Proinflammatory Cytokines in the Embolic Model of Cerebral Ischemia in Rat

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ABSTRACT

Increased levels of proinflammatory cytokines have been recorded after the onset of transient or permanent brain ischemia and are usually associated with exacerbation of ischemic injury. Embolic stroke model is more relevant to the pathophysiological situation in such patients, because the majority of ischemic injuries in humans are induced by old thrombi that originate from the heart and carotid arteries. Therefore, the aim of the present study was to investigate changes of inflammatory cytokines after embolic stroke.

Rats were subjected to embolic stroke, induced by a natural old clot which was injected in Middle Cerebral Artery (MCA), or sham stroke, which the same volume of saline was injected into the MCA. At 48 h after stroke induction, the levels of 5 cytokines (IL-1α and β, IL-6, IFN-γ and TNF-α) were determined in 500 µg of total protein using the Bio-Plex Rat Cytokine Array (BioRad), according to the manufacturer's instructions in ischemic and non-ischemic cortices.

While stroke animals showed infarctions and neurological deficits, we did not observe any cerebral infarction and neurological deficits in sham-operated animals. The levels of IL-1α (p=0.000) and -β (p=0.004), IL-6 (p=0.008), TNF-α (p=0.000) and IFN-γ (p=0.044) were significantly increased compared to sham treated animals.

The findings of the present study suggest that part of ischemic injury in the embolic stroke may be mediated through the increased levels of inflammatory cytokines.

Keywords: Brain ischemia; IFN-γ; IL-1; IL-6; TNF-α

INTRODUCTION

Inhibition of inflammation has been suggested for some time as a therapeutic target for stroke, especially because of the delayed time course over which it occurs leaving a long therapeutic window for intervention.1,2 There is increasing evidence from clinical studies and experimental animal research that inflammation contributes not only to the deleterious consequences of ischemic stroke but also to its recovery and repair.3-5
Increased levels of cytokines including tumour necrosis factor alpha (TNF-α), interleukin-1 (IL-1), Interleukin-6 (IL-6) and interferon gamma (IFN-γ) have been recorded after the onset of ischemia in rodents and are usually associated with exacerbation of ischemic injury. Conversely, anti-inflammatory cytokines are also up-regulated in experimental stroke. Increased interleukin-10 (IL-10) levels have been linked with an increased risk of stroke. Less is known about granulocyte macrophage-colony stimulating factor (GM-CSF), IL-2 and IL-4, however, all have been reported to have neuroprotective effects when administered after experimental stroke. Hence, the inhibition of anti-inflammatory mediators would be detrimental.

Embolic stroke model, induced by natural old clots, is more relevant to the pathophysiological situation in patients, because the majority of ischemic injuries in humans are induced by old thrombi that originate from the heart and carotid arteries. Changes of pro-inflammatory cytokines have not yet been investigated following embolic model of stroke. Since the increased levels of the above proinflammatory cytokines were associated with brain injury, the aim of the present study was to investigate changes of cytokine production following the embolic model of cerebral ischemia.

MATERIALS AND METHODS

Surgical Preparation, Animals and Experimental Groups

All procedures used in this study were approved by the animal ethic committee of Rafsanjan University of Medical Sciences. Male wistar rats (weighing 300 to 350g) were anesthetized with 5% isoflurane (5% in 30% O2 and 70% N2O) and subjected to embolic stroke or sham operated. Body temperature was maintained normothermic (37°C) throughout. Cytokine levels, infarct volume and behavioural outcome were determined in 16 animals randomly assigned to stroke (n=8) or sham stroke (n=8).

Induction of Embolic Stroke Model

Embolic stroke was induced by placing a preformed clot into the Middle Cerebral Artery (MCA). We used the technique that achieves stable occlusion with less failed embolizations (by using laser Doppler) and a low percentage of early recanalizations by a selective MCA occlusion. In this method, infarctions are consistent in both size and distribution within the MCA perfusion territory. Briefly, a longitudinal incision of 1.5cm in length was made in the midline of the ventral cervical skin. The right common carotid artery, internal carotid artery, and external carotid artery were exposed. The distal portion of the external carotid artery was ligated and cut. The modified PE-50 tubing with the 20mm clot was connected to a 50µl Hamilton lock syringe, and advanced 17–19mm in the internal carotid artery until its tip was inside of the MCA. The clot was then injected, and the catheter was removed. The wound was closed and the animal was returned to its cage. A minimum initial reduction of 70% in the laser Doppler reading was considered a successful occlusion of MCA perfusion territory. Animals that did not show 70% reduction in the laser Doppler reading, were deemed to have had failed successful clot placement and were excluded from any further study. For sham-operated animals, the surgery was the same except 5µl saline was injected into MCA. The stroke surgeon was blinded to the drug treatment regimen throughout the experiments. The duration of the surgery did not exceed 30min in any case. All animals were sacrificed at 48h after MCA occlusion (MCAo).

Cerebral Blood Flow and Blood Gases

Arterial blood was obtained from the tail artery and blood gases (Pao2, Paco2), pH and glucose were analysed 5min before and after embolization (Radiometer Medical A/S, Copenhagen, Denmark). Relative regional cerebral blood flow was monitored with laser Doppler monitor (LD-CBF) extradurally (1mm posterior to the bregma and just lateral to the linea temporalis) according to Dinapoli et al. Recordings were taken at a frequency of 2Hz for a minimum of 10min prior to embolization, continuously throughout the surgery and for a minimum of 5min after embolization.

Behavioural Testing

Neurological deficits were recorded at 24, 48 and 72 hr after embolic stroke and determined with a modified 6-point scoring system as follows: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion plus decreased resistance to lateral push; 3, unidirectional circling; 4, unidirectional circling plus
Measurement of Infarct Volume
At 48h after stroke, rats were decapitated, brains removed and cut into 6 x 2mm coronal sections using a rat brain matrix. A small part of the ischemic tissue from each brain was immediately isolated, frozen on dry ice and stored at -80°C for determining cytokines level. Infarct volume was quantified in sections stained with 2% 2, 3, 5- triphenyltetrazolium chloride using a color flatbed scanner and image processing software as previously described. The total volume of each hemisphere and infarction were determined by integration of the distance of the 6 sections. Infarctions were adjusted to the size of contralateral hemisphere by applying the following formula: Infarct volume= (volume of left hemisphere-(volume of right hemisphere-measured infarct volume))/ volume of left hemisphere. Analysis of images was conducted by a blinded observer.

Quantification of Cytokine Concentrations in the Injured Cortex
To determine the concentrations of cytokines and leukocyte-attracting chemokines in the injured cortex, rats were killed at 48h after embolization by decapitation. The frozen ischemic (stroke) or non-ischemic (sham) cortical parts of brains were homogenised in ice-cold extraction buffer containing Tris-HCl (50mM, pH 7.2), NaCl (150mM), Triton-X (1%) and 1 tablet of protease inhibitor cocktail per 10ml solution (Boehringer Mannheim) at a ratio of 1:4 (tissue:buffer). Samples were homogenized, shaken for 90min on ice and centrifuged at 10,000g for 15min at 4°C, aliquoted and frozen until analysis. Protein from tissue specimens were extracted by using a cell lysing kit (Bio-Rad) according to the manufacturer's recommendations. Then, the proteins extracts were stored frozen at -80°C until analyzed. Protein homogenates were prepared from the cortex and total protein concentrations were measured with the Bradford assay. The levels of 5 cytokines (IL-1α, IL-β, IL-6, IFN-γ and TNF-α) were determined in 500µg of total protein using the Bio-Plex Rat Cytokine Array (BioRad), according to the manufacturer’s instructions. In brief, the premixed standards were reconstituted in 0.5ml of a Bio-Plex rat tissue standard diluent, generating a stock concentration of 50,000pg/ml for each cytokine. The standard stock was serially diluted in the Bio-Plex rat tissue standard diluent to generate eight points for the standard curve. The assay was performed in a 96-well filtration plate supplied with the assay kit. Premixed beads (50µl) coated with target capture antibodies were transferred to each well of the filtration plate and washed twice with Bio-Plex wash buffer. The samples were diluted 1:4 in the Bio-Plex tissue sample diluent. Premixed standards or diluted samples (50µl) were added to each well containing washed beads. The plate was shaken and incubated at room temperature for 30min at low speed (300rpm). After incubation and washing, premixed biotin conjugated detection antibodies were added to each well. Then the plate was incubated for 30min with shaker at low speed (300rpm). After incubation and washing, streptavidin-phycocerythrin was added to each well. The incubation was terminated after shaking for 10 min at room temperature. After washing, the beads were resuspended in 125µl of Bio-Plex assay buffer. Beads were read on the Bio-Plex suspension array system (Bio-Rad), and the data were analyzed using Bio-Plex Manager software version 3.0 with 5PL curve fitting.

Statistical Analysis
Data are expressed as mean ± SEM. Physiological parameters, cytokine concentrations and infarct volume were compared by Student’s t-test. Neurological deficits were reported as medians and interquartile ranges (25th and 75th percentiles) and were analyzed with Kruskal–Wallis test. A value of p<0.05 was considered to be significant.

RESULTS
There were no significant differences between groups in any of the physiological parameters measured (Table 1). We did not observe any cerebral infarction in sham-operated animals but embolization of MCA with the clot induced a huge infarction as shown in figure1. While no neurologic change observed in the sham operated animals, all stroke animals showed neurological deficits 24 and 48h post-ischemia. There were no significant differences in initial mean laser Doppler readings between treatment groups. Percentage laser Doppler reductions were 77 ± 5% and 5 ± 2% for stroke and sham-operated groups, respectively.
Table 1. Summary of physiological values pre- and post-stroke or sham surgery

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH</th>
<th>Paco₂ (mmHg)</th>
<th>Pao₂ (mmHg)</th>
<th>Glucose (Mmol/l)</th>
<th>MAP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-surgery</td>
<td>7.40 ± 0.04</td>
<td>35.1 ± 1.4</td>
<td>174.0 ± 8.4</td>
<td>14.0 ± 0.4</td>
<td>94.9 ± 1.9</td>
</tr>
<tr>
<td>Post-surgery</td>
<td>7.40 ± 0.02</td>
<td>36.8 ± 3.5</td>
<td>155.7 ± 10.3</td>
<td>15.2 ± 1.8</td>
<td>93.9 ± 3.3</td>
</tr>
<tr>
<td>Sham (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-surgery</td>
<td>7.40 ± 0.01</td>
<td>37.7 ± 2.0</td>
<td>147.9 ± 15.6</td>
<td>14.6 ± 0.6</td>
<td>94.5 ± 2.1</td>
</tr>
<tr>
<td>Post-surgery</td>
<td>7.38 ± 0.20</td>
<td>42.0 ± 3.6</td>
<td>143.5 ± 10.2</td>
<td>16.6 ± 0.8</td>
<td>86.2 ± 2.6</td>
</tr>
</tbody>
</table>

Physiological parameters are shown as mean ± SEM. There was no significant difference between groups before or after stroke surgery. N= 5 in each group.

The level of inflammatory cytokine IL-6 in stroke and sham groups is presented in Figure 2. Compared to the sham group, IL-6 level was significantly increased in the ipsilateral hemisphere of stroke animals (p<0.01) (Figure 4). Our data also showed that embolic stroke insult significantly increased IFN-γ level in the ipsilateral hemisphere of stroke animals (Figure 5; p<0.01). No significant change was observed between sham and stroke animals in the cytokine levels of contralateral hemispheres.

Figure 2. IL-6 concentrations in the ischemic and non-ischemic brains of rats. ** p<0.01 compared to sham at the same hemisphere. N= 8 for each group.

The level of inflammatory cytokine IL-6 in stroke and sham groups is presented in Figure 2. Compared to the sham group, IL-6 level was significantly increased in the ipsilateral (p<0.01) hemisphere of stroke animals. Level of IL-1α and β were also significantly increased in the ipsilateral hemisphere of stroke animals (Figure 3; p<0.001). Compared to the sham group, TNF-α level was significantly increased in the ipsilateral hemisphere of stroke animals (p<0.001) (Figure 4). Our data also showed that embolic stroke insult significantly increased IFN-γ level in the ipsilateral hemisphere of stroke animals (Figure 5; p<0.01). No significant change was observed between sham and stroke animals in the cytokine levels of contralateral hemispheres.

Figure 3. IL-1α and β concentrations in the ischemic and non-ischemic brains of rats. ** p<0.001 compared to sham at the same hemisphere. N= 8 for each group.
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Figure 4. TNF-α concentration in the ischemic and non-ischemic brains of rats. ** p<0.001 compared to sham at the same hemisphere. N= 8 for each group.

Figure 5. IFN-γ concentration in the ischemic and non-ischemic brains of rats. ** p<0.01 compared to sham at the same hemisphere. N= 8 for each group.

Table 2. Neurological deficits of sham or stroke at 24 and 48 hr following the onset of ischemia

<table>
<thead>
<tr>
<th>hr</th>
<th>Stroke</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>3 (1.75-3.25)</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>3 (2-3.5)</td>
<td>0</td>
</tr>
</tbody>
</table>

Neurological deficits were measured by a six-score scale at 24 and 48hr following the embolic cerebral ischemia onset. The data are presented as median, 25th and 5th percentiles (percentiles in the parentheses).

DISCUSSION

In the present study, we investigated effect of embolic stroke on pro-inflammatory cytokines in rats’ ischemic hemisphere. Our results demonstrated that IL-6, IL-1α, IL-1β, TNF-α and IFN-γ were increased in ischemic hemisphere following embolic stroke.

Mechanisms resulting in cell death and tissue injury during cerebral ischemic insult are complex and include excitotoxicity, oxidative stress and inflammation. In recent years, it has become increasingly clear that brain injury following ischemia is highly associated with the inflammatory response, involving the infiltration of mononuclear phagocytes and the activation of microglia. Recruitment of polymorphonuclear cells is one of the initial events of cerebral ischemia and triggers the release of reactive oxygen species (ROS) which ultimately result in brain injury. In addition, it is widely accepted that microglia-induced neuro-inflammation has a clear role in ischemia-induced brain injury. After stroke, many of the inflammatory cells migrate from the blood vessels and accumulate in the infarct zone to contribute to neuroinflammation.

It has been recently reported that inflammatory cytokines such as TNF-α, IL-1α, IL-1β and IL-2 are increased after brain ischemia and contributed to neuronal damage. In addition, PRE-084, a selective sigma-1 receptor agonist, has reversed this neuronal injury by blocking these inflammatory cytokines. It has been shown that IL-6, IL-1α, IL-1β and TNF-α are the first secreted cytokines during inflammation. These cytokines induce several parameters of inflammation including expression of selectins (E and P selectins), integrines and chemokines on the endothelial cells of inflamed vessels to recruit effector immune cells to the inflammation sites. They also are the main causes for release of neutrophil from bone marrow to the peripheral blood. Additionally, IL-6, IL-1α, IL-1β and TNF-α can affect hypothalamus and hepatocytes to induce fever and acute phase response proteins secretion, respectively. On the other hand, IFN-γ is the main inflammatory cytokine that is produced by T lymphocytes and natural killer cells. IFN-γ has receptors on the antigen processing cells, such as macrophages and dendritic cells, and lead to activation of the cells to produce inflammatory cytokines, including IL-12, and also increased expression of type 2 MHC. All of these phenomena lead to inflammation in the target tissues.

In summary these results suggest that in embolic model of stroke, similar to other models of ischemia, pro-inflammatory cytokines are increased in ischemic areas and may contribute to the pathogenesis of ischemia.

ACKNOWLEDGEMENTS

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REFERENCES


