

Research Report

Upregulation of intercellular adhesion molecule 1 (ICAM-1) on brain microvascular endothelial cells in rat ischemic cortex

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Abstract

The expression of intercellular adhesion molecule 1 (ICAM-1) was studied in rat focal ischemic cortex. A significant increase in ICAM-1 mRNA expression in the ischemic cortex over levels in contralateral (nonischemic) site was observed by means of Northern blot analysis following either permanent or temporary occlusion with reperfusion of the middle cerebral artery (PMCAO or MCAO with reperfusion) in spontaneously hypertensive rats. In the ischemic cortex, levels of ICAM-1 mRNA increased significantly at 3 h (2.6-fold, $n = 3$, $P < 0.05$), peaked at 6 to 12 h (6.0-fold, $P < 0.01$) and remained elevated up to 5 days (2.5-fold, $P < 0.05$) after PMCAO. The profile of ICAM-1 mRNA expression in the ischemic cortex following MCAO with reperfusion was similar to that following PMCAO, except that ICAM-1 mRNA was significantly increased as early as 1 h (6.3-fold, $n = 3$, $P < 0.05$) and then gradually reached a peak at 12 h (12-fold, $P < 0.01$) after reperfusion. ICAM-1 mRNA expression in ischemic cortex following PMCAO was significantly greater in hypertensive rats than in two normotensive rat strains. Immunostaining using anti-ICAM-1 antibodies indicated that upregulated ICAM-1 expression was localized to endothelial cells of intraparenchymal blood vessels in the ischemic but not contralateral cortex. The data suggest that an upregulation of ICAM-1 mRNA and protein on brain capillary endothelium may play an important role in leukocyte migration into ischemic brain tissue.

Keywords: Intercellular adhesion molecule 1; Focal brain ischemia; Stroke; Reperfusion; Inflammation

1. Introduction

Polymorphonuclear leukocyte (PMN) infiltration into the ischemic brain tissue has been demonstrated in several animal models of stroke [1,7,11,13,18]. The recruitment of circulating PMN into ischemic tissue initially requires the interaction of microvascular endothelial cells with these inflammatory cells via specific adhesion molecules [4,26]. Several adhesion molecules and their counter-parts have been characterized and found to play critical roles in the cell surface interactions between endothelial cells and leukocytes. A number of these cell surface molecules have been described so far with mutually adhesive properties on either

endothelial cells or leukocytes. Some of the best-known adhesion molecules found on the endothelial cell surface include intercellular adhesion molecule 1 (ICAM-1), ICAM-2, vascular cell adhesion molecule 1 (VCAM-1), endothelial-leukocyte adhesion molecule 1 (ELAM-1, E-selectin), and P-selectin [4,26]. The adhesion molecules on leukocytes include L-selectin, CD14, and molecules belonging to the β_1 - (e.g. VLA-4) or β_2 -integrin (CD11/CD18) (e.g. LFA-1, Mac-1, and p150,95) subfamily [4,26].

ICAM-1 is a single-chain glycoprotein of the immunoglobulin supergene family. The extracellular domain of ICAM-1 comprises five Ig-like domains with ligand binding sites for LFA-1 (CD11a/CD18) and for Mac-1 (CR3; CD11b/CD18) on the surfaces of leukocytes [9,21]. ICAM-1 has been found constitutively expressed on endothelial cells and upregulated by en-

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dotoxin (e.g. lipopolysaccharide) and several cytokines including TNF- α , IL-1, and IFN- γ both in vitro and in vivo [10,25,31]. Recently, increases in ICAM-1 expression have been observed in the rabbit pulmonary artery occlusion and reperfusion model [14] and in the ischemic and reperfused canine myocardium [16]. Furthermore, Okada et al. [23] reported that focal cerebral ischemia and reperfusion in the baboon stimulated the expression of endothelial P-selectin and ICAM-1 peptides.

Based upon our observations that occlusion of the rat middle cerebral artery (permanent or with reperfusion) increases PMN infiltration into the focal ischemic tissue [1,3,7] and the recent demonstration that increased expression of cytokines (TNF- α , IL-1 β , IL-6) and chemokine (CINC/KC) are produced early in ischemic brain [18,19,20,30], we postulated that adhesion molecules on the endothelial cells are upregulated prior to and/or temporarily associated with the infiltration of the inflammatory cells into the ischemic tissue. Therefore, as an initial step to study the molecular mechanisms involved in the interaction between endothelial cells and leukocytes in focal stroke, we evaluated the kinetic pattern of ICAM-1 expression in rat ischemic brain tissue following both PMCAO and MCAO with reperfusion.

2. Materials and methods

2.1. Focal brain ischemia

Cerebral focal ischemia or sham surgery was carried out in male spontaneously hypertensive rats (SHR; Taconic Farms, Germantown, NY) and in three normotensive rat strains (Fisher-344, Wistar-Kyoto, or Sprague-Dawley; Charles River, Danvers, MA), at 18 weeks of age weighing 250–330 g, by permanent occlusion of the middle cerebral artery as described in detail previously [1,2,3]. Briefly, the middle cerebral artery (MCA) was occluded and cut dorsal to the lateral olfactory tract at the level of the inferior cerebral vein using electrocoagulation (Force 2 Electrosurgical Generator, Valley Lab Inc., Boulder, CO). For temporary MCAO with reperfusion, the MCA was lifted from the brain surface to occlude blood flow for 160 min and then reperfused as described in detail previously [2,3]. In sham-operated rats the dura was opened over the MCA but the artery was not occluded. Rats were overdosed with pentobarbital and forebrains were removed and dissected at various times following PMCAO or after MCAO with reperfusion. The ischemic cortex (i.e. the cortex ipsilateral to surgery) was dissected from ipsilateral frontoparietal cortical hemisphere; the contralateral cortex was dissected as the non-ischemic control from the same rat [1,3]. The cortical samples were immediately frozen in liquid nitrogen and stored at -80°C .

2.2. Isolation of RNA and Northern hybridization analysis

Total cellular RNA was prepared from cortical samples by using a method of acid guanidinium thiocyanate, phenol, and chloroform extraction procedure [6]. RNA samples (40 $\mu\text{g}/\text{lane}$) were electrophoresed through formaldehyde-agarose slab gels [17], and trans-

ferred to GeneScreen Plus membranes (DuPont-New England Nuclear). Rat ICAM-1 cDNA was generated by reverse transcription and polymerase chain reactions (RT/PCR) using 5' and 3' synthetic oligonucleotide primers according to the published sequence [15]. RNA isolated from ischemic tissue following 12 h PMCAO was reverse transcribed from 3' antisense primer (5'-GGAATTCCTGTTCAGAAGCACCACC-3', complementary to bases 1047 to 1064 of the cloned cDNA and with an *EcoRI* restriction site at the 5'-end), and then PCR amplified between this oligonucleotide and a sense oligonucleotide (5'-GGAATTCCTCAGGTATCCATCCATCCC-3', identical to bases 112 to 129 and with an *EcoRI* site at the 5'-end). The RT/PCR was carried out according to the conditions described previously [29]. The PCR amplified DNA fragment was digested with *EcoRI* restriction enzyme, electrophoresed and isolated, and ligated into a pGEM3Zf(-) vector (Promega). The identity of the cloned cDNA insert was confirmed by DNA sequencing. For Northern blot analysis, the 0.95-kb rat ICAM-1 cDNA insert released with *EcoRI* digestion was uniformly double-labeled with [α - ^{32}P]dATP and [α - ^{32}P]dCTP (3000 Ci/mmol, Amersham Corp.), or a ribosomal protein L32 cDNA [22] (a gift of Perry RP, Institute for Cancer Research, Fox Chase, PA) was labeled with a single isotope using a random-priming DNA labeling kit (Boehringer Mannheim). Hybridization of each probe was carried out overnight with 1×10^6 cpm/ml of probe at 42°C in $5 \times \text{SSPE}$ (750 mM NaCl, 50 mM NaH_2PO_4 , pH 7.6, 5 mM EDTA), 50% formamide, $5 \times$ Denhardt's solution, 2% SDS, and 200 $\mu\text{g}/\text{ml}$ boiled salmon sperm DNA. The membranes were washed in $2 \times \text{SSPE}$, 2% SDS at 65°C for 1–2 h with a change every 30 min, then autoradiographed at -80°C with a Cronex Lightning-Plus intensifying screen for various times depending upon the signal intensity. A probe was stripped from the membranes by boiling in 10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, 1% SDS for 20 min prior to re-hybridization with the other probe.

PhosphorImager (Molecular Dynamics, Sunnyvale, CA) was used to quantitate the band intensities of the Northern blots and ImageQuant-TM Software v3.0 (Molecular Dynamics, Sunnyvale, CA) was used to analyze the results. The relative mRNA was calculated by normalization to the rpl32 signal in each sample. The rpl32 mRNA expression is relatively constant under these experimental conditions and therefore provides the means of correcting and standardizing for any loading differences between samples [29,30].

2.3. Immunohistochemistry

After ischemia, rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.) and the tissues were perfusion fixed (through cardiac perfusion with 10% formalin-PBS), cryoprotected with 20% sucrose immersion for 2 days and frozen in isopentane over dry ice. Cryostat sections (16 μm) of brain samples were mounted on gelatine-coated slides. The sections were incubated for 48 h at 4°C with a mouse monoclonal antibody against rat ICAM-1 (Clone IA29; Seikagaku, Rockville, MD). After three washes (10 min each) with 0.2% Triton-PBS, the sections were exposed to a FITC-labeled goat anti-mouse antibody for 30 min at room temperature. Thereafter, the sections were washed with 0.2% Triton-PBS 2 times (10 min each) and incubated with a polyclonal rabbit antibody against human Factor VIII-related antigen (Dako, Carpinteria, CA) at 4°C for 24 h for staining of the endothelial cells in each section. The sections were then washed three times (10 min each) in 0.2% Triton-PBS and exposed to a rhodamine-labeled goat anti-rabbit antibody for 30 min at room temperature. Incubations with the primary antibody were done in 0.3% Triton-PBS with 2% normal goat serum. The primary antibodies were diluted 1:1000, rhodamine-labeled second antibodies were diluted 1:100 and FITC labeled antibodies 1:300. The specificity of the staining was confirmed by comparing the antibody staining to that of a normal mouse IgG, or by exclusion of the

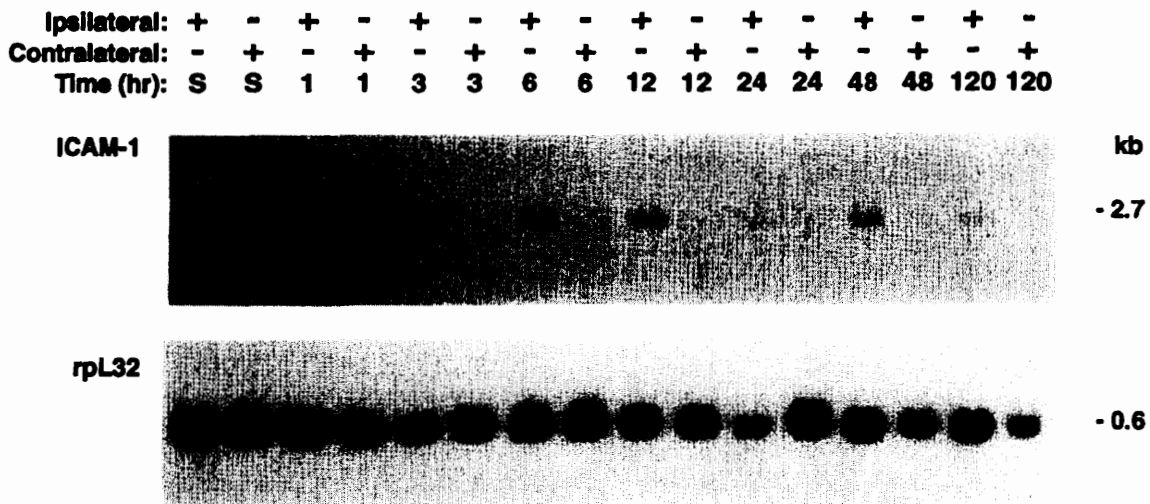


Fig. 1. Northern blot analysis of ICAM-1 mRNA induction in SHR cortex post-PMCAO. Total cellular RNA (40 μ g/lane) was resolved by electrophoresis, transferred to a nylon membrane, and hybridized to rat ICAM-1 and ribosomal protein L32 (rpl32) cDNA probes as detailed in Materials and methods. The mRNA size was determined by comparing with the migration of RNA ladder (BRL) and the number of kilo bases (kb) marked on the right. Ipsilateral and contralateral cortex samples (denoted by +) from individual rats following sham surgery (S) or following 1, 3, 6, 12, 48, and 120 h of PMCAO are depicted.

primary antibody. After washing, the sections were coverslipped and examined under fluorescence microscope.

2.4. Statistical analysis

Statistical evaluation was performed using three complete sets of cortical samples at various times using a Student *t*-test. The results were expressed as mean \pm standard error. Significance was accepted for $P < 0.05$ by comparing the relative mRNA levels in the ischemic cortex with non-ischemic cortex or comparing the ischemic cortex with sham-operated cortex.

3. Results

3.1. ICAM-1 mRNA expression in spontaneously hypertensive rat cortex following PMCAO

Fig. 1 illustrates a representative Northern blot for the ICAM-1 mRNA in the focal ischemic and non-ischemic cortex, and in sham-operated samples. Quantitated Northern blot signals ($n = 3$), after normalizing to a rpl32 probe, are represented graphically in Fig. 2. A low level of ICAM-1 mRNA was detected in the sham-operated animals or in the contralateral (non-ischemic) cortex. ICAM-1 mRNA was significantly induced in the ipsilateral (ischemic) cortex compared to the nonischemic cortex at 3 h (2.6-fold increase in the mean value, $P < 0.05$, $n = 3$), peaked at 6 (5.6-fold, $P < 0.01$) to 12 h (6.0-fold increase, $P < 0.01$), and maintained a significantly elevated level up to 5 days (2.5-fold increase) post PMCAO (Figs. 1 and 2).

3.2. ICAM-1 mRNA expression in spontaneously hypertensive rat cortex following temporary MCAO with reperfusion

Fig. 3 shows a representative Northern blot for the increase in ICAM-1 mRNA levels following temporary MCAO with reperfusion. Quantitated data ($n = 3$) of ICAM-1 mRNA expression exhibited an overall similar profile to that of the PMCAO model, with the exception that ICAM-1 mRNA following temporary MCAO with reperfusion was upregulated somewhat earlier: 1

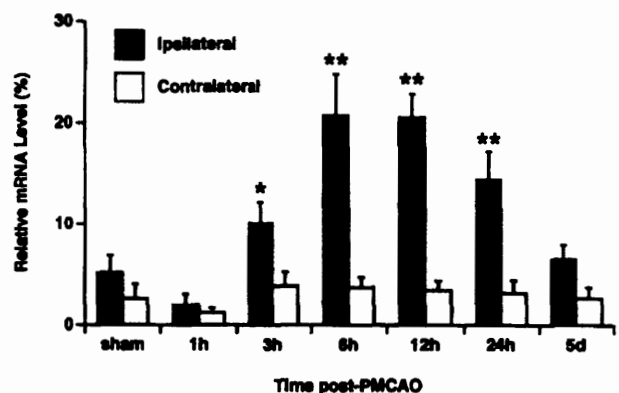


Fig. 2. Time course of the relative ICAM-1 mRNA levels quantitated from Northern blot analysis following PMCAO. The mRNA bands of ICAM-1 on Northern blot were quantitated by PhosphorImager analysis. The samples loaded in each lane were normalized with the values of rpl32 mRNA signals. Data are presented as the mean values of three separate experiments in spontaneously hypertensive rats ($n = 3$) for each time point. * $P < 0.05$, ** $P < 0.01$, compared to contralateral cortex as determined with Student's *t*-test.

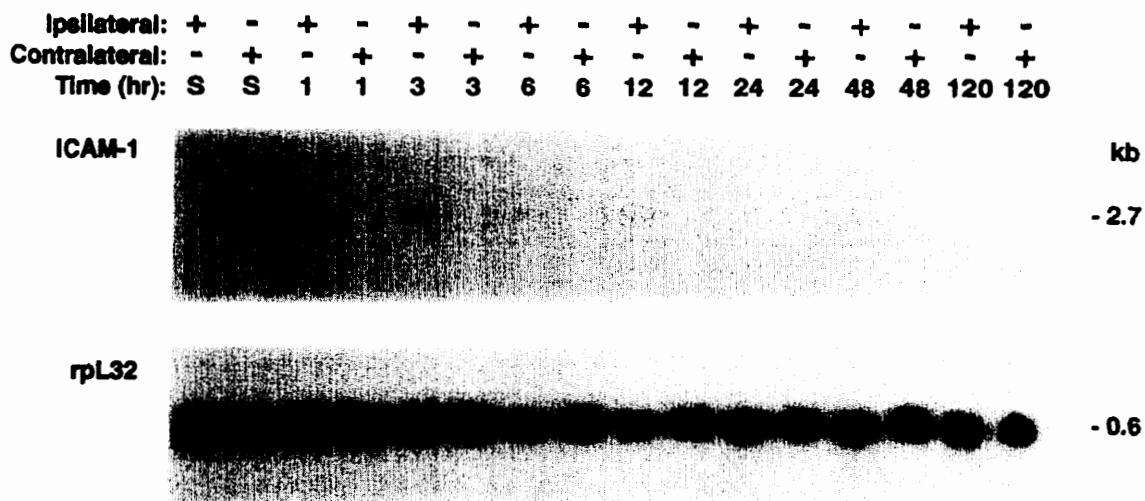


Fig. 3. Northern blot analysis of ICAM-1 mRNA induction in SHR cortex following temporary MCAO with reperfusion. The figure is illustrated as described in Fig. 1 legend except that temporary occlusion (160 min) of the cerebral middle artery was utilized. The time point indicated refers to the time after reperfusion.

h after reperfusion, the mRNA was already 6.3-fold over control ($P < 0.05$). The elevated ICAM-1 mRNA gradually reached a peak at 12 h (12.0-fold increase, $P < 0.01$) and then slowly decreased to an almost basal level by 5 days (1.7-fold increase) (Figs. 3 and 4).

3.2. Differential induction of ICAM-1 mRNA in the ischemic cortex of hypertensive and normotensive rats following PMCAO

To evaluate whether the elevated levels of ICAM-1 mRNA in the ischemic cortex are particular to a specific rat strain or the presence of a stroke risk factor such as hypertension, we compared the hypertensive strain (SHR) with three normotensive rat strains (Fisher-344, Wistar-Kyoto, and Sprague-Dawley) at a single time point (12 h) post PMCAO. A representative

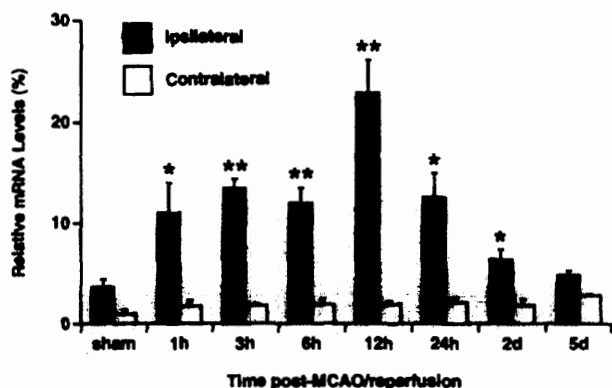


Fig. 4. Time course of the relative ICAM-1 mRNA levels quantitated from Northern blot analysis following temporary MCAO with reperfusion. Details for the illustration see the Fig. 2 legend.

Northern blot for ICAM-1 mRNA expression is shown in Fig. 5 and the quantitated group data ($n = 4$) are illustrated in Fig. 6. Northern blot analysis revealed that PMCAO induced a high level of ICAM-1 mRNA expression in the ipsilateral (ischemic) cortex of all four rat strains ($P < 0.01$) and in particular the SHR. The order of ICAM-1 mRNA expression was SHR > Fisher-344 (F-344) > Sprague-Dawley (SD) > Wistar-Kyoto (WKY) (Fig. 6). The elevated ICAM-1 mRNA level was significantly lower in WKY (0.53-fold of the mean value, $P < 0.01$) and SD (0.67-fold, $P < 0.05$) compared with that in SHR (Fig. 6).

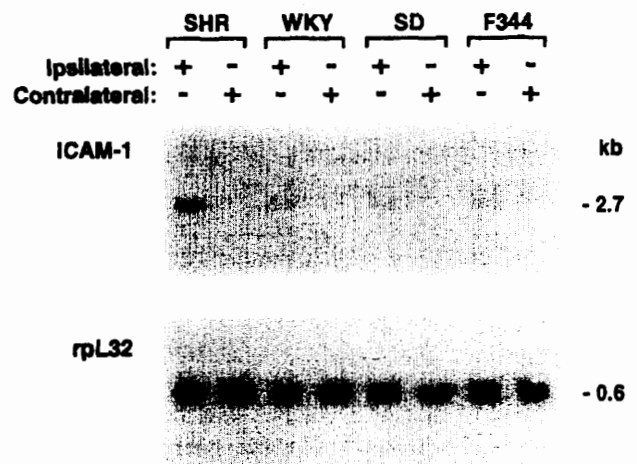


Fig. 5. Northern blot analysis of ICAM-1 mRNA expression in SHR and normotensive rats at 12 h following permanent MCAO. Total cellular RNA was isolated from the ischemic (ipsilateral) and non-ischemic (contralateral) cortex of hypertensive (SHR) and normotensive (WKY, SD, and F344) rats at 12 h following PMCAO. Northern blot was carried out as described in Materials and methods. The figure is illustrated as in Fig. 1 legend.

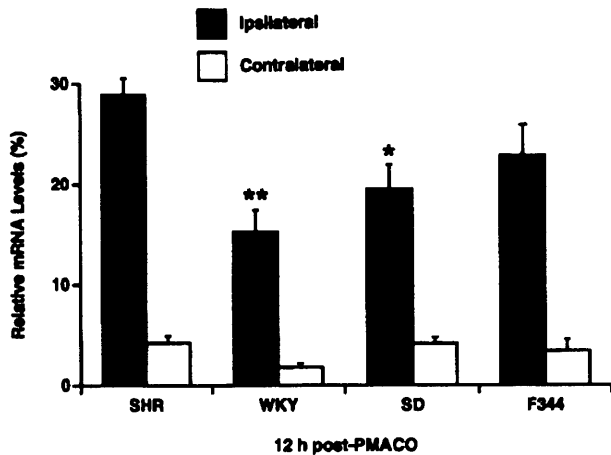


Fig. 6. Quantitative analysis of ischemic cortex ICAM-1 mRNA production in hypertensive (SHR) and normotensive (WKY, SD and F344) rats at 12 h post permanent MCAO. The data were quantitated as described in Fig. 2 legend. Ribosomal protein L32 mRNA was used to normalize the samples loaded in each lane. Data are presented as the mean values of four animals ($n = 4$) from SHR, WKY, SD, and F344 strains at 12 h post PMCAO. A significant induction for ICAM-1 mRNA expression was observed in the ischemic (ipsilateral) cortex compared to the nonischemic (contralateral) cortex of all four strains ($n = 4$, $P < 0.01$). * $P < 0.05$, ** $P < 0.01$, compared with SHR.

3.3. Immunostaining of ICAM-1 in the ischemic cortex following PMCAO

To define the cellular component for the induced ICAM-1 mRNA expression in the focal ischemic cortex and to examine the upregulation of ICAM-1 in response to ischemia, immunohistochemical techniques were applied using a mouse monoclonal antibody against rat ICAM-1. A strong and specific immunostaining was observed on the endothelial cells of intraparenchymal blood vessels in the ipsilateral (ischemic) cortex (Fig. 7), while this staining was almost undetectable in the contralateral (nonischemic) cortex at 12 h following PMCAO in SHR. Control sections stained with mouse IgG, or exclusion of the primary antibody did not demonstrate detectable staining. The microvascular endothelial staining was confirmed by double-labeling with antibody against Factor VIII related antigen which stains exclusively endothelial cells (Fig. 7).

4. Discussion

Previous studies have demonstrated that focal brain ischemia results in PMN infiltration into the ischemic cortex establishing an inflammatory reaction [1,3,7,11,13]. The expression of adhesion molecules on both endothelial cells and leukocytes is required for the recruitment of leukocytes from the circulating blood into the injured tissue [26]. A number of adhesion

molecules including ICAM-1, VCAM-1, ELAM-1 and PECAM-1 have been implicated in leukocyte interaction with microvascular endothelial cells [4,26,27]. In the present work we demonstrated a significant induction of ICAM-1 mRNA in the rat ischemic cortex following both PMCAO and MCAO with reperfusion (Figs. 1–4). These data are consistent with recent reports demonstrating increased expression of ICAM-1 in several different models of ischemic tissue injury including pulmonary injury after occlusion and reperfusion in rabbits [14], ischemia and reperfusion of the canine myocardium [16], and focal brain ischemia and reperfusion in baboons [23]. So far, only the latter report examined brain ischemia where endothelial P-selectin and ICAM-1 expression in brain microvessels in the ischemic zone was studied by quantitative immunohistochemistry. That study, however, only provided a partial time course of ICAM-1 immunoreactivity following focal ischemia. In this report, we utilized two different ischemia models (i.e., both permanent MCAO and temporary MCAO with reperfusion in rats) and quantitative Northern blot analysis. More importantly, the present study provides an extended time course and reveals the kinetic profile of the ICAM-1 mRNA induction in both ischemia models. Comparatively, ICAM-1 mRNA expression shows a similar profile in rats following PMCAO and MCAO with reperfusion. It is also noted that ICAM-1 mRNA levels in the MCAO with reperfusion were increased as early as 1 h after reperfusion and remained at higher levels for the duration of the experiment as compared to PMCAO. In both models a similar peak in ICAM-1 mRNA expression was observed at about 12 h post ischemia, which is quite different from the previous report in the baboon model [23], where significant ICAM-1 expression was observed at 1 h post ischemia followed by rapid decrease by 4 h after reperfusion. The differences between these studies may reflect species differences, or different methods used for the quantitation because ICAM-1 mRNA was monitored by Northern analysis in the present study while the protein was analyzed by immunohistochemical method in previous report by Okada et al. [23]. It is possible that rapid translational regulation in the baboon leads to early upregulation of ICAM-1 but this early event is not sustained by transcriptional regulation.

Applying double staining techniques using antibodies against ICAM-1 and factor VIII-related antigen [32] (a specific marker for endothelial cells) we localized the upregulated ICAM-1 to microvascular endothelial cells in the ischemic cortex (Fig. 7). The detection of ICAM-1 immunoreactivity in the ischemic but not in the nonischemic cortex following focal ischemia suggests that the ischemic damage not only results in the increase in the ICAM-1 mRNA (Figs. 1–4), but also the translation and the generation of the

active cellular surface peptide, which is critical for leukocyte-endothelial cell adhesion and the infiltration of leukocytes into focal ischemic tissue.

The data presented in this report also demonstrates that SHR expressed more ICAM-1 mRNA than normotensive rats (Figs. 5 and 6), which is in accord with previous data on the increased sensitivity of hypertensive rats to focal stroke compared to normotensive animals [2,12]. However, our data also demonstrates that increased ICAM-1 mRNA expression following focal ischemia is not specific to the SHR strain, as significant expression was found in three normotensive rat strains. In fact, the level of ICAM-1 mRNA in the Fisher 344 strain was statistically indistinguishable from that of SHR (Fig. 6).

It is of interest that the temporal expression profile of ICAM-1 mRNA (Figs. 1 and 2) following focal ischemia and elevated expression of ICAM-1 mRNA (Figs. 5 and 6) in SHR compared to normotensive rats strikingly parallel that of TNF- α and IL-1 β mRNA expression [18,19], and the increase in neurological deficits and the degree of cortical infarction that occur in different strains following PMCAO [2]. In addition, the induction profile of ICAM-1 mRNA following

MCAO with reperfusion (Figs. 3 and 4) also showed an identical profile to that of TNF- α and IL-1 β mRNAs (Wang et al., manuscript in preparation) in that specific model. This parallelism suggests a functional association of ICAM-1 with the inflammatory cytokines and the involvement of these molecules in the inflammatory response in the ischemic tissue. The induced expression of ICAM-1 on endothelial cells by endotoxin (LPS) and cytokines, TNF- α , IL-1 and IFN- γ has been reported previously in vitro and in vivo [10,24,31]. Recently, upregulation of ICAM-1 expression has been shown in human brain microvessel endothelial cells by LPS, TNF- α , IL-1 β and IFN- γ in a concentration- and time-dependent manner [33]. Therefore, it is possible that the increased expression of the proinflammatory cytokines such as TNF- α and IL-1 β in focal ischemic tissue following MCAO may result in the elevated expression of adhesion molecules such as ICAM-1 and ELAM-1 on microvascular endothelial cells. The increases in the expression of these adhesion molecules can further facilitate the recruitment of circulating leukocytes into the focal ischemic tissue. The present work provides evidence for the involvement of ICAM-1 in the inflammatory reaction following focal ischemia.

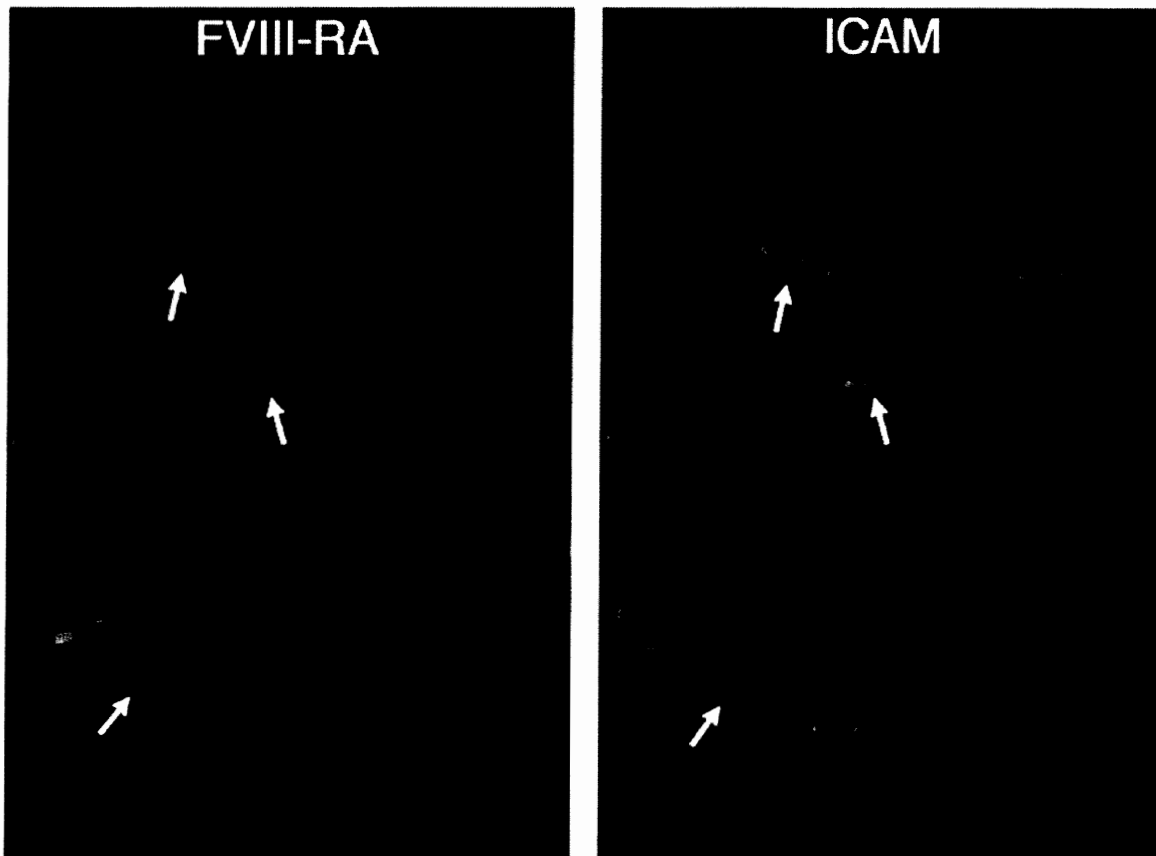


Fig. 7. Immunohistochemical detection of ICAM-1 expression in ischemic cortex following 12 h permanent MCAO in SHR. Left panel depicts a section through the lesion area and demonstrates staining with rhodamine-labeled antibodies against Factor VIII related antigen (FVIII-RA) of the endothelial cells of intraparenchymal blood vessels. The right panel demonstrates the same area after exposure to fluorescein-labeled monoclonal antibodies against rat ICAM-1. Arrows indicate blood vessels positive for both ICAM-1 and FVIII-RA. Magnification $\times 500$.

The significance of ICAM-1 expression following focal ischemia was further supported by recent demonstrations that treatment with an anti-ICAM-1 antibody reduces neurologic deficits after reperfusion following spinal cord ischemia and embolic stroke in the rabbit [5,8]. Very recently, Zhang et al. [34] found that an anti-ICAM-1 antibody can significantly reduce the ischemic injury after TMCAO in the rat.

In conclusion, the present data demonstrate the induction profile for ICAM-1 mRNA and protein on the microvascular endothelial cells in the ischemic cortex following focal ischemia. This profile is remarkably parallel to that of TNF- α and IL-1 β induction in ischemic tissue, suggesting that the induction of these proinflammatory cytokines may upregulate the expression of ICAM-1 and possibly other adhesion molecules, which in turn facilitate leukocyte infiltration into the cerebral ischemic tissue. The data suggest a critical role for ICAM-1 in this inflammatory response to cerebral ischemic tissue damage, and the potential utility of ICAM-1 antagonists as therapeutic agents in stroke.

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