INTERNATIONAL SYMPOSIUM

Polymorphonuclear Leukocytes and Microcirculatory Perfusion in Acute Stroke in the SHR

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(Received for publication on May 29, 1996)

Abstract. In order to determine the effect of depleting circulating polymorphonuclear neutrophils (PMN's) on brain microcirculation and lesion size in an acute stroke model, Spontaneously Hypertensive Rats (SHR) were injected intraperitoneally with either 2 ml RP-3 antineutrophil antibody followed in 4 hours by MCAO (n = 5), 2 ml saline followed in 4 hours by middle cerebral artery occlusion (MCAO) (n = 6), or 2 ml saline followed in 4 hours by sham operation (n = 3). After 4 hours of ischemia or a 4 hour interval (sham-operated animals), microvascular perfusion was assessed by means of an intravascular fluorescent tracer technique: FITC-dextran and Evans blue were injected intravenously 10 seconds and 5 seconds, respectively, before decapitation. Lesion volume was calculated by interpolation from histologic sections cut from 8 predefined stereotactic levels. MCAO with the normal complement of neutrophils led to significant impairment of perfusion in nutrient vessels and a maximal ischemic lesion volume. Depletion of circulating leukocytes by RP-3 significantly attenuated the microvessel perfusion impairment and reduced the volume of ischemic brain injury. (Keio J Med 45 (3): 248–253, September 1996)

Key words: polymorphonuclear leukocytes, microcirculatory perfusion, ischemia, stroke, spontaneously hypertensive rat

Introduction

It is becoming increasingly clear that inflammatory mediators acting at the blood-endothelial interface participate in progressive brain damage in acute stroke.¹ Brain injury arising from the interaction of multiple mediators at the blood-endothelial interface during acute ischemia has been termed "blood-damaged tissue interaction"² and leukocytes appear to have a multifaceted role in this process.³ Activated leukocytes are large, stiff, viscoelastic cells that can adhere to endothelium, obstruct capillaries in an ischemic injury zone and increase diffusion distances for oxygen within the microenvironment of individual neurons.^{4,5} Leukocytes also possess an impressive armamentarium of biochemical mediators of tissue injury. For example, leukocytes can generate a variety of reduced oxygen products that are capable of initiating lipid peroxidation in cell membranes and dam-

aging key intracellular components.⁶ The release of granule constituents such as elastase, collagenase and gelatinase from neutrophils can potentially mediate tissue destruction and extend tissue damage in an ischemic injury zone.^{7,8} Activation of phospholipases initiates a cascade of reactions producing vasoactive substances such as prostaglandins, prostanoids, lipoxins, leukotrienes, and platelet activating factor.^{9,10} Polymorphonuclear neutrophils (PMN's) as well as monocytes can synthesize and release an impressive array of cytokines including TNF- α , IL-1 β , IL-1ra, IL-8, and TGF- β 1.^{11,12} Finally, PMN's can activate platelets and induce their aggregation.^{13–15}

Several of these mediators have the potential to profoundly impair brain microcirculation. However, in order to directly assess leukocyte interference in microcirculatory function, it is necessary to reliably measure the density of perfused capillaries in ischemic brain. Several

methods have been employed to evaluate brain capillary filling defects following experimental ischemia. One of the most widely used techniques to visualize patent microcirculatory vessels involves postmortem infusion of substances such as carbon and gelatin under pressures that approximate mean arterial pressure.^{16–18} However, this technique suffers from several limitations that can produce artifactual defects in microvessel filling.^{19,20} For example, aggregation of the carbon suspension, promotion of aggregation of blood elements, or, in most perfusion fixations, the absence of normal pulsatile flow may all affect the adequacy of perfusion. Also, the different flow rates that accompany different routes of perfusate administration can influence the pressure drop across the infusion needle and alter actual perfusion pressure.²¹ Many of these potential pitfalls are circumvented by another technique involving intravenous infusion of horseradish peroxidase 20 minutes prior to death,²² but this necessitates many cycles of recirculation. A fluorescent tracer technique described by Weiss²³ and critically refined by Kuschinsky²⁴ appears to offer the most sensitive index of capillary perfusion in injured brain with a temporal resolution that approaches one-pass kinetics.²⁵ In this study, we have utilized this technique to assess the role of PMN's in the development of postischemic impairment of microcirculatory perfusion. Specifically, the effect of PMN depletion by RP-3 antibody²⁶ on brain capillary perfusion and lesion size was assessed 4 hours after MCAO in SHR.

Methods

Adult (300–350 g) male SHR were anesthetized with halothane in nitrous oxide; oxygen (70:30). One femoral artery was cannulated to obtain blood samples for white blood cell counts. A baseline blood sample was collected, then the surgical site was closed. Either RP-3 antibody or saline was administered i.p. Anesthetics were withdrawn and rats returned to home cage.

Four hours following antibody/saline administration, under halothane anesthesia, the left middle cerebral artery was permanently occluded by electrocoagulation between the olfactory tract and the inferior cerebral vein. For sham-operated controls, the middle cerebral artery was exposed but not occluded. Following surgery, anesthetics were once again withdrawn. Finally, 4 hours post-MCAO, microvascular perfusion was assessed using the fluorescent tracer technique (see below) under halothane anesthesia.

The following groups were studied; 1) saline – 2 ml i.p. followed by sham operation (n = 3); 2) saline – 2 ml i.p. followed by MCAO (n = 6); 3) RP-3 antibody (murine IgM mouse ascites – kindly supplied by Dr Warren Lo, Ohio State University OH, USA) 2 ml intraperitoneally (i.p.) followed by MCAO (n = 5).

Blood samples (90 µl) were collected (under halothane anesthesia) at baseline and immediately prior to administration of fluorescent tracers (4 h post-MCAO and 8 hours after the baseline sample). White blood cell counts were determined manually on cresyl violet-stained cells with a hemocytometer.

Microvascular perfusion was assessed by means of an intravascular fluorescent tracer technique based on the methods described by Kuschinsky and co-workers.²⁴ Briefly, 2 fluorescent tracers – Evans Blue (2% in saline; 0.2 ml/100 g body weight; Sigma, St. Louis, MO, USA) and FITC-dextran (10% in saline; 0.2 ml/100 g body weight; 71000 kD; Sigma, St. Louis, MO, USA) – were used to obtain 2 measurements of microvascular perfusion within each animal. Following 4 h of MCAO, the tracers were sequentially administered into separate femoral veins 10 seconds (FITC-dextran) and 5 seconds (Evans Blue) prior to decapitation. The brain was rapidly removed, frozen in 2-methylbutane (–42°C), and sectioned on a cryostat (–21°C). Six µm sections were cut for fluorescence imaging, while 20 µm sections were taken for subsequent histological staining.

To quantify the number of perfused microvessels, sections were examined by fluorescence microscopy and images acquired and processed using the Metamorph image processing system (Universal Imaging, West Chester, PA, USA). Images were acquired from 3 regions of cortex at each of 2 levels ipsilateral to the MCAO (Fig 1). The images were manually thresholded prior to automatic calculation of the number of microvessels per field of view. Final results are expressed as the mean (±S.D.) number of perfused microvessels per mm² for each region of interest. Statistical comparisons were by one-way ANOVA followed by Dunnett's test.

For volumetric assessment of the ischemic lesion, sections were cut at 8 pre-defined stereotactic levels²⁷ and stained with either cresyl violet or hematoxylin and eosin. Sections were examined by light microscopy and the borders of the ischemic lesion delineated. Lesion areas were then measured from the sections using an image analyzer (NIH image) and converted to total volume of ischemic damage. Lesion volumes were corrected for tissue swelling by means of a formula adapted from Swanson:²⁸

$$\left[\frac{\left(\text{contralateral hemisphere mm}^3 \right) - \left(\text{ipsilateral non-infarcted mm}^3 \right)}{\text{contralateral hemisphere mm}^3} \right] \times 100$$

Statistical comparisons of lesion volumes in the drug and vehicle groups were by unpaired t-tests.

Results

PMN counts were comparable at baseline among the

Saline/Sham, Saline/MCAO, and RP-3/MCAO groups ($1.44 \pm 0.27 \times 10^6/\text{ml}$, $1.70 \pm 0.45 \times 10^6/\text{ml}$, $1.33 \pm 0.38 \times 10^6/\text{ml}$, respectively). In blood samples collected 8 hours later, 4 hours after MCAO, PMN counts reflected a strong RP-3 effect that counteracted the substantial in-

crease in circulating leukocytes noted in the other two groups (Saline/Sham $- 3.77 \pm 1.68 \times 10^6/\text{ml}$, saline/MCAO $- 3.44 \pm 1.12 \times 10^6/\text{ml}$, RP-3/MCAO $- 0.24 \pm 0.21 \times 10^6/\text{ml}$).

The mean number $\pm$ S.D. of perfused microvessels per mm^2 brain tissue in each of the 3 regions at each of the 2 levels for each of the 3 groups are presented in Table 1. A highly significant reduction in microvessel filling at both the 5 second and the 10 second circulation intervals was apparent in the Saline/MCAO group compared to Saline/Sham animals for both the ischemic core (Region C) and perifocal/penumbral (Region B) regions. This impairment of microcirculatory perfusion was significantly reduced in the perifocal/penumbral region by RP-3 depletion of PMN's. In the ischemic core, RP-3 only induced a trend toward improvement in microvessel perfusion. Microvascular perfusion in non-ischemic Region A did not differ significantly between the 3 experimental groups.

In the Saline/Sham group, there was no essential difference between the number of brain microvessels that filled after either 5 seconds or 10 seconds of indicator circulation. In contrast, both MCAO groups had an increase in the number of microvessels perfused after 10 seconds in both ischemic regions at both coronal levels studied (the increase in perfusion ranged from 12–116 microvessels per mm^2 brain tissue). Thus, delayed filling indicative of a reduced perfusion velocity rather than total obstruction occurred in some of the microvessels in the groups subjected to MCAO.

The percent of the ipsilateral hemisphere that developed an ischemic lesion was $23.7 \pm 5.1\%$ in the Saline/MCAO group and $20.2 \pm 3.1\%$ in the RP-3/MCAO group. A modified Swanson calculation²⁸ was applied to adjust for tissue swelling and the corrected lesion volume

Fig 1 Schematic diagram of the 2 coronal neuroanatomic levels, and the 3 cortical regions analyzed for quantitation of microvascular perfusion.

Table 1 Microvascular Perfusion Following Middle Cerebral Artery Occlusion (MCAO)

LEVEL 1	A: Non-ischemic		B: Perifocal/penumbral		C: Ischemic core	
	5s	10s	5s	10s	5s	10s
Saline/Sham	347 $\pm$ 12	333 $\pm$ 7	381 $\pm$ 32**	377 $\pm$ 15**	360 $\pm$ 30**	358 $\pm$ 20**
Saline/MCAO	342 $\pm$ 38	342 $\pm$ 29	110 $\pm$ 99	147 $\pm$ 93	11 $\pm$ 8	66 $\pm$ 51
RP-3/MCAO	327 $\pm$ 24	328 $\pm$ 22	283 $\pm$ 40**	295 $\pm$ 37*	18 $\pm$ 10	134 $\pm$ 53

LEVEL 2	A: Non-ischemic		B: Perifocal/penumbral		C: Ischemic core	
	5s	10s	5s	10s	5s	10s
Saline/Sham	350 $\pm$ 22	352 $\pm$ 7	330 $\pm$ 27**	352 $\pm$ 26**	324 $\pm$ 7**	324 $\pm$ 13**
Saline/MCAO	337 $\pm$ 52	333 $\pm$ 42	80 $\pm$ 90	139 $\pm$ 95	27 $\pm$ 20	96 $\pm$ 34
RP-3/MCAO	346 $\pm$ 40	339 $\pm$ 30	271 $\pm$ 57**	290 $\pm$ 32**	66 $\pm$ 41	150 $\pm$ 36

Data are presented as the mean number $\pm$ S.D. of perfused microvessels per mm^2 brain tissue. Statistical comparisons by one way ANOVA, followed by Dunnett's t-tests.

** $p < 0.01$, * $p < 0.05$ comparison with saline/MCAO group.

Fig 2 Graph depicting the percent of the ipsilateral hemisphere that developed an ischemic lesion in the Saline/MCAO (Sal) and RP-3/MCAO (RP-3) groups. Values are presented both without and with the Swanson correction for edema.

in the Saline/MCAO group was $22.4 \pm 5.4\%$ compared to $16.5 \pm 2.5\%$ in the RP-3/MCAO group. This difference was significant at the $p < 0.05$ level. These results are illustrated in Figure 2.

Discussion

The data shows that relative to sham-operated animals, MCA occlusion for 4 hours in the SHR with the normal complement of circulating neutrophils produced a significant impairment of perfusion in the nutrient vessels. The reduction in capillary filling was most pronounced in the core lesion area, but was also substantial in the perifocal/penumbral region at both levels studied. Depletion of circulating leukocytes by RP-3 antineutrophil antibody prior to MCAO attenuated this impairment of capillary perfusion and, relative to the saline control group, reduced the volume of brain injury caused by 4-hour exposure to ischemia when the modified Swanson correction for edema was applied. A previously reported reduction of neutrophil accumulation and lesion size following RP-3 pre-treatment assessed 24 hours after 1 hour of transient focal ischemia in Wistar rats, is in general agreement with our findings.²⁹ Whether the benefits of leukocyte depletion observed in this study are lasting can only be determined by longer periods of follow-up.

The magnitude of the reduction of microcirculatory perfusion impairment in animals subjected to neutrophil depletion by RP-3 appears to be reflected in the degree of neuroprotection achieved with the antibody. Relative to animals with normal circulating neutrophil counts, capillary filling defects were reduced 65–70% in the perifocal/penumbral regions and 20–25% in the lesion

core in neutrophil-depleted animals. Since the core region accounts for more than 80% of the lesion volume, these results accord with the 26% overall reduction in tissue injury observed after RP-3 treatment. Therefore, the sparing of nutrient perfusion may account for most of the cytoprotection observed with RP-3 treatment in this model.

The mechanism by which RP-3 depletes circulating PMN's is not firmly established. Based on observations of neutrophil phagocytosis by peritoneal macrophages and a rapidly detectable decline in circulating leukocytes after RP-3, antibody coating of neutrophils leading, respectively, to phagocytosis and transfer to the marginating pool have been proposed as mechanisms for neutrophil depletion by RP-3.²⁶

Although the theoretical arguments presented in the introduction suggest that most of the techniques used heretofore to assess microcirculatory patency are subject to experimental artifact and should be interpreted with caution, our results are in general agreement with the earlier studies of Del Zoppo⁵ and Garcia²² that demonstrate obstruction of the brain microcirculation after ischemia and implicate leukocytes in that process. Our results, somewhat paradoxically, differ from the findings of Kuschinsky's group³⁰ in that they found only infrequent, widely scattered foci of impaired capillary filling in the Sprague-Dawley rat 1 hour after MCA occlusion, which stands in marked contrast to the profound reduction in local cerebral blood flow measured in the MCA distribution in the same animals. Two factors that could account for this discrepancy are; 1) we studied a strain of rat (SHR) in which acute ischemia was superimposed on a chronic vasculopathy secondary to chronic hypertension in contrast to a strain (Sprague-Dawley) that is free of stroke-risk factors and 2) we used a 4-hour time point instead of a 1-hour time point to assess the patency of brain capillaries. In a previous study investigating the time course for the development of microcirculatory perfusion changes after MCA occlusion in SHR (submitted), filling defects were prevalent at 5 minutes, decreased substantially by 1 hour, but then increased to become prominent by 4 hours post-MCAO. The secondary deterioration of microvascular perfusion would not be detected at the 1 hour time point.

In conclusion, RP-3 significantly attenuated impairments in microcirculatory perfusion following MCAO and reduced tissue injury. These results provide further support for the hypothesis that leukocytes, and PMN's in particular, are involved in the pathogenesis of focal ischemic injury and can profoundly influence microcirculatory function.

Acknowledgements: This work was partially supported by a grant from NINDS 2 P50 NS 30318–04A1.

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Discussion (John M Hallenbeck)

Johansson: The infarct volume did not change by edema correction when you blocked TNF- α . Do you interpret this observation as indicating that blocking TNF- α also reduced the edema? There is other evidence to suggest that TNF- α provokes brain edema. If so, blocking TNF- α could have a stronger anti-edema effect than blocking leukocytes.

Hallenbeck: I think that you are making a very reasonable interpretation of the data.

Asano: 1) Is the decrease of capillary perfusion immediately after recirculation correlated with the alteration in the rCBF? 2) What is the major cause for the initial perfusion defect?

Hallenbeck: 1) We did not measure blood flow in this series, but there is considerable published evidence that blood flow (measured by the iodoantipyrine autoradiographic technique for example) is reduced in the areas of microcirculatory perfusion impairment at the time points studied. 2) Of the various possible mechanisms for impeding microcirculatory flow, our data permit the inference that PMNL are involved.

Uchiyama: You have previously demonstrated that removal of von Willebrand factor reduces the size of infarct. Do you have any direct evidence that TNF- α induces or increases the release of von Willebrand factor from endothelial cells?

Hallenbeck: There are reports that TNF- α does increase the release of von Willebrand factor among its many

procoagulant and proinflammatory effects on the endothelium.

Yamasaki: What are the possible sources of TNF- α in the reperfusion model? Do you have any data for TNF- α production after RP-3 treatment?

Hallenbeck: Possible sources of TNF- α would include monocytes, PMNL, macrophages, astrocytes, and microglial cells. We did not measure the TNF- α production after RP-3 treatment in these studies, but it would be interesting to do so.