Proinflammatory cytokines in serum of patients with acute cerebral ischemia: kinetics of secretion and relation to the extent of brain damage and outcome of disease

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Summary

The release of the proinflammatory cytokines IL-1β, IL-6, TNF-α and soluble TNF-receptors p55 and p75 in peripheral blood was serially determined in 19 patients with acute cerebral ischemia. Only patients admitted within 4 h following onset of symptoms were studied. In contrast to serum levels of IL-1β, TNF-α and TNF-receptors, which did not exhibit a significant response, IL-6 showed a significant increase of serum levels already within the first hours following onset of disease and reached a plateau at 10 h until day 3 and returned to baseline by day 7. The increase of levels of this cytokine was significantly (P < 0.05) correlated with increasing volumes of brain lesion and was also significantly (P < 0.005) associated with poor functional and neurological outcome. The increase of levels of IL-6 despite a considerable dilution in peripheral blood shown in this preliminary study suggests an early inflammatory response in ischemic brain lesion.

Introduction

Ischemic stroke is characterized by brain tissue damage of variable extent and development, which are both difficult to predict soon after onset. Since hyperacute and potentially hazardous treatment currently investigated is aimed to restore perfusion and to protect neuronal tissue from necrosis within a “therapeutic window” of still unknown duration, the demonstration of “markers” of tissue lesion could be of considerable value for an appropriate estimate between risk and benefit.

Recently, the release of proinflammatory cytokines, e.g. tumor necrosis factor-α (TNF-α) or interleukin-6 (IL-6), was shown consecutive to tissue lesion caused by surgery or trauma (Nishimoto et al. 1989; Fassbender et al. 1993 a,b). These mediators, including also the proinflammatory cytokine interleukin-1β (IL-1β), are produced by monocytes, fibroblasts, endothelial cells and microglia (Akira et al. 1990; Libby et al. 1986; Woodroofe et al. 1991). Inhibitory proteins which bind TNF-α and lymphotoxin (TNF-β) have been identified as soluble fragments of the TNF-receptor extracellular domains. Two types of soluble TNF-receptors with molecular masses of 75 kDa and 55 kDa (sTNF-R type A and type B, respectively) have been described. These may limit the toxic effect of circulating TNF (Engelmann et al. 1990). Intracranial synthesis of proinflammatory cytokines has been observed following infections (Waage et al. 1989; Grau et al. 1987) and upon mechanical or hypoxic injury of the CNS (Woodroofe et al. 1991; Minami et al. 1992). This group of cytokines has multiple local and systemic effects which may modulate the disease course, including cytotoxicity (Araujo et al. 1990), astrocyte proliferation (Giulian and Lachman 1985) or leucocyte activation (Stricker et al. 1989).

The aim of this study was to investigate whether such immune responses can be studied in peripheral blood despite a dilution effect after acute cerebral infarction, and if so, to evaluate the kinetics of their release in relation to the extent of brain damage and clinical outcome.

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Patients and methods

Patients
This series included 24 consecutive patients admitted for hyperacute treatment of an episode of acute cerebral ischemia, lasting less than 4 h as diagnosed by history, neurological exam and computerized tomography (CT). Onset of symptoms had to be accurately determined. General history, physical examinations, chest x-rays, and standard laboratory investigations (e.g., erythrocyte sedimentation rate, white blood cell count, and urinalysis) were performed to exclude patients with coincident diseases or conditions interfering with the aim of the study (e.g., infections, malignancies, surgical procedures within the last 3 weeks, or treatment with immunosuppressive drugs). Also, patients with nosocomial infections or other inflammatory complications detected by daily clinical examinations and further laboratory tests were excluded. The neurological and functional deficits were determined at admission and at the 2nd and 3rd week according to standard criteria (Scandinavian Stroke Scale, Scandinavian Stroke Study Group 1985; Barthel's Score, Mahoney and Barthel 1965).

Quantification of the volume of brain damage
CT scans were reviewed by two of the investigators (M.D., S.W.) who were blinded with regard to the clinical data. The extent of the brain lesions was calculated from CT scans using a computer assisted planimetry algorithm. Scans were obtained between days 3 and 14 to demonstrate the final lesion due to the present disease. The lesion area of the CT slices was then manually determined; and regarding the slice thickness, the lesion volume was calculated. The measurements included all actual lesions but excluded older lesions.

Blood sampling and quantification of IL-6, IL-1β, TNF-α, TNF-R type A and B
Blood was obtained from an intravenous cannula at 4, 6, 8, 10 and 14 h and on days 1, 3, 5 and 7 following onset. Blood was allowed to clot at room temperature for 1 h, and after centrifugation the serum was stored at −80°C until used. Serum levels of IL-1β, IL-6 and TNF-α were estimated with a commercially available quantitative “sandwich” enzyme immunoassays (R & D Systems, Minneapolis, MN, USA) using an antibody specific for these cytokines coated onto a microtiter plate and an enzyme-linked polyclonal specific antibody added after washing. The lower limits of detection were 0.3, 0.35 and 7.5 pg/ml, respectively. sTNF-R concentrations were measured in diluted sera (1:5) by an enzyme linked binding assay (ELIBA) with a TNF-α horseradish peroxidase conjugate as detecting agent described previously (Digel et al. 1992). Briefly, 96 well microtiter plates were coated with non-inhibitory mAbs directed against either the TNF-R type A (75 kDa) or the TNF-R type B (55 kDa). In a single step reaction, sera were incubated together with the TNF-α enzyme conjugate in the microtiter plates. The bound TNF-α enzyme was measured enzymatically with the tetramethyl benzidine as substrate. The lower limits of detection of both were 65 pg/ml. No cross-reaction with TNF-α was observed.

Statistics
Results are expressed as mean ± SEM. Response of cytokines is expressed as the maximum net cytokine increase (ΔMAX): the difference of the maximum IL-6 value and the value obtained at 4 h. For statistical analysis, the Friedmann two-way ANOVA, the Wilcoxon matched rank test, the Mann-Whitney test and Spearman’s rank correlation were used.

### Table 1

<table>
<thead>
<tr>
<th>Time following onset of symptoms</th>
<th>IL-6 (pg/ml)</th>
<th>IL-1β (pg/ml)</th>
<th>TNF-α (pg/ml)</th>
<th>TNF-R type A (ng/ml)</th>
<th>TNF-R type B (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 h</td>
<td>5.32 ± 1.41</td>
<td>0.00 ± 0.00</td>
<td>7.99 ± 1.88</td>
<td>1.91 ± 0.26</td>
<td>5.11 ± 0.58</td>
</tr>
<tr>
<td>6 h</td>
<td>8.06 ± 2.44</td>
<td>0.02 ± 0.02</td>
<td>9.88 ± 2.37</td>
<td>1.77 ± 0.36</td>
<td>4.81 ± 0.76</td>
</tr>
<tr>
<td>8 h</td>
<td>10.14 ± 2.70</td>
<td>0.22 ± 0.20</td>
<td>7.07 ± 1.39</td>
<td>1.82 ± 0.37</td>
<td>5.03 ± 1.02</td>
</tr>
<tr>
<td>10 h</td>
<td>12.66 ± 3.22</td>
<td>0.16 ± 0.14</td>
<td>7.10 ± 1.35</td>
<td>1.91 ± 0.32</td>
<td>4.96 ± 0.86</td>
</tr>
<tr>
<td>14 h</td>
<td>10.67 ± 2.78</td>
<td>0.14 ± 0.14</td>
<td>6.80 ± 1.43</td>
<td>1.72 ± 0.32</td>
<td>4.37 ± 0.90</td>
</tr>
<tr>
<td>1 day</td>
<td>12.65 ± 3.63</td>
<td>0.05 ± 0.04</td>
<td>10.32 ± 1.93</td>
<td>1.84 ± 0.31</td>
<td>4.41 ± 0.67</td>
</tr>
<tr>
<td>3 days</td>
<td>11.22 ± 3.05</td>
<td>0.19 ± 0.19</td>
<td>7.13 ± 1.67</td>
<td>1.99 ± 0.37</td>
<td>5.00 ± 0.90</td>
</tr>
<tr>
<td>5 days</td>
<td>9.61 ± 2.96</td>
<td>0.00 ± 0.00</td>
<td>8.80 ± 1.62</td>
<td>2.06 ± 0.38</td>
<td>6.02 ± 1.01</td>
</tr>
<tr>
<td>7 days</td>
<td>7.82 ± 2.64</td>
<td>0.00 ± 0.00</td>
<td>8.97 ± 2.90</td>
<td>1.74 ± 0.24</td>
<td>4.47 ± 0.77</td>
</tr>
</tbody>
</table>

* P < 0.05 compared to serum concentrations at 4 h.
** P < 0.005 compared to serum concentrations at 4 h.
Results

Patients

5 Patients had to be excluded from further analysis because of preexisting or nosocomial infections. Thus, 19 patients (8 men, 11 women) aged between 39 and 89 years (median age 73 years) were analyzed in this study. Of these, 17 patients had suffered an ischemic stroke, and 2 patients suffered transient ischemic attacks without demonstrable cerebral infarction. Ischemia was confined to the carotid arterial territory in 16 patients, and to the vertebrobasilar territory in 3 patients. The diagnostic subgroups were: large vessel occlusive disease (*n* = 4), cardioembolic (*n* = 6), small vessel disease (*n* = 2), and unknown or other (*n* = 7). Standard therapy consisted of intravenous heparin (*n* = 10), and subcutaneous high (*n* = 4) or low dose (*n* = 5) heparin. One patient died on day 5, and one on day 7.

Kinetics of release of proinflammatory cytokines

Table 1 shows serum concentrations of IL-6, IL-1β, TNF-α, TNF-R type A and B at different times after acute cerebral ischemia. At 4 h, serum concentrations of IL-6 were lowest at 5.32 ± 1.41 pg/ml. Levels then significantly increased peaking 10 h after onset with a slightly fluctuating plateau until day 3. On day 7, values were back to baseline without significant difference from initial values. In contrast, concentrations of IL-1β, TNF-α, sTNF-R type A and B did not significantly change immediately after or following cerebral ischemia (Table 1) and were within the normal range. Concentrations of cytokines did not differ at any time in regard to the sex of the patients. No significant correlation between cytokine levels and age of the patients was observed (data not shown).

Extent of brain damage

The volume of brain lesion correlated significantly with ΔMAX IL-6 (*r* = 0.61, *P* < 0.05), as well as with concentrations of IL-6 at 4 h (*r* = 0.78, *P* < 0.005), 6 h (*r* = 0.62, *P* < 0.05), 10 h (*r* = 0.59, *P* < 0.05), day 1 (*r* = 0.62, *P* < 0.05), day 3 (*r* = 0.63, *P* < 0.05) and day 5 (*r* = 0.68, *P* < 0.05). Patients with lesions > 5 cm³ (*n* = 10) showed significantly increased values of ΔMAX IL-6 when compared with those having brain tissue lesion of < 5 cm³ (*n* = 9; *P* < 0.05) (Fig. 1).

Clinical outcome

ΔMAX of IL-6 significantly correlated with the functional outcome established by the evaluation of the activities of daily living (Barthel’s Score) between day’s 14 and 21 (*r* = −0.70, *P* < 0.005). Similarly, a significant correlation between ΔMAX and the neurological outcome (Scandinavian Stroke scale) was found (*r* = −0.76, *P* < 0.005). Further, ΔMAX IL-6 correlated with the initial scores of the Scandinavian Stroke Scale (*r* = −0.65, *P* < 0.05) but not with the initial Barthel’s scores (*r* = −0.48, n.s.). The values of concentrations IL-6 (pg/ml) of the 2 patients with TIA at the 9 intervals studied were 2.3, 2.5, 6.7, 5.8, 6.2, 4.7, 2.4, 2.5, 3.6 and 1.8, 2.4, 2.8, 2.1, 2.7, 1.7, 1.6, 2.6, 1.9, respectively.

Discussion

Serial measurements revealed a rapid response of the proinflammatory cytokine IL-6 to ischemic brain lesion, although a high interindividual variation of levels was observed. Compared to the initial serum concentrations, levels of IL-6 were already significantly increased at 6 h and returned to basal values on day 7. Normal values established earlier using the this ELISA were 1 ± 4 pg/ml (Daniel et al. 1992). In regard to the large dilution of IL-6 in peripheral blood, our results suggest a release of considerable amounts of this cytokine and point toward an activation of mononuclear phagocytes during the early phase of brain damage. The source of the IL-6 remains unknown. Monocytes

![Graph](image-url)
in blood, endothelial cells, or brain derived microglia may be responsible for this cytokine production. Increased concentrations of IL-6 in stroke have recently been reported in only 10 patients without serial measurements (Coull et al. 1993). The present study of kinetics of release of these rapidly reacting cytokines shows an important correlation with the extent of brain damage and outcome, which has not been investigated before in acute stroke. These observations confirm previous experimental reports by Woodroofe et al. (1991), who showed an early production of IL-6 and IL-1β following mechanical injury by microdialysis of intracerebral tissue with peaks of release within the first 48 h. Our observations are also consistent with recent reports of response of proinflammatory cytokines consecutive to surgical trauma, which was similarly characterized by an increase of levels already within the first few hours (Nishimoto et al. 1989; Fassbender et al. 1993a). The lack of increase of levels of IL-1β, TNF-α and sTNF-R types A and B may be explained by the large dilution in peripheral blood. This interpretation is supported by other studies which failed to detect these proinflammatory cytokines in peripheral blood even following extensive inflammatory stimuli (Van Deventer et al. 1992).

Because of the significant correlation between the response of IL-6 and the volume of the brain lesion, serial determination of levels may indicate the extent of the brain lesion within hours after onset of disease when CT or MRI fail to estimate the extent the brain lesion. Further studies with a greater number of patients and CT or MRI after 1–2 months, when demarcation of infarction is completed, are necessary to define its predictive value. Functional outcome of disease as established by the Barthel’s Score and the neurological outcome as quantified by the Scandinavian Stroke Scale were correlated with the increase of this cytokine at a high significance level (P < 0.005).

Due to their rapid response to infections (Waage et al. 1989; Fassbender et al. 1993b) and because of the recently shown association between cerebral infarction and infections (Ameriso et al. 1991), increased levels of proinflammatory cytokines may also be the result of preexisting or nosocomial infections. However, their increase within the initial hours following onset of stroke symptoms allows with high probability attribution of this cytokine response to the brain lesion. Further, patients with concomitant inflammatory processes and postoperative patients were excluded in this study by careful examination and follow-up.

The number of functions ascribed to proinflammatory cytokines is increasing. These include cytotoxicity (Araujo et al. 1990), an ability to stimulate astrocyte proliferation (Giulian and Lachman. 1985), induction of production of nerve growth factor by astrocytes (Frei et al. 1989), changes in coagulation and fibrinolysis (Beviacqua et al. 1985), or induction of hepatic synthesis of acute phase proteins including fibrinogen (Gauldie et al. 1987). Moreover, monocyte activation results in the release of interleukin-8, a cytokine that activates and attracts neutrophils (Strieter et al. 1989) which may lead to further obstruction of the ischemic damaged lesion and to reduction of oxygen diffusion increasing the area of injury (Engler et al. 1983). Therefore, their release may influence the disease course in stroke.

In conclusion, this preliminary study showed a response of IL-6 to ischemic stroke suggesting an inflammatory reaction even in the early stage of ischemic brain lesion. Monitoring of serum levels of IL-6 may possibly allow the estimation of lesion extent within the first hours of stroke since normal levels of IL-6 may be suggestive for none or miniature brain lesion.

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