Evolution of Cerebral Tumor Necrosis Factor-α Production During Human Ischemic Stroke

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Background and Purpose—Tumor necrosis factor-α (TNF-α) is detected in ischemic brain cells in experimental animal models and is believed to play an important role in apoptosis. However, the natural expression of TNF-α during human stroke is not known.

Methods—We examined TNF-α immunohistochemistry and terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) in brain samples of stroke victims (n=16) after variable survival (15 hours to 18 days). Systemic TNF-α content from a separate cohort including severe or lethal stroke cases (n=26) was also assayed.

Results—Neuronal TNF-α was demonstrated from 0.6 to 5.4 days after the onset of stroke symptoms, peaking bilaterally during days 2 and 3. Bilateral glial TNF-α immunoreactivity was detected during the acute phase, with the astrocytic TNF-α expression dominating in later phases and persisting contralaterally to the infarct in more matured phases (17 to 18 days). Invading inflammatory cells were TNF-α immunopositive beginning on the third day. Besides, vascular wall structures showed immunoreactivity sporadically. TNF-α levels were mostly nondetectable in peripheral blood. TUNEL labeling and TNF-α staining overlapped, although not completely, during the first days.

Conclusions—The data support the hypothesis that TNF-α may be involved both in the acute propagation of inflammatory processes and cell death and possibly in the more delayed reconstitutive processes of human ischemic stroke. (Stroke. 2001;32:1750-1758.)

Key Words: apoptosis ■ cytokines ■ ischemic brain damage ■ neuronal death ■ stroke ■ TUNEL

Tumor-necrosis factor-α (TNF-α) is a cytokine with potent stimulatory actions in immune and vascular responses. It has been suggested to play a role in a legion of neurological disease, including infectious and immunological diseases such as multiple sclerosis,1,2 bacterial meningitis,3 cerebral malaria,4 and AIDS,5,6 as well as noninfectious acute brain insults like ischemic stroke.7,8 Molecular data support the participation of TNF-α in neurodegenerative diseases such as Parkinson’s disease9 and Alzheimer’s disease (reviewed elsewhere10). Apoptosis, a form of cell death by “suicide,” is under genetic control and can be triggered by many factors, including cytokines.11 The list of central nervous system disorders with evidence for increased apoptosis overlaps TNF-α–associated diseases and includes Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis, retinitis pigmentosa, cerebellar degeneration, and ischemic injury (reviewed elsewhere12,13).

TNF-α is activated in experimental ischemia at both the mRNA and protein levels14–17 (Sairanen et al, unpublished data, 2001), but no data on the time course and cellular distribution are available from acute ischemic stroke in humans. In 2 systemic autoimmune diseases, rheumatoid arthritis and Crohn’s disease, TNF-α antagonists have been shown effective in randomized, double-blind settings,18,19 and these regimens are in clinical use. There is growing interest in anti-TNF approaches in cerebral ischemia as well. Before therapeutic modulation of TNF-α signaling can be pursued clinically, as suggested by Bruce et al,20 we must know the pattern and cell types responsible for the TNF-α response exhibited by the human brain during ischemia. It is crucial to test the applicability of animal data from cerebral ischemia in human specimens before clinical trials are planned and timed. This led us to investigate whether TNF-α is produced in humans and whether it is correlated with apoptotic or necrotic neuronal death.

Materials and Methods
We studied autopsy specimens (postmortem delay, 17±3 hours, mean±SE) from 16 patients with ischemic stroke with symptom duration of 15 hours to 18 days before death treated at the Department of Neurology, Helsinki University Central Hospital.
Acute infectious disorders were detected in 5 patients (patient 10, moderate bronchitis; patient 14, cholecystitis; patients 12, 15, and 16, pneumonia; Table 1). Three patients who died of nonneurological causes were used as control subjects. The mean autopsy delay for control subjects was 16.5 hours (14, 14.5, and 21 hours). The study protocol was approved by the institutional review committee of the Helsinki University Central Hospital. Informed consent was given by relatives. Clinical characteristics are outlined in Table 1.

**TABLE 1. Characteristics and Results of the Immunohistochemical Examination of Neuronal TNF-α Expression of the Succumbed Stroke Patients Studied Postmortem**

<table>
<thead>
<tr>
<th>Patient*†</th>
<th>Sex</th>
<th>Age, y</th>
<th>Risk Factors</th>
<th>Cause of Death, Agonal Phase‡</th>
<th>Survival Time, d</th>
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<td>M</td>
<td>63</td>
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<td>2/F</td>
<td>F</td>
<td>89</td>
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<td>Stroke</td>
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<td>H</td>
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<td>67</td>
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<td>MCA/TE</td>
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<td>F</td>
<td>79</td>
<td>CAD, H, HF</td>
<td>PE (AMI, VF), stroke</td>
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<td>++</td>
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<td>F</td>
<td>72</td>
<td>AS (EA), CAD</td>
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<tr>
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<td>DM, H, HF</td>
<td>CA</td>
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<td>–</td>
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<tr>
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<tr>
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<td>AMI</td>
<td>…</td>
<td>…</td>
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</tbody>
</table>

* Ipsi indicates ipsilateral; Contra, contralateral; AF, atrial fibrillation; AS, generalized arteriosclerosis; DM, diabetes mellitus; H, hypertension; HF, heart failure; VF, ventricular fibrillation; ICA, internal carotid artery; TE, thromboembolism; CAD, coronary artery disease; MCA, middle cerebral artery; E, embolism; T, thrombosis; HC, hypercholesterolemia; IE, chronic ischemic encephalopathy; BA, basilar artery; PE, pulmonary embolism; AMI, acute myocardial infarction; EA, carotid endarterectomy; and CA, cardiac arrest. The immediate cause of death is mentioned first; contributing cause is second. Neuronal immunoreactivity (cytoplasms and neuronal processes) was analyzed by counting 5 fields per section from 4 to 6 sections for each patient and control subject. The grading of immunoreactivity is based on the mean of positive structures per millimeter squared and is marked as follows: –, no immunoreactivity; ++, trace (corresponding to <7 cell cytoplasms or <33 neuronal processes per millimeter squared); +, moderate (7 to 26 or 33 to 66, respectively); ++, strong (27 to 66 or 67 to 132); and ++++, marked (>67 or >133).

†Diagnosis was based on autopsy findings.

In neuropathological autopsy, dense multifocal hemorrhagic transformation was identified in infarct patients 2, 7, and 16, whereas in patients 3, 4, 11, and 15, more subtle hemorrhagic transformation was evident. No hemorrhagic transformation was detected in the rest of the patients. Spontaneous, complete recanalization was not evident in any cases of thromboembolic infarction. Neither was recanalization achieved in a patient who received 1.1 mg/kg alteplase according to the European Cooperative Acute Stroke Study (ECASS) trial protocol (patient 1).

**Immunohistochemistry**

Aceton-fixed fresh-frozen sections were permeabilized in 0.3% Triton X-100 for 20 minutes before quenching in 1% H2O2 and blocking with 10% normal sera for 1 hour with phosphate-buffered saline (PBS) washes in between. The avidin-biotin complex/horseradish peroxidase method (Vectastain Elite Kit, Vector Laboratories,
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cells, their TNF-α heterogenous immunoreactivity in glial, endothelial, and phagocytic as contralateral (Table 1). Because of less frequent and more reactivity (Table 2).

included as control experiments besides preabsorption of the TNF-α antibody suitable for blotting experiments (Genzyme) with aminoethylcarbazole as chromogen (AEC; Sigma Chemical Co) was applied to detect rabbit polyclonal anti-human TNF-α antibody (IP-300, Genzyme Diagnostics). A dilution series (1:50, 1:100, 1:250, 1:500) was run before 1:100 with 1-hour incubation was chosen as the working dilution. Replacement of TNF-α antibody with normal rabbit serum and omission of the primary antibody were included as control experiments besides preabsorption of the TNF-α antibody with 10 μg/mL recombinant human TNF-α (Genzyme) overnight. Staining of the adjacent sections was used to identify granulocytes with a monoclonal antibody against CD15 epitope (IP-300, Genzyme). Human recombinant TNF-α (Genzyme) was separated on gel electrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), transferred to nitrocellulose filter by a semidry blotting system, and analyzed as described earlier.

Analysis of TNF-α Immunoreactivity

TNF-α immunoreactivity was evaluated in 5 randomly selected, consecutive fields of 0.152 mm² from each section by a blinded investigator (T.S.). As the number of TNF-α immunoreactive neuronal processes (Figure 1A) and neuronal cytoplasms (depicted by black arrows in Figure 1E and 1F) were counted, no statistically significant differences were evident between different time intervals of infarct duration. Also, no statistically significant differences between cell structures (cytoplasms or processes) in the core and peri-infarct area or between the ipsilateral and contralateral hemispheres were evident. Thus, a more illustrative semiquantitative scoring (−, +, +, + +, + + + +) was applied, as shown in Table 1. Results from the infarction core and peri-infarct region were grouped together as ipsilateral TNF-α immunoreactivity in the summary table, and homologous regions of the noninfarcted hemisphere were presented as contralateral (Table 1). Because of less frequent and more heterogenous immunoreactivity in glial, endothelial, and phagocytic cells, their TNF-α expression was graded as follows: no immunoreactivity, trace of immunoreactivity, and increased TNF-α immunoreactivity (Table 2).

Western Blotting

Western blotting was performed to confirm specificity with a polyclonal rabbit anti-human TNF-α antibody suitable for blotting experiments (IP-310, Genzyme), giving identical immunohistochemical staining results with the TNF-α antibody used for immunohistochemistry (IP-300, Genzyme). Human recombinant TNF-α (Genzyme) was separated on gel electrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), transferred to nitrocellulose filter by a semidy blotting system, and analyzed as described earlier.

Double-Fluorescent Labeling

Double-fluorescent labeling was used to confirm the identity of TNF-α-positive cells with cellular markers for neurons, neuronal filament 200 (mouse monoclonal anti-neurofilament 200 kDa, Boehringer Mannheim GmbH), or astrocytes (mouse monoclonal antiglial fibrillary acidic protein [anti-GFAP]; Dako A/S). After acetone fixation, the specimens were blocked with 0.5% bovine serum albumin/Tris-buffered saline for 5 minutes and permeabilized with 0.3% Triton X-100/PBS for 20 minutes. TNF-α antibody (1:50 dilution for 1 hour) was succeeded by biotinylated anti-rabbit antibody (Vectastain Elite Kit) that was visualized by Neutralite avidin, a Texas Red-X conjugate (A6377, Molecular Probes Europe BV). The second primary antibody against neurofilament (NF-200, 1:50 dilution for 1 hour) was succeeded by biotinylated anti-rabbit antibody (Vectastain Elite kit, Vector) in 1:200 dilution for 30 minutes. The terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) reaction

In Situ Cell Death Detection by TUNEL

Double-fluorescent labeling of TNF-α and in situ cell death was carried out on fresh-frozen brain sections. After 20 minutes’ fixation in formalin, subsequent blocking with 0.5% with bovine serum albumin in PBS, and permeabilization with 0.3% Triton X-100/PBS for 20 minutes, the sections were incubated for 1 hour with rabbit anti-human TNF-α antibody (IP-300, Genzyme) in 1:50 dilution. After incubation with biotinylated anti-rabbit antibody (Vectastain Elite kit, Vector) in 1:200 dilution for 30 minutes, the sections were incubated with Texas Red conjugate (Molecular Probes Europe BV) in 1:200 dilution for 30 minutes. The terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) reaction

Figure 1. TNF-α immunoreactivity during different phases of infarction. A, Typical TNF-α immunoreactivity pattern consisted of cytoplasmic staining together with punctate and swollen filamentlike structures depicted by reddish AEC peroxidase staining in a section from infarct core (case 2; Table 1). B, TNF-α immunoreactivity was evident along the endothelium (thick white arrow) and in glial cells (thin white arrow) among other nonneuronal and neuronal cells in the peri-infarct region of an acute infarction (23 hours). C, Composition photomicrograph from the peri-infarct area of an acute infarction (case 2) showing TNF-α immunoreactivity in activated microglial cells as determined by their morphology. D, Immunoreactivity was abolished by preabsorption of the polyclonal TNF-α antibody with recombinant human TNF-α. E, Cytoplasmic TNF-α immunoreactivity was evident in multinucleated cortical cells, including cell bodies with triangular shapes consistent with neuronal morphology (black arrows), in a brain of an individual who survived for 3 days (case 8). The section was taken from the hemisphere contralateral to the infarction (location homologous to peri-infarct area). F, Neuronal TNF-α immunoreactivity (black arrows) persisted in the edematous cortical tissue even at relatively late post-ischemic phase (infarction core at 5.4 days). G, TNF-α immunoreactivity was evident on the basement membrane of the vessel wall...
was carried out subsequently according to the manufacturer’s instructions (Boehringer Mannheim). Control sections were incubated with label solution only.

**Immunofluorometric TNF-α Assay**

Peripheral blood samples were drawn from a separate group of severe or lethal stroke cases on days 1 (n = 26, including 3 lethal), 2 (n = 7), 3 (n = 7), and 4 (n = 12) and assayed immunofluorometrically with a detection limit of 6 ng/L as described earlier. 24 Cerebrospinal fluid (CSF) sampling was attained from 11 of these patients (1 lethal) on day 1 of hospitalization in the absence of a mass effect in CT scanning. Control blood samples were drawn from 7 persons without ischemic neurological disorders or stroke risk factors such as hypertension, diabetes mellitus, or cigarette smoking. The study protocol was approved by the institutional review committee of the Helsinki University Central Hospital. All participants gave informed consent.

**Statistical Analysis**

Differences between the number of either TNF-α-immunoreactive neuronal cytoplasms or neuronal processes in different brain infarct regions and between different time intervals of acute (0.6 to 1 days), subacute (1.2 to 6.3 days), and chronic (8.5 to 18 days) infarct duration were analyzed by the Mann-Whitney rank-sum test.

Systemic TNF-α values below the detection limit were ascribed the lowest detectable level (6 ng/L). Fisher’s exact test was used to compare the proportions of observations (detectable versus nondetectable). The effect of prior or in-hospital use of ASA or other NSAIDs on the detection of TNF-α immunoreactivity was also tested with Fisher’s exact test.

**Results**

**TNF-α Immunoreactivity**

Already in the most acute postischemic phase (0.6 to 1.0 days; n = 2), TNF-α was expressed widely in the core and peri-infarct areas (Tables 1 and 2). A typical neuronal TNF-α immunoreactivity pattern consisted of cytoplasmic staining together with punctate and swollen filamentlike structures (Figure 1A), a pattern similar to an earlier description suggested to represent protein distributed in neural processes of postischemic brain.25 In ischemic cerebral cortex, neuronal cell bodies and processes and small cells with rounded shapes and sparse processes (possibly oligodendrocytes) were TNF-α positive. Astrocytes and axons were stained in the subcortical white matter. TNF-α immunoreactivity was detected in sporadic neurons and glial cells of the contralateral hemisphere in locations homologous to peri-infarct areas (Tables 1 and 2). Occasionally, vessel wall structures showed TNF-α immunoreactivity (Table 2 and Figure 1B).

Preabsorption of TNF-α antibody with recombinant TNF-α reduced immunoreactivity remarkably (Figure 1C and 1D). Replacing TNF-α antibody with normal rabbit serum resulted in loss of the specific staining, as did omission of the primary antibody (data not shown). In addition, a band of ~17 kDa was seen on the autoradiograms from Western blotting experiments. An ~6-fold signal intensity difference was evident between the recombinant samples of the quantities of 0.4 and 4.0 ng in image

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**TABLE 2. Nonneuronal TNF-α Immunoreactivity**

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<tr>
<th>Patient</th>
<th>Duration of Symptoms, d</th>
<th>Astrocytes</th>
<th>Oligodendrocytes</th>
<th>Microglia</th>
<th>Vascular Wall Structure</th>
<th>Phagocytic Cells</th>
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</table>

The grading of immunoreactivity is as follows: ○ indicates no immunoreactivity (corresponds to control-level immunoreactivity); ●, trace immunoreactivity (above control-level immunoreactivity); and ●, increased TNF-α immunoreactivity (clearly above control-level immunoreactivity). Analysis of immunohistochemical staining is based on evaluating 5 fields/section from 4 to 6 sections from each patient and control subject.
analysis. In a parallel filter incubated with normal rabbit serum, no signal was detected (data not shown).

During the second day (deaths at 1.6 and 1.8 days as a result of herniation), TNF-α immunoreactivity was located predominately in sporadic neurons of the infarct core and in contralateral locations homologous to the peri-infarct region (Table 1). Invading inflammatory cells showed TNF-α immunoreactivity in the infarct core from the second day on (Table 2). The strikingly bilateral pattern of neuronal TNF-α immunoreactivity was further strengthened in the infarctions aged 2.5 to 3 days (n=3; Table 1). The topography was similar to the more acute cases, namely in infarct core and in section of the contralateral hemisphere homologous to the peri-infarct region (Figure 1E).

From the third day on, contralateral neuronal immunoreactivity attenuated, whereas neuronal TNF-α expression was still evident at 5.4 days in the infarct core and peri-infarct areas (Figure 1F). Astrocytes exhibited a second wave of TNF-α expression and the most intense glial TNF-α immunoreactivity in a bilateral manner from the third day on (Table 2). In 2 cases with basilar artery occlusion (6.3 and 8.5 days), neuronal and astroglial TNF-α expression was seen in the infarcted areas only (Tables 1 and 2 and Figure 1G).

In infarctions of prolonged duration (17 and 18 days), TNF-α immunoreactivity was detected only in invading inflammatory cells in the affected hemisphere (Figure 1H and II) and to a minute extent in astrocytes in remote contralateral areas, which did not mirror the infarct core or peri-infarct regions (contralateral pons and contralateral temporal lobe, respectively; Table 2). The control cases with no neurological disorder did not show TNF-α immunoreactivity (Tables 1 and 2 and Figure 1J).

NSAIDs or ASA was used occasionally before hospitalization and/or in hospital (except in patient 14; indomethacin 50 mg twice daily regularly before hospitalization) or at a low dose aimed at secondary cardiovascular prevention (ASA 100 or 250 mg daily). In our study, there were no cases without any cerebral TNF-α immunoreactivity. Furthermore, 100% of patients using an NSAID other than ASA regularly before hospitalization showed TNF-α immunoreactivity (either in neuronal or nonneuronal cells). Seventy-five percent of patients with regular ASA intake before hospitalization were TNF-α positive. Sixty percent of patients with in-hospital NSAIDs showed TNF-α immunoreactivity, as did 50% of patients who received ASA in hospital. The proportion of cases showing TNF-α immunoreactivity compared with those with no TNF-α immunoreactivity did not differ significantly between patients who had an NSAID or ASA before hospitalization or received either of these medications during their hospital stay compared with patients not receiving them (Fisher’s exact test).

**Double-Fluorescent Labeling**

Colocalization of TNF-α with both GFAP (for astrocytes) and NF-200 (for neurites) was evident. TNF-α and NF-200 immunofluorescence colocalized in thicker and longer neuronal processes (Figure 2A and 2B), whereas TNF-α-positive thinner and shorter cell processes were not double labeled with NF-200, thus preferentially representing glial TNF-α. The glial component was further confirmed by overlapping immunofluorescence of TNF-α and GFAP (Figure 2C and 2D).

**Double-Fluorescent Labeling of In Situ Cell Death (TUNEL) and TNF-α**

Nuclear TUNEL positivity without TNF-α immunofluorescence was revealed in cell somas in the infarction core and peri-infarct areas of the most acute stroke case (0.6 day). After 1.6 days, TUNEL staining was evident in peri-infarct areas and to a lesser extent in infarct core areas and the contralateral hemisphere. TNF-α fluorescent cellular processes and cell somas mostly colocalized with TUNEL fluorescence in these locations, but the overlap was not complete because TUNEL staining in cell somas was also evident without TNF-α fluorescence (Figure 2E and 2F). In 2 cases of basilar artery occlusion (2.5 and 3.3 days), TNF-α–fluorescent cytoplasm mostly colocalized with TUNEL-positive nuclei in the peri-infarct region (Figure 2G and 2H). However, the numerous TNF-α–fluorescent cellular processes and cell somas did not always colocalize with intensively TUNEL–fluorescent cell nuclei (Figure 2I and 2J). Lack of TUNEL labeling accompanied the absence of TNF-α fluorescence in noninfarcted areas. In 3 control brains, occasional TNF-α–fluorescent cell processes were seen in the absence of TUNEL staining. No TUNEL fluorescence was detected with labeling solution only (Figure 2K).

**Assay of TNF-α in CSF and Peripheral Blood**

In serial peripheral blood samples of a separate cohort with severe or lethal stroke, only 1 of 26 cases showed a detectable level of TNF-α on day 1 of hospitalization (45 ng/L; detection limit, 6 ng/L). On day 2, all patients (n=7) had undetectable TNF-α levels; on day 3, 2 of 7 patients had levels above the detection level (16±1 ng/L, mean±SD); and on day 4, 1 of 12 showed a detectable TNF-α level (10 ng/L). Of 11 patients, 4 had detectable TNF-α levels in their CSF on day 1 (14.8±2.2 ng/L). No TNF-α protein was detected in the peripheral blood samples of control subjects (n=7). In comparisons of the proportions of observations (detectable versus nondetectable TNF-α) in the peripheral blood of stroke patients (1 versus 25) and in the CSF (4 versus 7) during the first hospitalization day in a contingency table, a significant difference of the proportions was evident (P=0.021). Despite more cases with detectable levels of TNF-α in the peripheral blood as a function of time, no statistically significant increase in proportions was evident (2 versus 5 on day 3, 1 versus 11 on day 4).

**Discussion**

In human victims of fatal ischemic stroke, cells throughout the brain participated in the induction of TNF-α immunoreactivity in the different phases of focal cerebral infarction with no or incomplete recanalization. This sustained, comprehensive cellular participation is different from that reported in experimental animal models of stroke, which have indicated a decline in TNF-α mRNA and protein by 12 to 24 hours after ischemia-reperfusion15–17 except for expression up to 5 days after permanent occlusion.14 The increased level of TNF-α observed more frequently in the CSF than in the plasma of stroke patients and no detection of significant fluctuations in plasma TNF-α levels of the severe or lethal stroke cases further support the central origin of TNF-α.
during stroke. Our results confirm that TNF-α production also is an element of acute pathophysiology of cerebral infarction in humans. The extended time evolution of TNF-α response in humans, peaking at 2 to 3 days and lasting up to weeks after stroke, raises several scenarios as to its potential role in the maturation of human cerebral infarcts.

The role of locally released TNF-α may depend on the timing and cellular sources of TNF-α expression in the tissue. In the present study, TNF-α immunoreactivity was most prominent in neuronal processes of the infarction core and peri-infarct area during the first day of infarction, possibly reflecting either de novo synthesis or accumulation and release by neurons. The first wave of biphasic TNF-α immunoreactivity in astroglia in both the ipsilateral and contralateral hemispheres during the first day indicates early, global production by them. This was followed by bilateral enhancement of TNF-α response in neuronal cytoplasms, presumably indicating new synthesis in the soma, together with detection in neuronal processes in the contralateral hemisphere fitting with either uptake or transportation in neurites. This may also indicate diaschisis phenomena during the second and third days. Yet we cannot rule out that contralateral TNF-α production may also be promoted by increased intracranial pressure resulting from cerebral herniation in some cases (Table 1). In patients 3 and 15, axonal TNF-α immunoreactivity was present in the cerebellum contralateral to infarction, fitting with the topography of crossed cerebellar diaschisis. This indicates that the expanding infarction in the ipsilateral hemisphere does not leave the contralateral brain unaffected but stimulates the release of a proinflammatory factor that also has been shown to render tolerance to ischemia. Neuronal immunoreactivity subsided in the contralateral hemisphere by the fifth day, whereas in the infarcted hemisphere, it was still detected in neuronal cytoplasms. Astrogial TNF-α was upregulated simultaneously in the ipsilateral hemisphere between the third day and >1 week, fitting with a role in scarring or acute plasticity in the survived brain. The subsequent detection of astrogial TNF-α immunoreactivity only in the contralateral hemisphere promotes a role in cytokine-stimulated astrogial proliferation reported to participate in the delayed regenerative processes of the brain, such as cell growth.

Several reports promote exacerbation of ischemic neuroinjury by TNF-α. On the other hand, TNF-α signaling has been suggested to mediate glial cell proliferation, promotion of growth factor release, and neuroprotection in the settings of neuroinjury in vitro and in ischemic brain damage in vivo. It has been suggested that these discrepancies reflect differences between acute and chronic modifications of TNF-α, that they are associated with differences in cellular composition of cell cultures, that they are due to differences in knockout versus naive brain responses to injury, or that they may even result from actions of as-yet-unknown TNF ligands or receptors.

In the view of our present and previous results, a fundamental role for TNF-α in the propagation of inflammatory reactions is put forward. In human ischemic stroke, a relationship between TNF-α immunoreactivity and the inducible form of cyclooxygenase (COX-2), the key enzyme of prostanoid synthesis, is
supported by parallel work from our laboratory.\textsuperscript{22} Although significant neuronal COX-2 protein expression was still seen between 8.5 and 18 days in the hemisphere contralateral to infarction, no neuronal TNF-\(\alpha\) was detected after 5.4 days. Transient neuronal TNF-\(\alpha\) might have incited the subsequent COX-2 expression because proinflammatory cytokines are potent inducers of COX-2.\textsuperscript{45} Results of this activation cascade would include the release of more proinflammatory prostaglandins with procoagulant and proadhesive vasoactive effects. Further promotion of inflammation may follow the endothelial and perivascular cell TNF-\(\alpha\) immunoreactivity reported in this study, although sporadic (Table 2) because it overlaps increased bilateral expression of intercellular adhesion molecule-1, which we have previously reported in these infarcted human brains.\textsuperscript{46} Indeed, TNF-\(\alpha\) production by endothelial and perivascular cells, a phenomenon also described in rat models of focal and global ischemia\textsuperscript{25} (Sairanen et al, unpublished data, 2001), might well be centrally involved in mounting the robust inflammatory cell infiltration reported also in these infarcted human brains.\textsuperscript{46}

Our results demonstrate overlap in TNF-\(\alpha\) immunoreactivity and TUNEL-labeled cells. In view of the finding that neuronal cell death, including apoptosis, in general peaks after 24 to 48 hours after transient middle cerebral artery occlusion,\textsuperscript{47} we concentrated on the most acute stroke cases to investigate the relation between TUNEL and TNF-\(\alpha\) immunofluorescence. Our results are in accord with the description of the bulk of apoptotic cells to be located in the peri-infarct area with some scattered cells found in the infarct core.\textsuperscript{48} Furthermore, we established TUNEL-depicted cell death to occur in the contralateral hemisphere and to co-exist with TNF-\(\alpha\) expression after focal ischemia in humans. However, the incomplete overlap between them differs from the previous finding that only TUNEL-positive neurons express TNF-\(\alpha\) in ischemic murine brain.\textsuperscript{45} TUNEL labeling of dying cells without the presence of TNF-\(\alpha\) implies that other mechanisms regulate apoptotic cell death as well.

Interestingly, heat shock protein expression in rat brain has been shown to inhibit nuclear factor-\(\kappa\B(\text{NF-\(\kappa\B)}\) activation, which is a transcription factor regulating the expression of a number of inflammatory genes such as the TNF-\(\alpha\).\textsuperscript{49} We have previously demonstrated HSP72 immunoreactivity in these brains only locally in peri-infarct area.\textsuperscript{22} Taken together, the heat shock elements or other neuroprotective factors that inhibit NF-\(\kappa\B\) were not activated in the contralateral hemisphere, where NF-\(\kappa\B\) presumably was uninhibited to regulate inflammatory gene expression, including COX-2\textsuperscript{22} and TNF-\(\alpha\) (the present study), which may have participated in apoptosis or remodeling of neural networks. It is of future interest to investigate in these infarcted brains the respective roles of NF-\(\kappa\B\) in TNF-\(\alpha\) signaling and their correlation with cell death as described in rodent brain.\textsuperscript{50}

In conclusion, sequential expression of TNF-\(\alpha\) was detected primarily in the neurons and glia of infarction core and potentially salvageable peri-infarct area. However, this was followed by peak expression in the contralateral hemisphere and subsequent glial TNF-\(\alpha\) expression in brain areas remote from the infarction. Furthermore, TNF-\(\alpha\) expression and TUNEL-labeled dying cells coemerged during the first days. Consequently, our data support a longer window of opportunity for anti-inflammatory approaches to limit secondary ischemic brain injury in humans compared with animal models but warn against neglecting reparative processes by mediators that possibly are harmful in the acute phase but may participate later in the recovery.

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References

The preceding article by Sairanen et al deals with the expression of tumor necrosis factor (TNF-α) after stroke in humans. There has been a great deal of interest in the role of inflammatory cytokines in cerebral ischemia and the possibility that inhibitors of the expression of cytokines such as TNF-α and interleukin (IL)-1β might provide a therapeutic approach to stroke patients.1–3 There are several lines of investigation that would indicate the potential for manipulation of inflammatory cytokines, and in particular TNF-α, in stroke therapy.

It has clearly been demonstrated in animals that TNF-α and other cytokines are upregulated relatively early after cerebral ischemia. Both TNF-α messenger RNA and the peptide are expressed in the brain after experimental focal stroke in the rat.4 The messenger RNA was detected as early as 1 hour, peaked at 12 hours, and remained elevated 5 days later in the ischemic role of tumor necrosis factor-α in Stroke. 1997;37:236–239.

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cortex. TNF-α protein was elevated at 6 and 12 hours but not at 5 days after the stroke in nerve fibers, whereas macrophages within the infarct were immunoreactive for TNF-α at 5 days after the stroke. Other investigations have shown that TNF-α can be detected in the circulation after focal cerebral ischemia. In addition to the observations of Sairanen et al in this issue of the journal, other clinical pathology studies have confirmed the findings from the experimental investigations indicating that TNF-α can be found in microglia or macrophages as early as 33 hours after and persisting up to 40 days after stroke, as well as cerebrospinal fluid elevations. However, the cerebrospinal fluid increases for IL-1α, but not TNF-α, were significantly correlated with evidence of neurological worsening of the stroke.

There is potentially controversial evidence regarding the neuroprotective role of TNF-α increases in cerebral ischemia. The bulk of the evidence suggests that inhibition of the action of TNF-α prevention of the increase provides a neuroprotection, which suggests that the increase in this inflammatory cytokine contributes to the degeneration after stroke. Several investigations have demonstrated that administration of anti-TNF-α antibodies results in a decrease in the neuronal degeneration after stroke, with up to an 85% decrease in the infarct volume reported for focal ischemia. Furthermore, direct administration of exogenous TNF-α into the cerebral ventricle can significantly increase the size of the infarct, a response that can be prevented by prior injection of TNF-α antibody. Finally, local administration of TNF binding protein also has been shown to reduce infarct volume.

The evidence that TNF-α is neuroprotective comes from a variety of sources. It has been shown that neuronal survival is increased after the induction of a toxic influx of calcium mediated through neuronal N-methyl-D-aspartate acid glutamate ion channels. Furthermore, apoptosis can be prevented by TNF-α in cultured neurons exposed to oxidative and metabolic insults. Some very convincing evidence for a neuroprotective role of TNF-α, and specifically p55 and not p75 receptors, in cerebral ischemia was provided in mice lacking one or both of these receptors. Neuronal damage in the mice lacking the p55 but not the p75 receptor was significantly increased after focal cerebral ischemia and reperfusion. Thus, although many investigations have indicated that blocking the action or release of TNF-α is neuroprotective, there is evidence that under some conditions TNF-α may in fact be beneficial in neurodegenerative conditions. Some insight into the protective mechanism of TNF-α is provided by an investigation which demonstrated that pretreatment with TNF-α intracereally resulted in a significant reduction in infarct size in mice that underwent a permanent middle cerebral artery occlusion. This effect is not dissimilar to that of other molecules such as IL-1β and nuclear factor (NF)-κB, which may evoke a protective cascade of events at low levels of expression for a short time period, especially as a preconditioning stimulus.

The investigation of Sairanen and colleagues raises an important consideration regarding clinical studies that examine changes in inflammatory cytokines in stroke patients. For example, it is known that the transcription factor NF-κB elicits expression of inflammatory cytokines such as IL-1β and TNF-α. However, it is has also been shown that nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, sodium salicylate, tepoxalin, and triflusal inhibit activation of NF-κB and, in turn, the inflammatory cytokines. Thus, patients who regularly use NSAIDs and who are being used for investigations of the expression of inflammatory molecules following cerebral ischemia can potentially skew the results of these studies. Furthermore, it would be most interesting to pursue this further by examining brains of stroke patients, such as those with arthritis who are regular users of high doses of NSAIDs, to compare the levels of inflammatory cytokines and apoptotic markers.

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