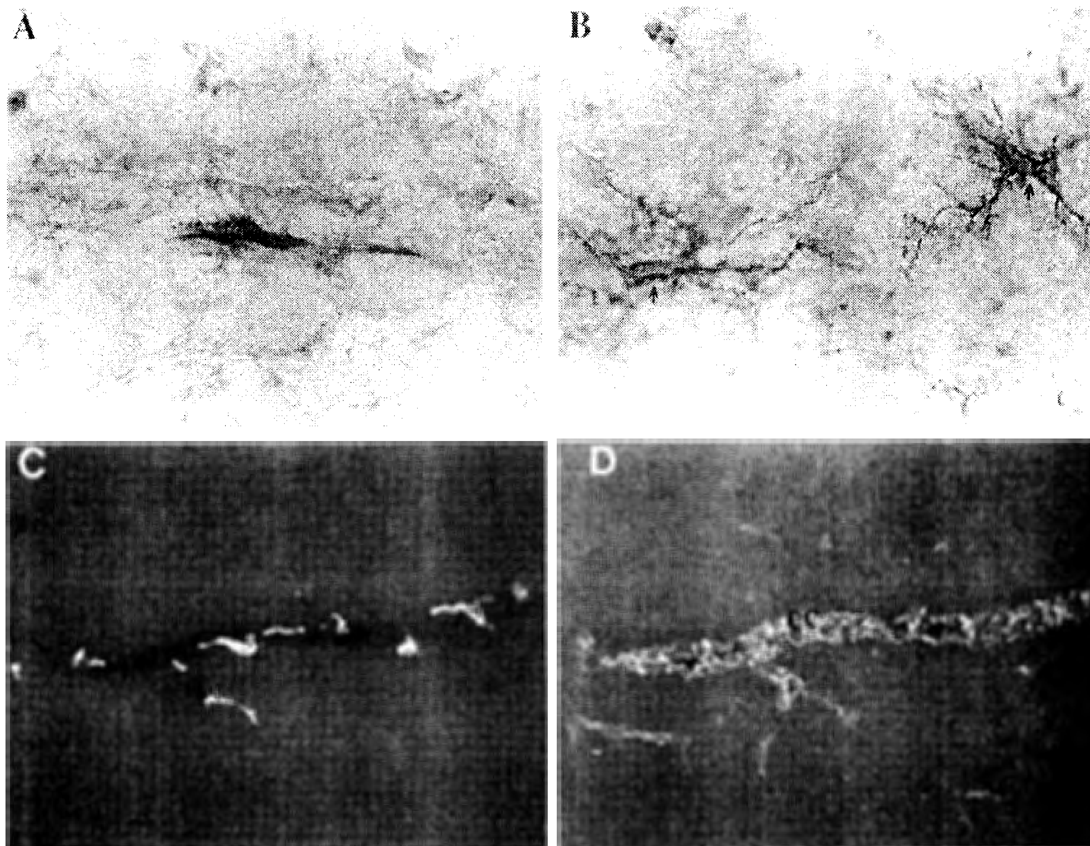

FIG. 1. **A** and **C:** Photomicrographs demonstrate ED2-positive perivascular macrophages around brain intraparenchymal blood vessels. **A:** A bright-field light microscopy image of a section stained with ED2 antibodies using the ABC method is seen. The asterisk denotes an ED2-labeled perivascular macrophage (original magnification ×1,000). **B:** A bright-field immunofluorescence image of a section stained with antibodies against rat CR3 (OX-42) demonstrates staining of two resident microglial cells (arrows) (original magnification ×1,000). **C:** A dark-field immunofluorescence image of a section stained with ED2 antibodies using fluorescein-labeled second antibody is seen. The asterisks denote ED2-labeled perivascular macrophages (original magnification ×120). **D:** The same section after staining with rhodamine-labeled factor VIII antibodies demonstrates staining of the endothelial cells (ec) (original magnification ×120).

dothelium of circulating inflammatory cells (Wekerle et al., 1986; Lossinsky et al., 1989; McCarron et al., 1991). Immunocompetent cells expressing adhesion molecules, antigens of the major histocompatibility complex (MHC), CD4 antigen, leukocyte-common antigen, and CR3 complement receptors have been detected using specific monoclonal antibodies in the brain parenchyma (microglia, astrocytes), surrounding blood vessels (perivascular macrophage, pericytes), subarachnoid space (meningeal macrophage), ventricular surfaces of choroid plexus (epiplexus cells), and the ventricular margin of ependymal epithelium (supraependymal cells), (Jordan and Thomas, 1988; Graeber et al., 1989; Akiyama and McGeer, 1990). The origin of these cells in the brain has been debated for decades. Stem cells of mesenchymal origin may enter the CNS during the embryonic period and exist in two interchangeable forms, ameboid and ramified microglia (Rio-Hortega, 1932). The perivascular cells around brain capillaries in both rodent and human brain are most probably bone marrow–derived (Hickey and Kimura, 1988; Peudenier et al., 1991) and express specific macrophage surface antigens (Peudenier et al., 1991). Labeled monocytes and lymphocytes have been found in brain after in vivo labeling of the bone marrow (Roessman and Friede, 1968; Schelper and Adrian, 1980, 1986; Wekerle et al., 1986), and the blood-borne monocytes, in addition to the microglial cells, seem to contribute to the immune response in CNS injury (Roessman and Friede, 1968; Schelper and Adrian, 1980, 1986; Gehrmann et al., 1992). Monocytes can adhere to, and penetrate across, brain endothelial cells via expression and binding of specific monocyte adhesion molecules to their specific adhesion molecule coun-

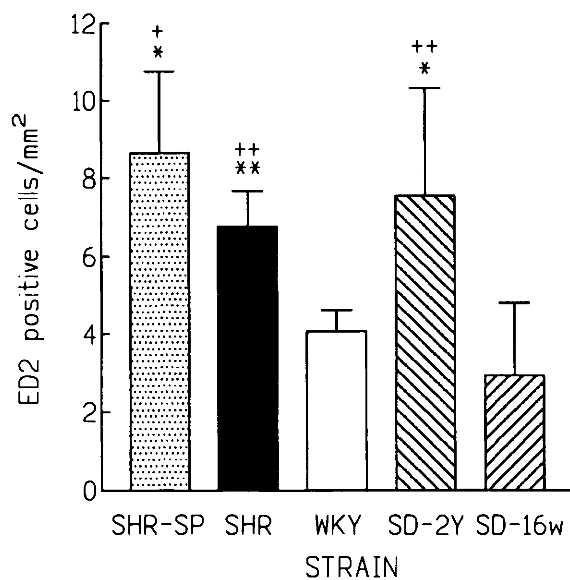

FIG. 2. Quantitation of perivascular macrophages in brain. Data (means ± SD) represent serial counts of ED2-positive cells/mm^2 from three complete sections from each rat (30 fields per section). Number of rats: SHR-SP, four; SHR, six; WKY, six; SD-16w, eight; SD-2y, nine; *, $p < 0.05$ versus WKY group (Mann–Whitney *U* test); **, $p < 0.01$ versus WKY group (Mann–Whitney *U* test); †, $p < 0.05$ versus SD-16w group (Mann–Whitney *U* test); ‡, $p < 0.01$ versus SD-16w group (Mann–Whitney *U* test).

terparts on endothelial cells (Pober and Cotran, 1990; Springer, 1990).

In our previous studies, intracerebroventricular injection of lipopolysaccharide induced greater increases in TNF-α activity in the CSF of hypertensive and aged rats than in normotensive or young controls (Hallenbeck et al., 1991; Sirén et al., 1992, 1993). The present study extends these previous findings in that it suggests that the increased numbers of perivascular macrophages in hypertensive and aged rats could account for the enhanced production of TNF-α in the brains of these rats, as monocytes and macrophages are a major source of TNF-α (Pober and Cotran, 1990). However, several other cell types in the CNS are capable of TNF-α expression. The ameboid microglia share many features with macrophages, including the production of interleukin (IL-1) and TNF-α (Sawada et al., 1989). Astrocytes may also be capable of transforming into macrophage-like cells (Kusaka et al., 1986) and have been shown to release cytokines in vitro (Sawada et al., 1989). Furthermore, macrophages within ependyma and meninges could also contribute to the TNF-α release into the CSF. A recent study demonstrated expression of IL-1 in ED2-positive perivascular cells, in ED2-positive macrophages of the meninges, and in the resident microglia after systemic challenge with lipopolysaccharide (Van Dam et al., 1992). However, the relative contribution of the various cell types to the release of TNF-α and IL-1 into the CSF in animals with the stroke risk factors hypertension and advanced age will not be clarified until studies using in situ hybridization and immunohistochemistry to reveal the exact cell types expressing mRNA for TNF-α after lipopolysaccharide stimulation in vivo become available.

The present data demonstrating increased numbers of perivascular macrophages in cerebral intraparenchymal vasculature of hypertensive and aged rats are in accord with our working hypothesis that an increased subendothelial accumulation of monocytes and macrophages and a cytokine-mediated interaction between these perivascular cells and endothelium may underlie the increased risk of stroke in hypertension and in advanced age (Hallenbeck et al., 1988, 1991; Sirén et al., 1992, 1993). According to this hypothesis, risk factors for stroke are initially associated with a change in endothelium and a hyperactive state of the monocytes and macrophages; whether the change in endothelium causes the monocytes to become hyperactive or vice versa is not established, but either case would lead to the interaction. The endothelial change involves, at a minimum, the expression of adhesion molecules that enable the monocytes to adhere to the vessel wall and undergo transendothelial migration in both large and small vessels, leading to local deposits of monocyte clusters in segments of the blood vessels. These monocyte clusters could then periodically signal the vessel endothelium via release of prothrombotic, proinflammatory, and chemotactic mediators, such as TNF-α, IL-1, and perhaps platelet-activating factor, to convert the endothelium to a procoagulant state and, in effect, prepare the vessel segments in a manner similar to the localized Shwartzman paradigm (Shwartzman, 1928; Movat

TABLE 1. *Perivascular macrophage counts in forebrain and hindbrain (medulla and cerebellum)*

Strain	Forebrain		Hindbrain	
	Macrophage count	No. of rats	Macrophage counts	No. of rats
SHR-SP	10 ± 2[a]	15	9 ± 1[a]	12
SHR	8 ± 2[a,b]	13	7 ± 1[a]	8
WKY	4 ± 1	14	5 ± 1	8
SD-2y	9 ± 2[a]	16	9 ± 2[a]	21

Values are means ± SD.
[a] Statistically significant compared with WKY rats ($p < 0.05$, Tukey's test).
[b] Statistically significant differences were seen between forebrain and hindbrain ($p < 0.05$, Tukey's test).

et al., 1987; Hallenbeck et al., 1988; Pober and Cotran, 1990). Complement activation or any stimulus leading to activation of the coagulation system (including natural oscillation of coagulation potential, stress, infection, trauma, and inflammation) could then precipitate a localized reaction of the prepared vessel segment and lead to a local thrombosis or hemorrhage.

Acknowledgment: This work was supported in part by PHS Grant NS-28225 and the Uniformed Services University Protocol R09232. The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or as necessarily reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences. The experiments reported herein were conducted according to the principles set forth in the "Guide for Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. NIH 85-23, 1985). The authors thank Ms. Monika Grojec for excellent technical assistance.

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