Ischemic Stroke Is Associated With a Systemic Increase of Blood Mononuclear Cells Expressing Interleukin-8 mRNA

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Background and Purpose—Ischemic brain injury secondary to an arterial occlusion is characterized by acute local inflammation. Blood polymorphonuclear leukocytes (PMNL), primarily neutrophils, adhere to endothelial cells and rapidly invade the injured brain after the arterial occlusion. This neutrophilic invasion might correlate with the production of certain chemoattractants by blood mononuclear cells (MNC). We evaluated mRNA expression of the CXC chemokine interleukin (IL)-8, and the CC chemokines monocyte chemoattractant protein-1, macrophage inflammatory protein (MIP)-1α, and MIP-1β in blood MNC from patients with ischemic stroke.

Methods—Peripheral blood was obtained at 8 AM on days 1 to 7 (mean, day 3) after onset of symptoms. In situ hybridization with radiolabeled synthetic oligonucleotide probes for the chemokines was adopted to measure chemokine mRNA expression in MNC. An enzyme-linked immunosorbent assay for IL-8 was used to measure IL-8 levels in plasma.

Results—Most patients with ischemic stroke had high numbers of IL-8 mRNA expressing blood MNC, regardless of the time interval between onset of clinical symptoms and examination. There was a marked difference between patients with ischemic stroke and healthy subjects (median, 6228 versus 885 positive cells per 10^5 MNC; P<.0001). IL-8 levels in plasma correlated positively to IL-8 mRNA expression in examined patients (n=7) with ischemic stroke (r=.78, P<.05). In contrast, mRNA expression for the CC chemokines showed no significant difference between patients with ischemic stroke and healthy control subjects.

Conclusions—This study demonstrated a systemic increase of IL-8 mRNA expressing MNC and IL-8 levels in plasma from patients with ischemic stroke, suggesting that IL-8 could be involved in recruiting blood PMNL to the sites of cerebral ischemia. (Stroke. 1998;29:462-466.)

Key Words: cerebral ischemia ■ chemokines ■ interleukins

Extensive research efforts are currently being made to identify molecules that may contribute to the frequently devastating effects of ischemic stroke. Accumulation of PMNL has been shown in animal models of cerebral ischemia, with a maximum at 24 to 72 hours after a transient occlusion of one branch of the middle cerebral artery. A role for PMNL has been implicated, especially in reperfusion injuries after acute ischemia where they can mediate tissue damage both by physical obstruction of vessels and by releasing oxygen radicals, proinflammatory cytokines, and cytolytic enzymes. Such a role is further supported by results from experimental studies directed at reducing or interfering with the PMNL. For example, antibodies blocking intercellular adhesion molecule-1 reduced tissue leukocyte infiltration and infarct size in rats with transient ischemia due to middle cerebral artery occlusion. Also, in human ischemic strokes, an accumulation of leukocytes has been detected in the brain during the acute and subacute stages. Several studies have shown changes in levels of cytokines and adhesion molecules in body fluids of patients with cerebral ischemia, reflecting activation of the immune system. For instance, serum concentrations of IL-6 and the IL-1 receptor antagonist were elevated in patients with cerebral ischemia and correlated to the magnitude of brain lesions.

In addition to adhesion molecules, factors that act as chemoattractants could play a pivotal role in the accumulation of leukocytes in ischemic areas of the brain. Chemokines are molecules with chemotactic activities on selective leukocyte subpopulations. Chemokines are subgrouped into the α- (or CXC) subfamily acting primarily on PMNL and the β- (or CC) subfamily attracting mainly lymphocytes and monocytes. Among the α-chemokines, members of the IL-8 family have previously been found to be upregulated during reperfusion in a rabbit model of pulmonary ischemia and after focal cerebral ischemia in rats. Here we describe the dramatic systemic increase of IL-8 mRNA expressing MNC and increased plasma levels of IL-8 in patients with brain ischemia, thereby introducing another molecule that could be targeted in future efforts to combat the infiltration of PMNL to the sites of cerebral ischemia.

Subjects and Methods

Patients

Eighteen patients (9 women) with acute ischemic stroke from the Stroke Center of the Department of Neurology, Huddinge University
Hospital, Stockholm, Sweden, were included. The patients’ ages varied from 52 to 84 years (mean, 71 years). Five patients had one or more TIA, defined as a complete recovery from clinical symptoms within 24 hours, whereas 13 patients had completed stroke defined as clinical symptoms persisting >24 hours.13 Eight additional patients with completed stroke (n=6) or TIA (n=2) were included for the IL-8 ELISA. The age of these 8 patients varied from 43 to 77 years (mean, 64 years). The diagnosis was confirmed by clinical history and neurological examination. One of the patients had symptoms confined to the vertebrobasilar territory, whereas the remaining 25 patients had symptoms from the carotid arterial territory. Brain CT was performed in all subjects within 24 hours after admission. No pathological lesions were demonstrated in the patients with TIA, whereas low-density areas were detected in all patients with completed stroke. Patients with verified intracranial hemorrhage, clinical evidence of acute infection, or evidence of myocardial infarction were excluded to avoid other potential sources of chemokine upregulation. Routine blood variables were assessed in all patients. None of our patients demonstrated increased blood leukocyte counts. Therefore, no differentiation between PMNL and MNC was made. Peripheral blood was obtained at 8 AM on days 1 to 7 (mean, day 3) after onset of symptoms.

Twenty-one healthy volunteers (8 women) from the Department of Neurology staff were included as control subjects. Their age range was 25 to 53 years (mean, 34 years).

Preparation of Blood MNC
Periperal blood MNC were obtained by density-gradient centrifugation on Lymphoprep (Nycomed). The cells from the interphase were collected, washed twice with Dulbecco’s modification of Eagle medium (Gibco), and ultimately washed once with PBS and counted. Cell viability as measured by trypan blue exclusion always exceeded 95%. Aliquots containing 1×10^6 blood MNC were dried onto electrically charged microscope slides (SuperFrost/Plus, Menzel-Gläser). Slides were kept at −20°C until hybridization.

In Situ Hybridization to Detect IL-8, MCP-1, MIP-1α, and MIP-1β mRNA in MNC
In situ hybridization was performed as previously described.14 For each chemokine, a mixture of four different synthetic oligonucleotide probes, each approximately 30 bases long, was used to increase the sensitivity of the method. The human IL-8, MCP-1, MIP-1α, and MIP-1β probes were purchased from R&D Systems. A constant guanine/cytosine ratio of approximately 60% was used. After in situ hybridization, slides were rinsed in SSC, dehydrated through gradient ethanol, dipped in Kodak NTB2 emulsion, and exposed at 4°C for 14 days. The emulsion-coated slides were developed in D19 (Kodak) and fixed in Unifix (Kodak). As control probe, the sense sequence to bases 4641 to 4688 of human interferon gamma was used in parallel, without revealing any positive cells. Coded slides were examined by dark-field microscopy for positive cells. The intracellular distribution of the grains was always checked with light microscopy at higher magnification (Fig 1). Positive cells always contained more than 15 (usually 50 to 100) grains in a star-like distribution, whereas negative cells almost always contained no or very few grains that were scattered randomly over the cell and not distributed in a star-like fashion. Consequently, it was only rarely difficult to differentiate between chemokine mRNA positive and negative cells. To compensate for cell losses, the total number of cells on the slides was regularly counted.

With the help of a microscope grid used as a measuring unit, the radius (r) of the surface area (A) covered by cells was determined. The area A was calculated by the formula A=π×r². Cells were usually counted in four grids at the periphery and one grid at the center of the surface covered by cells. In case of uneven distribution, cells in additional grids were counted. The mean value of the number of the cells per grid was determined and multiplied by A. The results were expressed as numbers of labeled cells per 10^5 MNC.

ELISA
Peripheral blood was collected in EDTA tubes and centrifuged within 30 minutes at 1500 g for 10 minutes at 4°C. The plasma was immediately stored at −20°C. IL-8 in coded plasma samples from 15 patients with acute ischemic stroke and 9 healthy control subjects was measured by an ELISA performed according to the manufacturer’s instructions (Biosource International). The detection limit was 0.39 pg/mL. Plasma samples were examined in duplicate. Peripheral blood MNC from 7 of these stroke patients and all 9 healthy control subjects were also examined for IL-8 mRNA by in situ hybridization.

Statistical Analysis
The nonparametric Mann-Whitney test was used for group comparisons. Linear regression was performed to correlate plasma IL-8 levels with numbers of IL-8 mRNA expressing MNC. Reported probability values are two-tailed and considered statistically significant at P<.05.

Results
Numbers of IL-8 mRNA expressing blood MNC were assessed with in situ hybridization in 14 patients with ischemic stroke and 11 healthy control subjects. Because of technical reasons, some slides had to be excluded; therefore, the number of patients examined for each of the chemokines under study is not identical. The numbers of patients examined for each chemokine are depicted in the Table.

All patients with ischemic stroke had high numbers of IL-8 mRNA expressing MNC in blood, regardless of the time interval between onset of clinical symptoms and examination. There was a marked difference between patients with ischemic...
stroke and healthy subjects (median, 6228 versus 885 IL-8 mRNA positive cells per $10^5$ MNC; $P<.0001$) (Table). Twelve of 14 patients with ischemic stroke had elevated levels of IL-8 mRNA expressing cells defined as $>$(mean $+2$ SD) of the healthy controls, ie, $>2868$ IL-8 mRNA positive cells per $10^5$ MNC, compared with 1 of 11 in the group of healthy control subjects. After subgrouping of the ischemic stroke patients, no differences could be detected between patients with cerebral infarction (n=10) and TIA (n=4), but the numbers of patients were small (Fig 2).

Soluble IL-8 was detected in plasma in all examined patients (n=15) with acute ischemic stroke (Table). Levels of IL-8 were higher in stroke patients than in healthy subjects (median, 4.2 versus 0.5 pg/mL; $P<.001$). There was a positive correlation between the numbers of IL-8 mRNA expressing MNC and IL-8 levels detected in plasma of the 7 stroke patients and the 9 healthy control subjects from whom samples were available for parallel examinations ($r=.83$, $P<.00005$) (Fig 3).

In parallel, mRNA expression for the $\beta$-chemokines MCP-1, MIP-1$\alpha$, and MIP-1$\beta$ was assessed. Numbers of MIP-1$\alpha$ and MIP-1$\beta$ mRNA expressing blood MNC were similarly high in blood from patients with ischemic stroke and healthy control subjects (Table). The frequencies of patients with elevated numbers of MIP-1$\alpha$ and MIP-1$\beta$ positive cells were similarly identical. For MCP-1, only sporadic stroke patients had transcripts detectable in their blood MNC, with no difference between patients and healthy controls. When subgrouping the patients with ischemic stroke regarding persistent damage (cerebral infarction, n=13) and transient symptoms (TIA, n=5), no differences were found for any of the $\beta$-chemokines under study (Fig 2).

Discussion

Accumulating inflammatory cells are considered to contribute to disability after cerebral ischemia. The mechanisms leading to leukocyte activation and migration through the blood-brain barrier into the central nervous system are, however, incompletely known. The inflammatory process is thought to be initiated by locally produced proinflammatory cytokines such as tumor necrosis factor-$\alpha$, IL-1$\beta$, and IL-6. These cytokines have the capacity to induce or enhance the expression, at least in cultured brain vascular endothelia, of several adhesion molecules including intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin. Furthermore, in vitro stimulation of astrocytes and endothelial cells by proinflammatory cytokines results in expression of chemokines,
which are low-molecular-weight proteins specialized to recruit specific subpopulations of leukocytes to areas of inflammation (for review, see Reference 7). Our results of dramatically elevated numbers of MNC expressing mRNA of the α-chemokine IL-8 systemically, together with elevated IL-8 levels in plasma, indicate a role for this chemokine in recruiting blood PMNL to the sites of cerebral ischemia.

IL-8 is a potent chemoattractant for PMNL both in vitro and in vivo.20–22 Intradermal injection of IL-8 in humans has been shown to induce perivascular infiltration of neutrophils lasting for several hours.19 In addition to recruiting PMNL, IL-8 also stimulates the release of neutrophil granules and the respiratory burst of these cells.20–22 The release of neutrophil granules is accompanied by upregulation of complement receptors and integrins in neutrophils, further facilitating cell adhesion.23 In a rat model, elevated concentrations of an IL-8–related neutrophil chemoattractant (cytokine-induced neutrophil chemoattractant) were detected in brain and serum after reperfusion after focal cerebral ischemia.12 The mechanisms behind cell recruitment to sites of inflammation in the brain are not completely understood. However, enhanced production of IL-8 locally in the ischemic brain could lead to a concentration gradient of IL-8 over the blood–brain barrier, which partly can be detected systemically, as our results show. In chemotaxis, the migration of cells in the direction along the concentration gradient results in a rapid influx of neutrophils to the brain parenchyma, thereby leading to a local inflammation.20–24 In a rabbit model, reperfusion of ischemic lung injury caused neutrophil infiltration and destruction of pulmonary tissue, together with local production of IL-8. Interestingly, the administration of a neutralizing antibody against IL-8 prevented cell infiltration and tissue damage, making a functional role for IL-8 probable.25 Compounds neutralizing IL-8 could provide future strategies in the ongoing battle against postischemic brain injury mediated by PMNL. Another potential agent of interest in this context is related to IL-1, a proinflammatory cytokine with the ability to induce IL-8 production.25 A human IL-1 receptor antagonist has been shown to influence the postischemic injury in murine studies by reducing the number of necrotic neurons, decreasing the number of leukocytes in the ischemic brain, and causing a significant decrease in the pallor area.20 Because the infiltration of the brain by PMNL during cerebral ischemia in rodents is prolonged, with a maximum at 24 to 72 hours after the insult, substantial time is provided for initiating the treatment, in contrast to available thrombolytic regimens.1

Numbers of MNC expressing mRNA for the β-chemokines MCP-1, MIP-1α, and MIP-1β, mainly attracting lymphocytes and monocytes, were not elevated in patients with ischemic stroke in this study, suggesting a selective importance of neutrophil leukocytes in early ischemic brain tissue damage. It will be of interest to study the expression of β-chemokines at later stages after the ischemic event, when the accumulation of PMNL in the damaged tissue is accompanied by MNC.27

In situ hybridization with radiolabeled oligonucleotide probes is a highly specific and sensitive method for cytokine and chemokine detection at the cellular level.14 Data must, however, be interpreted cautiously because chemokine mRNA expression may not necessarily equal protein secretion. However, in the case of IL-8, the numbers of mRNA expressing MNC correlated well to the measured IL-8 levels from the few patients from whom plasma was available. Another dilemma with chemokine measurements in body fluids, regardless of method used, is the extent to which the results reflect ongoing processes in the target organ. Therefore, measurement of chemokines, in particular IL-8, in cerebrospinal fluid over time is an ongoing study.

In conclusion, our study shows clearly elevated numbers of circulating MNC expressing IL-8 mRNA, together with a significant elevation of IL-8 levels in plasma, during the first week after ischemic stroke. The importance of this finding lies in the potent chemotactic properties of IL-8, which could mediate PMNL accumulation in damaged tissue after cerebral ischemia. The future will show whether regimens neutralizing IL-8 will be of benefit in the treatment of stroke.

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References


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