Increased Expression of ICAM-1 During Reoxygenation in Brain Endothelial Cells

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Methods

We isolated and cultured human brain microvascular endothelial cells and subjected them to hypoxia (Po2<10 mm Hg) in an anaerobic chamber followed by variable periods of reoxygenation.

Results

Twenty-hour periods of hypoxia did not lead to endothelial cytotoxicity as measured by a chromium-release assay. By Northern blot analysis, ICAM-1 mRNA transcripts were dramatically increased at 4 hours of reoxygenation but fell toward baseline (normoxia) by 12 and 24 hours. Hypoxia alone did not lead to an increase in mRNA levels. Western blot analysis showed an increased expression of ICAM-1 at 4, 12, and 24 hours of reoxygenation. The 4-hour increase in mRNA levels was not attenuated by pretreatment with 100 μmol/L allopurinol but was reduced by 30% with the addition of 20 mmol/L N-acetyl-l-cysteine at the time of reoxygenation and completely prevented by pretreatment with N-acetyl-l-cysteine.

Conclusions

Hypoxia-reoxygenation leads to an increase in ICAM mRNA levels that peaks at 4 hours in human brain microvascular endothelial cells. Pretreatment with N-acetyl-l-cysteine can completely block the increase in ICAM-1 mRNA levels. (Stroke. 1994;25:1463-1468.)

Key Words • cerebral circulation • endothelium • hypoxia • neutrophils
Specimens in which malignancy or tumor were found were excluded. The tissue was washed in M199 with penicillin (50 U/mL) and streptomycin (50 μg/mL) and minced into pieces of approximately 1 mm³ with a scalpel blade. The tissue was then homogenized with 10 to 20 strokes of a Dounce homogenizer. The homogenate was then mixed with a volume of 27.3% dextran to a final concentration of 15% and centrifuged at 5800g at 4°C in a swinging bucket rotor to separate the microvessels from the neuropil. The microvessels were then resuspended in M199 and successively passed through 210-μm and 149-μm nylon mesh and subsequently trapped on a 40-μm nylon mesh. The microvessels were then treated with a mixture of 1 mg/mL collagenase and dispase (Boehringer/Mannheim) at 37°C overnight. The following morning, the treatment was terminated by washing with M199, pelleting at 800g, and resuspending in M199 with 10% fetal bovine serum. Isolated clumps of endothelial cells were then seeded in 100-mm plates and maintained in medium consisting of 10% fetal bovine serum in M199 with 15 U/mL heparin and 20 U/mL endothelial cell growth factor (Upstate Biotechnology). The medium was changed every 2 to 4 days. The endothelial cells attached to the bottom of the plate, and 3 days later colonies were evident (Fig 1). Contaminating cell types were removed by mechanical weeding with a sterile 25-gauge needle. In addition, the culture dishes were treated with pancreatin in Hanks' buffer just before confluence. This treatment resulted in early detachment of endothelial cells while other contaminating cells (pericytes) remained attached to the dish. Cells were passaged every 5 to 7 days with pancreatin-ethylenediaminetetraacetic acid (EDTA) and used in the first 5 passages (Fig 2). Endothelial cells were identified by von Willebrand factor immunofluorescence and angiotensin-converting enzyme activity.21

von Willebrand Factor Immunofluorescence

Endothelial cells were grown to subconfluence on glass coverslips and then washed once with phosphate-buffered saline (PBS). The cells were then fixed for 10 minutes in methanol at −20°C. After three washes with PBS, the cells were overlaid with rabbit anti-human von Willebrand factor (Sigma) at a 1:50 dilution for 30 minutes at 37°C. After another three washes with PBS, goat anti-rabbit immunoglobulin (Sigma) at a 1:40 dilution was added for 30 minutes. After another three washes with PBS, the slides were read. Controls consisted of omitting the first antibody or substituting dilute normal rabbit serum. In addition, smooth muscle from bovine aorta was run in parallel (Fig 3).

Hypoxia Chamber

Endothelial cells were subjected to conditions of severe hypoxia in a Forma Scientific Anaerobic Glove Box at 37°C. Endothelial cells were grown to confluence in 100-mm plates. Just before placement in the anaerobic chamber, the medium was removed and exchanged with medium that was deoxygenated by bubbling 100% nitrogen for 45 minutes. The plates were then placed in the chamber for 20 hours and medium PO₂ was measured with an oxygen microelectrode (Diamond Electronics). Within 3 hours the medium PO₂ fell below 12 mm Hg, and by the end of the period the PO₂ was in the range of 3 to 8 mm Hg. Control cells were subjected for an identical period to conditions of normoxia in a Forma Scientific CO₂ incubator at 37°C. In some experiments the endothelial cell monolayers were treated with either 100 μmol/L allopurinol, 20 mmol/L N-acetyl-L-cysteine, or 20 mmol/L 4,5-dihydroxy-1,3-benzenedisulfonic acid (all from Sigma) before exposure to hypoxia or in some instances just before reoxygenation. When 20 mmol/L N-acetyl-L-cysteine was added to the monolayers, the pH of the medium was corrected to 7.3.

Cytotoxicity Assay

Endothelial cell cytotoxicity was measured by a chromium-release assay. Endothelial cell monolayers in 30-mm plates were incubated overnight with Na²⁴CrO₄ (5 μCi per plate). The following morning the medium was aspirated, and the wells were washed twice with serum-free medium. The cells were exposed to hypoxia-reoxygenation for varying time periods. The supernatant was collected, and the wells were washed once with serum-free medium (wash). The monolayers were
The density of each lane on the film was scanned (Shimadzu CS-900) at a wavelength of 600 nm. The quantitation of each lane was based on the normalized area against the human \( \beta \)-actin or human glyceraldehyde phosphate dehydrogenase (GAPDH) expression in the same lane.

**Probe Preparation**

A clone containing the human ICAM-1 insert was the gift of Brian Seed, PhD (Harvard Medical School, Massachusetts General Hospital). The ICAM-1 insert was cloned into the pHS3 vector. The host competent cells were 1061/P3 (Invitrogen). Restriction enzyme \( KhoI \) was used to excise the ICAM-1 insert from the vector. The human \( \beta \)-actin and GAPDH cDNA probes were obtained from the American Type Culture Collection.

The probes were labeled with \( \alpha \)-32P-dCTP (New England Nuclear) using a commercially available kit (Oligolabeling, Pharmacia). The labeled probes were purified by Spin Bind DNA extraction unit (FMC Corp) following the manufacturer's protocol.

**Results**

Cytotoxicity studies demonstrated that there was no specific endothelial cell cytotoxicity with periods of hypoxia of less than 24 hours of duration. Durations longer than 24 hours resulted in endothelial cell cytotoxicity evident immediately on removal from the chamber and during reoxygenation. All experiments were subsequently carried out with 16- to 20-hour periods of hypoxia and variable periods of reoxygenation.

With 20 hours of hypoxia there was dramatic increase in ICAM-1 mRNA transcripts at 4 hours of reoxygenation, which then rapidly decreased toward baseline by 12 and 24 hours (Fig 4). Immunoblots demonstrated an increase in ICAM-1 expression at 4, 12, and 24 hours of reoxygenation+100 \( \mu \)mol/L allopurinol (Sigma), hypoxia with 24 hours of reoxygenation, and hypoxia with 24 hours of reoxygenation+allopurinol. ICAM-1 is the 3.3- and 2.4-kb transcript, and \( \beta \)-actin is the 2.0-kb transcript.

**Immunoblotting**

Endothelial cells from one 100-mm plate were washed twice with tris(hydroxymethyl)aminomethane (Tris) buffer solution (TBS) (150 mmol/L NaCl, 50 mmol/L Tris, pH 7.5). The pellet of cells was lysed in NP-40 lysis buffer, and the mixture was put on ice for 30 minutes and then spun at 10,000g for 10 minutes at 4°C. The supernatant was removed, and the concentration of protein was determined using a Bio-Rad protein assay.

The blotted filter was washed two times at room temperature with TBS and blocked for 1 hour in block solution (5% nonfat dry milk in TBS). The filter was then incubated for 2 hours with the primary monoclonal antibody, anti-ICAM monoclonal antibody clone LB-2 (Becton Dickinson) diluted 1:125 in block solution. After washing, the second antibody, anti-mouse immunoglobulin alkaline phosphatase–labeled conjugate (Sigma), was added in a 1:1,000 dilution. The blot was then developed using a Bio-Rad alkaline phosphatase-detection system.

**Northern Blot**

Total RNA was extracted from \( 1 \times 10^4 \) cells using the UltrapecTM RNA kit (Biotex) following the manufacturer's instructions. Fifteen micrograms of total RNA from each sample was loaded in different lanes of the same gel and electrophoresed through a 0.9% agarose formaldehyde gel followed by transfer to a nylon filter (Bio-Rad). Binding was optimized by ultraviolet irradiation at 1200 \( \mu \)J/100 cm² in a UV crosslinker (Stratalinker 2300). Hybridization with the specific probes (with specific activity greater than \( 1 \times 10^6 \) cpmp) was carried out in 0.2 mol/L NaPO₄ (pH 7.2), 1 mmol/L EDTA, 1% bovine serum albumin, 7% sodium dodecyl sulfate (SDS), and 15% formamide at 65°C for 24 hours. The filter was washed in 40 mmol/L NaPO₄, 1 mmol/L EDTA, and 1% SDS followed by autoradiography at -70°C for 24 to 48 hours.

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levels (Fig 4). Treatment of the monolayers at the time of reoxygenation with 20 mmol/L N-acety-L-cysteine and 20 mmol/L 4,5-dihydroxy-1,3-benzenedisulfonic acid (a free-radical scavenger) led to a partial reduction in mRNA transcripts (30%) (Fig 6). Pretreatment with 20 mmol/L N-acetyl-L-cysteine completely blocked the increase in mRNA levels (Fig 7).

Discussion

Our studies indicate that there is an increase in ICAM-1 mRNA transcripts in human brain microvascular endothelial cells during reoxygenation after a period of severe hypoxia. These levels peak at 4 hours and then rapidly return to baseline. These increases can be blunted with the introduction of N-acetyl-L-cysteine at the time of reoxygenation and can be prevented with introduction of N-acetyl-L-cysteine at the time of severe hypoxia.

There have been no other published studies of the effect of severe hypoxia on the expression of adhesion molecules by human brain endothelial cells. Studies using human umbilical vein endothelial cells have produced varying results. Yoshida et al22 did not find an upregulation of ICAM-1 by enzyme-linked immunosorbent assay, but the periods of hypoxia (30 minutes) and reoxygenation (<3 hours) were both short. Shreenivas et al23 found an increased expression of ICAM-1 by Western blot during reoxygenation with periods and severity of hypoxia similar to ours, but mRNA levels were not studied. Since there is increasing evidence that endothelial cells are heterogeneous, with variation between species and also between organs within the same species,24-28 and that they differ in their expression of adhesion molecules,29,30 it is important to study the endothelial cells from the organ and vasculature (micro or macro) of interest. Our cells were derived largely from capillary segments. These cells may behave differently than cells derived from postcapillary venules. While our cells maintain von Willebrand factor expression in culture, they may lose other brain-specific characteristics.

The mechanism of the ICAM-1 upregulation during reoxygenation involves the generation of free radicals. N-acetyl-L-cysteine, but not allopurinol, blocked the increase in mRNA levels. At the concentration of 100 μmol/L, allopurinol is an inhibitor of xanthine oxidase.31 The inability of allopurinol to block the increase in mRNA levels argues against a significant role for xanthine oxidase, although in our experiments it is possible that allopurinol did not actually enter the cells in sufficient concentration. N-acetyl-L-cysteine increases levels of intracellular glutathione, which protects against free radicals.32 N-acetyl-L-cysteine is a potent inhibitor of the ubiquitous transcription factor, nuclear factor-κB (NF-κB).33 The presence of reactive oxygen intermediates correlates directly with the activation state of NF-κB in certain lymphocyte lines.32 There is also evidence that the activation of the AP-1 transcription factor is controlled by a redox mechanism.34 The ICAM-1 gene 5′-flanking region contains both NF-κB and AP-1 binding sites, indicating that these transcription factors may be involved in the regulation of ICAM-1 gene expression.33,35 ICAM-1 transcription therefore, may be sensitive to oxidant stress. In our system, N-acetyl-L-cysteine may be blunting the generation of free radicals at reoxygenation or may be acting "downstream" by inhibiting an intracellular signaling step involving reactive oxygen intermediates. The precise transcription factors and signal transduction mechanisms involved will require further study.
Increased expression of ICAM-1 during reoxygenation may lead to an increase in neutrophil infiltration into the brain. Limited studies have already indicated that ICAM-1 is expressed in the microvasculature of human cerebral infarctions.\textsuperscript{40} Upregulation of the expression of ICAM-1 would have adverse consequences as it could lead to microvascular plugging, breakdown of the blood-brain barrier with the development of edema, and early infiltration of the neutrophil by neutrophils with their toxic products.

There are strong parallels and similarities between inflammation and ischemia-reperfusion. Undoubtedly, there are other factors besides ICAM-1 upregulation that promote neutrophil accumulation in the brain. Moreover, there are other genes that are likely to be activated during ischemia or reperfusion which promote neutrophil aggregation and transform the endothelium into a procoagulant and proinflammatory state. A clearer understanding of the inflammatory components of stroke and their molecular mechanisms will be required before stroke therapy can be optimized.

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References

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