Tumor Necrosis Factor-α Expression in Ischemic Neurons

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Background and Purpose  Tumor necrosis factor-α (TNF-α) is a cytokine with diverse proinflammatory actions, including endothelial leukocyte adhesion molecule expression. Since leukocytes infiltrate into ischemic brain lesions, the present study was conducted to examine whether TNF-α messenger RNA (mRNA) and peptide are expressed in the brain after experimental focal stroke and before leukocyte accumulation.

Methods  TNF-α mRNA and protein expression were monitored in the ischemic and nonischemic cerebral cortex of rats after focal ischemia produced by permanent middle cerebral artery occlusion. The effect of TNF-α administered by microinjection into the brain cortex on leukocyte adherence to brain capillaries was also studied.

Results  Induction of TNF-α mRNA, normalized to a standard reference rat macrophage TNF-α mRNA, was detected as early as 1 hour after middle cerebral artery occlusion. TNF-α mRNA was elevated by 3 hours (29±6% versus 2±1% in sham-operated rats) only in the ischemic cortex, with peak expression at 12 hours (104±8%; P<.01). Five days after middle cerebral artery occlusion, TNF-α mRNA levels in ischemic cortex were still significantly elevated (38±5%; P<.05). Also, TNF-α mRNA expression was greater in the ischemic cortex of spontaneously hypertensive rats than in normotensive rats (P<.05). Double-labeling, immunohistochemical studies revealed the presence of TNF-α protein localized within nerve fibers in the evolving infarct at 6 and 12 hours after ischemia and further expression in the tissues immediately adjacent to the infarct 24 hours after ischemia. After 5 days, the neuronaically localized peptide had diminished greatly, but macrophages located within the infarcted tissues were immunoreactive. Cortical microinjections of TNF-α (10 ng in 1 μL) produced a significant neutrophil adherence/accumulation in capillaries and small blood vessels 24 hours later.

Conclusions  These results represent the first demonstration that focal cerebral ischemia in rats results in elevated TNF-α mRNA and protein in ischemic neurons. The neuronal expression of peptide appears to facilitate the infiltration of inflammatory cells that can further exacerbate tissue damage in cerebral ischemia and might contribute to increased sensitivity and risk in focal stroke. (Stroke. 1994;25:1481-1488.)

Key Words  • cerebral ischemia, focal • cytokines • rats

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Material and Methods

Focal ischemia was produced in male spontaneously hypertensive rats (SHR) and two strains of normotensive rats (Wistar-Kyoto [WKY] and Fisher-344; weight, 250 to 350 g) under sodium pentobarbital anesthesia (60 mg/kg IP) by occluding the middle cerebral artery (MCA) as described previously. Animals were housed and cared for in accordance with Guide for the Care and Use of Laboratory Animals (Bethesda, Md: Office of Science and Health Reports, DRR/NIH; 1985. US Dept of Health, Education, and Welfare [Dept of Health and Human Services] publication 85-23). Procedures using laboratory animals were approved by the Institutional Animal Care and Use Committee of SmithKline Beecham Pharmaceuticals, plc. Briefly, after a 2- to 3-mm craniotomy over the right MCA and removal of the dura, the hooked tip of a platinum-iridium wire (0.0045 in diameter) was mounted on a micromanipulator was placed under the MCA, and the artery was pulled 0.5 to 1.0 mm away from the brain surface. The artery then was simultaneously occluded and cut (Force 2 Electrosurgical Generator, Valley Lab Inc) dorsal to the lateral olfactory tract at the level of the inferior cerebral vein. In sham-operated animals the same surgical procedures were exercised, but the artery was not occluded. Later, rats that had recovered were administered pentobarbital, and forebrains were removed and dissected as described previously at different times after MCA occlusion (MCAO). A segment of the frontoparietal cortex was dissected from the ipsilateral hemisphere (ischemic cortex), and an identical segment was dissected from the contralateral cortical hemisphere (nonischemic cortex). The segments were immediately frozen in liquid nitrogen and stored at -80°C.

Rat TNF-α complementary DNA (cDNA) was cloned from cultured rat peritoneal macrophages stimulated with lipopolysaccharide (5 ng/mL for 4 hours as described previously for interleukin [IL]-1β4) via RT-PCR using synthetic oligonucleotide primers (5'-CGAGGGGGCCAGCAAACATCTCCTCTCGGAAAGGAGC-3' and 3'-GACCGCACAAGTAGGCAAGGATGCGGCCCGG-5'), based on the published sequence25 and including a NotI restriction site. The 0.75-kb fragment obtained by RT-PCR was digested with NotI, ligated into Bluescript KS+, and sequenced to confirm identity to the published sequence. A 0.8-kb EcoRI, cyclophilin cDNA fragment was prepared according to Borgna et al.26 The rat TNF-α and human cyclophilin cDNA fragments were labeled with an oligolabeling kit (Pharmacia LKB Biotechnology), purified with a Nuctrop push column (Strategene), and used as radioactive probes on Northern blots. RNA was isolated from the frozen ischemic and nonischemic rat cortical segments (above) as described previously and quantitated using a betascope counter (Betagen Corp), using as a standard reference. After hybridization to the rat TNF-α probe, blots were stripped and rehybridized with human cyclophilin to control for loading.

These data are expressed as mean±SEM. Statistical analyses were performed using ANOVA followed by Tukey or Dunnett’s multiple comparison tests. Statistical significance was accepted at P<.05.

Also after ischemia, brains were removed from the animals and prepared for cryosectioning by coating 2-mm coronal slices with embedding medium (Tissue-Tek, Miles Inc) and immediately freezing in 2-methylbutane chilled in liquid nitrogen and then stored at -80°C. Six-micrometer coronal forebrain sections from those rats killed at 6 and 24 hours and 1 and 5 days after MCAO (n=6 SHR per time point) were prepared and fixed in absolute methanol at -20°C. Indirect immunohistochemistry was performed using rabbit anti-mouse TNF-α (Genzyme) as the primary reagent and fluorescein-conjugated goat anti-rabbit IgG, heavy- and light-chain specific (Organon Teknika), as the secondary reagent. Appropriate concentrations of antibody were determined through checkerboard titration. Specificity was determined by absorption of the primary reagent with recombinant mouse TNF-α (Genzyme) before incubation on tissues sections. Results were also confirmed through additional immunohistochemistry using hamster anti-murine TNF-α monoclonal antibody (Genzyme) followed by goat anti-hamster IgG (Organon Teknika). Specificity was further confirmed through application of secondary antibody on sections to which primary antibody had not been applied. Double-label immunofluorescence was performed, in which the rabbit anti-TNF-α immunodetection was combined with immunodetection of cell-type–selective intermediate filaments on the same tissue section. To identify neurons, monoclonal mouse anti-bovine 200K neurofilament (NF) (ICN Immunobiologicals) was used. Monoclonal mouse anti-bovine glial fibrillary acidic protein (GFAP) (Boehringer Mannheim) was used to identify astroglia. Each of these primary reagents cross-reacts with rat intermediate filaments and was followed by rhodamine-conjugated goat anti-bovine mouse IgG, heavy- and light-chain specific. Selectivity of secondary reagents in double-labeling studies was confirmed through application of anti-rabbit secondary antibody to sections that had been exposed to the anti–intermediate filament mouse monoclonal antibodies only and application of anti–mouse secondary antibodies to sections that had received only the anti-TNF-α rabbit antisemur. Slides were read on an Olympus BH2 microscope equipped with UV epi-illumination. Further analysis was performed using the OPTIMAS image analysis system and software (Fernon Electronic Imaging).

MCAO results in the development and resolution of an ipsilateral cortical infarction.28 TNF-α mRNA was induced in ischemic cortex after permanent MCAO as indicated by Northern blots (Fig 1), whereas no significant expression of TNF-α mRNA in the contralateral
nonischemic cortex was observed. Fig 2 depicts the time course of TNF-α mRNA expression quantified in the ischemic and nonischemic cortices. There was no significant induction of TNF-α mRNA in sham-operated ipsilateral cortex. TNF-α mRNA induction was observed in ischemic cortex as early as 1 hour and was significantly increased from 3 hours to 5 days after MCAO, with peak expression at 12 hours. In the contralateral cortex no significant TNF-α mRNA was observed above that which occurred after sham operation. To examine whether the TNF-α mRNA response to ischemia is specific to the SHR strain, two other rat strains were studied. Although a full time course was not carried out, in both WKY and Fisher-344 normotensive rat strains elevated expression of TNF-α mRNA in the ischemic cortex at 12 hours after MCAO was clearly observed, but to a lesser extent than at this same time point of maximum expression in SHR (Fig 3).

Examination of brain sections immunolabeled with a rabbit antiserum directed against murine TNF-α revealed strong, specific immunoreactivity observed as a fine, punctate to linear filamentous pattern within and adjacent to the developing infarct. At 6 hours, the immunoreactivity was observed in a small area of superficial cortex adjacent to the meninges (Fig 4a). The TNF-α immunoreactive area greatly increased in size by 12 hours and corresponded to the developing infarct (identified by phase-contrast microscopy) and the tissue immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it. By 24 hours after MCAO, the filamentous immunoreactivity was lost from within the infarcted tissue but remained in the cortex immediately adjacent to it.

To confirm that the immunoreactive epitope(s) were located on TNF-α, recombinant mouse TNF-α was added to the primary antibody and incubated for 1 hour at 23°C before application on tissue sections. This greatly reduced or eliminated the filamentous immunoreactivity observed within and adjacent to the ischemic cortex in a concentration-dependent manner (Fig 4c). A hamster monoclonal antibody directed against mouse TNF-α also gave results similar to those obtained with rabbit antiserum (data not shown).

Double-label immunofluorescence was used to identify the cell type in which the filamentous anti-TNF-α immunoreactivity was observed. Monoclonal antibodies directed against NF, the intermediate filament type of neurons, and GFAP, the intermediate filament type of astroglia, were used in conjunction with the rabbit anti-TNF-α antiserum. The anti-NF antibodies identified nerve fibers, and the antibody directed against GFAP identified astroglia. Double-labeling with the anti-TNF-α antibody clearly indicated that TNF-α was colocalized with NF in nerve fibers (Fig 4d through 4f). No colocalization with GFAP was observed (Fig 5a through 5c). Incubation of sections with rabbit anti-TNF-α antiserum only followed by anti-mouse secondary antibody, or mouse monoclonal anti-intermediate filament antibodies only followed by anti-rabbit secondary antibody, resulted in no detectable immunofluorescence. At 5 days after MCAO very little filamentous TNF-α immunoreactivity was observed within the infarct zone, adjacent to the infarct, or from white matter tracts. However, the macrophages present in the lesion at this point showed cytoplasmic TNF-α immunoreactivity (data not shown).

Animals that received direct cortical microinjections of TNF-α and saline revealed vascular alterations due to TNF-α administration. Specifically, capillaries and small blood vessels in the TNF-α-injected hemisphere...
FIG 4. Photomicrographs and computer-captured images of ischemic rat cerebral cortex immunolabeled with antiserum directed against tumor necrosis factor-α (TNF-α) (a through d, f) and neurofilaments (e, f). Ischemic cortex at 6 or 12 hours after middle cerebral artery occlusion (MCAO) demonstrated intense immunoreactivity in a linear or granular filamentous pattern (a; 6 hours after MCAO). This fluorescence was restricted to the tissues undergoing ischemic injury. At 24 hours after MCAO, the filamentous immunoreactivity was primarily restricted to the cortex immediately adjacent to the infarct (b, area labeled cor) and white matter tracts near the ischemic zone, such as the corpus callosum (b, area labeled cc) and striatal white matter. When the anti-TNF-α antiserum was preincubated with TNF-α, the immunoreactivity was diminished or eliminated (c, field similar to that shown in b). Computer-captured images of brain tissue 24 hours after MCAO (d, e, f) allow demonstration of double-label immunofluorescence for TNF-α (green fluorescence) and neurofilaments (red fluorescence). TNF-α is found in a filamentous pattern (arrows in d), with some filaments extending toward a blood vessel (bv). Immunolabeling of neurofilaments demonstrates the presence of nerve fibers throughout the section (e). When double labeled (f), many nerve fibers are present that express TNF-α in addition to neurofilaments (arrows in f), although nerve fibers in which there is no detectable TNF-α are also present. Note the double labeling of blood vessel-associated fibers (double arrows). The neurofilament–TNF-α double-labeled images (d, e, f) were captured using an OPTIMAS image analysis system and software (Fermon Electronic Imaging). Bars=100 μmol/L.

were laden with leukocytes, many of which were along the vessel wall (Figs 5d and 6). Neutrophils were the most common leukocyte present, many of which were attached to the endothelium in a manner similar to that observed and quantified in focal ischemia.22,23,28 Leukocytes were essentially absent within the vasculature of the saline-injected hemisphere (Figs 5e and 6). No additional changes were found in the TNF-α injection site.

Discussion
This study represents the first evidence that TNF-α mRNA is induced in rat ischemic cortex after permanent MCAO. In a previous study24 we showed marked
MCAO. Other cells studied at early time points were found not to contain any significant TNF-α immunoreactivity. For example, TNF-α immunoreactivity was not observed in astrocytes, microglial cells do not stain for NF, and macrophages are not observed in the infarcted tissue until 5 days after MCAO.28 Definitive proof that neurons are the source of TNF-α will require in situ hybridization experiments. Also, we certainly have not demonstrated in these studies that no other cellular elements produce TNF-α under these conditions. The presence of TNF-α immunoreactivity in nerve fibers within and adjacent to the developing infarct suggests that TNF-α expressed in ischemic neurons is probably delivered via axonal transport. It may be important to identify the neurons that express TNF-α in response to injury and to determine the location of their axon terminals to identify any potential role for this protein in either recovery from ischemic injury or in exacerbation of that injury. TNF-α stimulates the expression of nerve growth factor in a variety of cell types including fibroblasts, glioblastoma cells, and astrocytes,29 which may contribute to neuronal survival in locations and around the ischemic site (ie, by signaling support cells of their condition and need for assistance, perhaps indirectly via nerve growth factor).

Previous studies have shown that neurons in normal mice express TNF-α mRNA, but in contrast to other tissues such as liver and kidney, they do not synthesize protein.30 Although no significant basal TNF-α mRNA was observed in the rat in the present study, the highly punctate TNF-α immunoreactivity we observed after focal ischemia suggests that MCAO may induce not only the increase in TNF-α message but also the translation and rapid secretion of TNF-α via axonal transport from the newly expressed mRNA. The regulation of TNF-α production at the translational level has been observed in vitro in macrophages,31 and the region of 3' untranslated TNF-α mRNA that modulates translation in macrophages also affects expression of TNF-α in vitro32 and in vivo in transgenic mice.33 In transgenic mice, the disregulated expression of TNF-α protein in peripheral tissues has significant pathophysiological consequences related to arthritis.33 Recently, increased neuronal TNF-α protein and mRNA have been demonstrated in the brain after mechanical injury,34,35 suggesting that neuronal TNF-α expression is not restricted to ischemic-damaged tissue.

To further elucidate the role of TNF-α in ischemic injury, we needed to examine what effects TNF-α might have within the brain by directly injecting saline/vehicle or TNF-α into the cerebral cortex. Blood vessels in hemispheres exposed to vehicle were similar to those we have previously described in normal SHR cerebral cortex.28 Neutrophils were not seen within these vessels located in the vehicle injection site. In marked contrast, the vasculature of TNF-α-treated cortex contained numerous neutrophils, many of which were attached to the endothelium, with some already extravasated into the subendothelial space. These results suggest that one potential role for TNF-α after cerebral ischemia is the recruitment of inflammatory cells from peripheral blood into the injured tissue. We have previously described in detail the inflammatory cell response that occurs in this model, and the expression of TNF-α mRNA and protein corresponds well with the inflammation stage, the second stage in the evolution of the infarct.28 The presence of TNF-α within 3 to 6 hours of MCAO precedes neutrophil infiltration, which is observed beginning at 12 hours after MCAO.28 Furthermore, the decreased immunolocalization of TNF-α in nerve fibers by the fifth day after MCAO correlates with decreased numbers of neutrophils observed at that point.28 Whether chemotactic cytokines (eg, IL-8, cytokine-induced neutrophil chemoattractant/gro) and/or adhesion molecules (eg, ICAM-1, vascular cell adhesion molecule [VCAM], endothelial leukocyte adhesion molecule [ELAM]-1) are induced after expression of TNF-α under these conditions remains to be determined but might be expected based on the role of TNF-α in stimulating adhesion to and diapedesis through endothelial cells layers.36-39 Interestingly, neurons have also been reported to synthesize IL-1β,40 whose mRNA expression is also increased after MCAO24 and which can also contribute to neutrophil infiltration.1,4,35,37,39 Hypertensive rats have increased sensitivity and risk for stroke.21,41,43 In this study SHR expressed more TNF-α mRNA than normotensive rats. Also, SHR exhibit increased frequency of brain stem thrombosis and hemorrhage and produce more TNF-α in the central nervous system and in the blood in response to lipopoly-saccharide than normotensive animals with lower stroke risk,44-46 which can contribute to the increased stroke sensitivity/risk in this strain.31,41,43 TNF-α activates the endothelium for leukocyte adherence and procoagulant processes that can exacerbate ischemic damage. Specifically, it increases tissue factor, platelet-activating factor, and von Willebrand factor and enhances adhesion of inflammatory cells via increased expression of adhesion receptor molecules.35,37-39 Reducing circulation neutrophil levels or their adhesion to blood vessels can significantly reduce ischemic damage and neurological dysfunction in central nervous system ischemia.47-56 Since exposure of the cerebral cortex to TNF-α results in neutrophil adhesion to microvascular endothelium similar to that seen in ischemia, it is possible that this pleiotropic cytokine may play a role in brain tissue response to ischemia by promoting inflammatory cell infiltration and perhaps contributing to increased sensitivity and risk in focal stroke, and that blocking TNF-α might be beneficial in focal ischemia.

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References


Liu et al investigated tumor necrosis factor-α (TNF-α) messenger RNA (mRNA) and protein expression in ischemic and nonischemic cerebral cortex and the effect of exogenously administered TNF-α on adherence of leukocytes to brain capillaries. Two major findings were reported. First, focal ischemia in rats results in elevated TNF-α mRNA and protein in ischemic neurons. In addition, hypertensive rats, which have increased sensitivity and risk for stroke, expressed more TNF-α than normotensive rats. Second, the neuronal expression of TNF-α appears to facilitate the infiltration of leukocytes after focal ischemia, which can possibly exacerbate tissue damage and contribute to increased sensitivity and risk in focal stroke.

TNF-α is a multifunctional cytokine initially identified as a macrophage-derived serum protein mediating necrosis of solid tumors in mice and lysis of several transformed cells in vitro. This cytokine has numerous biological activities, such as immunoregulatory, antimicrobial, and inflammatory effects. It is produced mainly by activated lymphoid and myeloid cells. However, nearly all cells express TNF-α in various ways, including induction of cellular genes, among them heat-shock proteins and the antioxidative enzyme manganese superoxide dismutase (MnSOD). Overexpression of MnSOD can protect the cells in culture from TNF-α, heat, radiation, and possibly other insults associated with production of oxygen free radicals. Although few reports on TNF-α in brain injury are available, TNF-α has been shown to stimulate acute-phase protein secretion, enhance endothelial permeability and cell adhesion molecules, and induce other inflammatory mediators and growth factors. The data on direct effects of TNF-α administration on ischemic injury are very interesting. The vascularature of TNF-α-treated cortex contained neutrophils, many of which were attached to the endothelium, with some already extravasated into the subendothelial space. These results strongly suggest a direct role for TNF-α in the recruitment of inflammatory cells from peripheral blood to the ischemic tissue.

It is also very interesting to note that TNF-α immunoreactivity after focal ischemia was observed after middle cerebral artery occlusion, suggesting that not only the TNF-α message but also the translation and rapid secretion of TNF-α occurred via axonal transport from the newly expressed mRNA. The immunohistochemical data suggested that neurons were the source of newly synthesized TNF-α. Although the evidence presented is compelling, it does not present proof. Proof that neurons were the only source would require proper in situ hybridization experiments and a demonstration that no other cellular elements produced TNF-α.

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