Inhibition of Intercellular Adhesion Molecule-1 Protein Expression by Antisense Oligonucleotides Is Neuroprotective After Transient Middle Cerebral Artery Occlusion in Rat

Raghu Vemuganti, PhD; Robert J. Dempsey, MD; Kellie K. Bowen

**Background and Purpose**—The present study was performed to determine whether antisense inhibition of intercellular adhesion molecule-1 (ICAM-1) protein expression decreases focal ischemic brain damage.

**Methods**—Male spontaneously hypertensive rats underwent 1-hour middle cerebral artery occlusion (MCAO) and 24-hour reperfusion. Rats were infused with ICAM-1 antisense or control oligodeoxynucleotides (ODNs) (48 nmol/d ICV) or vehicle, starting 24 hours before MCAO and continuing until the time of death. ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) mRNA levels were measured by real-time polymerase chain reaction. ICAM-1 protein knockdown was confirmed by Western blotting. Infarct volume was quantified by the use of cresyl violet–stained brain sections. Neurological deficits were evaluated. Mean arterial blood pressure was recorded by laser Doppler. Tissue penetration of antisense was confirmed by the use of fluorescent ODNs.

**Results**—Transient MCAO upregulated ICAM-1, but not VCAM-1, mRNA expression in the ipsilateral cortex between 3 and 72 hours of reperfusion. ICAM-1 antisense infusion prevented ischemia-induced ICAM-1 protein expression and reduced total infarct volume (by 53%; \( P<0.05 \); 226±35 mm\(^3\) in control ODN group and 104±27 mm\(^3\) in antisense ODN group; \( n=8 \) each) and mean neurological deficit score (by 44%; \( P<0.05 \); 2.4 in control ODN group and 1.3 in antisense ODN group; \( n=8 \) each). Neither control nor antisense ODN had any effect on mean arterial blood pressure and the physiological parameters monitored during MCAO. Compared with noninfused control, intracerebroventricular infusion of artificial cerebrospinal fluid or antisense or sense ODN had no significant effect on the regional cerebral blood flow changes that accompanied ischemia and reperfusion.

**Conclusions**—Increased ICAM-1 expression is implicated in the pathogenesis of focal ischemia since ICAM-1 protein knockdown decreased ischemic brain damage. The mechanism by which ICAM-1 inhibition offers neuroprotection is independent of blood pressure modulation. (*Stroke*. **2004;35:179-184.)

**Key Words:** inflammation ■ intercellular adhesion molecule-1 ■ ischemia ■ neuroprotection ■ oligonucleotides, antisense ■ stroke

In rodents, increased expression of proinflammatory genes including cytokines (interleukin [IL]-1\(\alpha\), IL-1\(\beta\), IL-6, and tumor necrosis factor-\(\alpha\) [TNF-\(\alpha\)]), chemokines (macrophage inflammatory protein-1\(\alpha\) [MIP-1\(\alpha\)] and monocyte chemotactic protein-1 [MCP-1]), and adhesion molecules (intercellular adhesion molecule-1 [ICAM-1], E-selectin, and P-selectin) contributes to the neuronal damage after focal cerebral ischemia.\(^1\)\(^\text{-}^4\) Cytokines formed immediately after ischemia stimulate the expression of adhesion molecules on endothelial cells and leukocytes, leading to leukocyte adherence and extravasation into brain parenchyma.\(^5\)\(^\text{-}^6\) Of the various types of leukocytes, neutrophils (the major subtype of polymorphonuclear leukocytes) are the first to enter the brain after ischemia. Extravasated polymorphonuclear leukocytes release reactive oxygen species and lipid peroxidation products and promote blood-brain barrier disruption, vascular plugging, edema, and cerebral infarction.\(^7\)\(^\text{-}^9\)

Previous studies showed a significant decrease in middle cerebral artery occlusion (MCAO)–induced brain damage in ICAM-1 knockout mice and anti–ICAM-1 antibody–treated rats (see review by Frijns and Kappelle\(^10\) for several studies). In rodents, hypothermic neuroprotection against focal ischemia is associated with attenuation of ICAM-1 induction and polymorphonuclear leukocyte infiltration.\(^11\) However, in a multicenter acute stroke trial with 625 patients, murine anti–ICAM-1 antibody (enlimomab) therapy resulted in a higher mortality rate than with placebo treatment.\(^12\) Furuya et al. (2001)\(^13\) observed that administration of enlimomab to rats stimulated the generation of rat anti-mouse antibodies and augmented focal ischemia–induced infarct size. Thus, the most likely reason for the failure of the enlimomab stroke trial is an inflammatory reaction in patients due to the use of a mouse antibody.
Since recanalization of an occluded cerebral artery assists in the recovery of reversibly ischemic tissue, anti-adhesion strategies that can be used in conjunction with thrombolytic therapy are beneficial for stroke patients. Recent studies showed the benefit of thrombolysis with prourokinase, recombinant tissue plasminogen activator (tPA), UV laser, and ultrasound.

Antisense oligonucleotides (ODNs) bind to a specific mRNA and prevent its translation to knockdown protein product locally in a time-dependent manner. Antisense therapy is currently being tested in cancer, inflammation, and viral diseases. Our laboratory established antisense as a powerful tool to elucidate the function of specific proteins in rat brain after focal ischemia. The present study evaluated the efficacy of ICAM-1 antisense to prevent infarct development and neurological deficiency after transient MCAO in adult rats.

Materials and Methods
Adult male spontaneously hypertensive rats (SHR) (weight, 280 to 320 g; Charles River, Wilmington, Mass) used in these studies were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services publication No. 86-23, revised 1986). The Research Animal Resources and Care Committee of the University of Wisconsin at Madison approved the surgical procedures.

Focal Ischemia
Transient MCAO was induced under halothane anesthesia by the suture occlusion method as described earlier. The left femoral artery was cannulated for continuous monitoring of arterial blood pressure and to obtain the measurements of pH, PaO2, PaCO2, hemoglobin, and blood glucose concentration. In all experiments, MCA was occluded for 1 hour, followed by reperfusion (confirmed by laser Doppler). During ischemia and 90 minutes of reperfusion, regional cerebral blood flow (rCBF) was continuously monitored by laser-Doppler flowmeter with the use of a probe placed on the surface of the ipsilateral cortex as described earlier. During MCAO, PaO2 (100 to 200 mm Hg), PaCO2 (30 to 40 mm Hg), body temperature (37°C to 38°C), and cranial temperature (36°C to 37°C) were maintained at the physiological level. Sham-operated rats served as control.

ICAM-1 Antisense Studies
Effect of ICAM-1 knockdown on transient MCAO-induced brain damage was studied as described earlier. Phosphorothioate antisense ODN (5'-TCA CCT CCA CTG AG-3') targeting nucleotides 852 to 865 of rat ICAM-1 mRNA (GenBank No. D00913) was custom designed with the use of R.A.D.A.R. (Rational Algorithmic Design of Antisense Reagents) software and manufactured by Biognostik Inc. Biognostik is the owner of the intellectual property rights of the antisense sequence. The control ODN sequence (5'-GAA CCA AGA GCA CC 3') was similar in length and GC content to the ICAM-1 antisense sequence, passed a homology search against all sequences in the GenBank database, and was observed not to contain any problematic motifs. Lyophilized ODNs were reconstituted in artificial cerebrospinal fluid (aCSF) and continuously infused (2 nmol/µL per hour) into left lateral ventricle (coordinates: –1.5 mm lateral; –0.8 mm anterior-posterior; –4.8 mm dorsal; based on the rat brain atlas of Paxinos and Watson, 1998) with the use of a brain infusion cannula connected to an osmotic minipump (model 2001; Alza Pharmaceuticals) placed in the neck fold. Three groups of rats infused with antisense ODN, control ODN, and aCSF for 1 day and a control group with no infusion (n=8 per group) were subjected to transient MCAO (1 hour) on the side homolateral to intracerebroventricular infusion. At 1 day of reperfusion, the rats were perfused transcardially with buffered paraformaldehyde, and the brains were serially sectioned (coronal, 40 µm thick at an interval of 320 µm), stained with cresyl violet, and scanned with the use of the NIH Image program, and the volume of the ischemic lesion was computed by the numeric integration of data from 12 to 14 sections with respect to sectional interval, as described earlier. The infarct volume was corrected to account for edema and shrinkage due to processing. Transient MCAO–induced neurological deficit was evaluated on a 6-point scale by an investigator blinded to the study groups, as described earlier. In a separate cohort of 3 rats, fluoroisothiocyanate (FITC)-conjugated antisense was infused for 1 day (2 nmol/h), the brains were sectioned, and the tissue distribution of the fluorescent ODN was evaluated microscopically.

Western Blotting
ICAM-1 knockdown was confirmed by Western blotting as described earlier. For this, a separate cohort of rats was subjected to transient MCAO and 24-hour reperfusion after aCSF, ICAM-1 antisense ODN, and ICAM-1 control ODN infusion (n=4 per group). Tissue homogenizes (25 µg protein equivalent each) from the entire contralateral cortex and the ipsilateral cortex from each rat were electrophoresed on polyacrylamide gels, transferred to nitrocellulose, and probed with monoclonal ICAM-1 antibodies (1:2000; Sigma Chemical Co) followed by horseradish peroxidase–coupled anti-mouse IgG. The blots were stripped and reprobed with monoclonal anti–β-tubulin antibody (1:6000; Sigma Chemical Co). The protein bands recognized by the antibodies were visualized by ECL Western blotting kit (Amersham Pharmacia Biotech). Before immunodetection, protein loading and transfer efficiency were confirmed by Ponceau-S staining.

Real-Time Polymerase Chain Reaction
ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) mRNA levels were estimated as described earlier. Twenty rats were subjected to transient MCAO and killed at 3, 6, 24, 48, and 72 hours of reperfusion (n=4 per group). Sham-operated rats (n=4) served as control. Total RNA was extracted from the entire contralateral and ipsilateral cortex with the TRIZol Reagent (Life Technologies), and 1 µg RNA from each sample was reverse transcribed with oligo(dT)15 and random hexamer primers with the use of M-MuLV reverse transcriptase (Life Technologies). Next, 10 ng of cDNA and a priming mixture were amplified for 30 cycles in a 25-µL volume, according to the following conditions: 2 min at 94°C; 30 sec at 94°C; 30 sec at 60°C; and 1 min at 72°C. The reaction was terminated by incubation for 5 min at 72°C. Each 25-µL reaction mixture contained 12.5 µL of AmpliTaq Gold DNA Polymerase Mix (Perkin Elmer) and 1 µL cDNA. Primers for ICAM-1 and VCAM-1 were designed with the use of Primer Express software (PE Applied Biosystems) and synthesized by PE Applied Biosystems (Foster City, Calif). Each primer was used in a concentration of 5 µM. Primers were designed to span exons and avoid intronic sequences. After optimization, specificities of ICAM-1 and VCAM-1 primers were assessed with the use of the DNA fragments of corresponding gene that were amplified according to the above-mentioned conditions. Both ICAM-1 and VCAM-1 primers were specific for target genes, whereas primer specificity was eliminated when the primer was extended. Amplification efficiency under the above-mentioned reaction conditions was determined by use of the standard samples prepared from a single cDNA sample by serial dilution 1:10, with the use of the ICAM-1 and VCAM-1 primers. The standard samples were amplified in triplicate, and the threshold cycle (Ct) was determined for each standard dilution. The results showed good amplification efficiency for both ICAM-1 and VCAM-1 primers. The Ct values for the standard samples were correlated in a linear fashion over a wide range of cDNA concentrations. Product purity was confirmed by gel electrophoresis. To ensure the specificity of each PCR product, pCR 4.0 TOPO cloning vector (Invitrogen) was used to clone the products of the following primers: ICAM-1 sense primer: 5’-TTT ATG GAT CCT GAT CCA GCT C-3’ and ICAM-1 antisense primer: 5’-GAG CAT CAC CAT CCT CAC ATC-3’; VCAM-1 sense primer: 5’-TCT CCC CTT TCA GTT TCG CTT G-3’ and VCAM-1 antisense primer: 5’-GTC TAC TCC AGG CAG GCG T-3’. The amplified fragments were subcloned into the vector and sequenced. The sequences of the amplified products were identical to those generated by the primers used. The Ct value of the standard sample was used to convert the Cq value into the copy number of target gene. The copy number of target gene in each sample was then amplified into each sample. The Cq value of the target gene was compared between the control and antisense groups. The data are presented as mean±SD. A statistically significant difference was considered to be present at a level of P<0.05 by the use of the 2-tailed Student t-test.
gene-specific primers were added to SYBR Green PCR Master Mix (SYBR Green I Dye, AmpliTaq DNA polymerase, dNTPs with dUTP and optimal buffer components; Applied Biosystems) and subjected to polymerase chain reaction (PCR) amplification (1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute) in a TaqMan Sequence Detection System (Applied Biosystems). For each transcript, real-time PCR was conducted 3 times in duplicate with the use of each RNA sample. 18S rRNA amplification was used as internal control. The amplified transcripts were quantified with the use of the comparative C\textit{\textsubscript{t}} method (http://pebiodocs.com/pebiodocs/04303859.pdf). The sequences of the primers (designed with the use of Primer Express software, Applied Biosystems) were CGC CGC TAG AGG TGA AAT TCT and CGA ACC TCC GAC TTT CGT TCT for 18S RNA (GenBank No. M11188); GGG CCC CCT ACC TTA GGA A and GGG ACA GTG TCC CAG CTT TC for ICAM-1 (GenBank No. D00913); and TGT GGA AGT GTG CCC GAA AT and TGC CTT GCG GAT GGT GTA C for VCAM-1 (GenBank No. M84488).

**Results**

**Increased ICAM-1 Expression After Transient MCAO**

Real-time PCR analysis showed a significant increase in ICAM-1 mRNA levels in the ipsilateral cortex compared with the contralateral cortex or sham cortex between 3 and 72 hours of reperfusion after transient MCAO (peak increase of 7- to 9-fold at 24 hours; \(P<0.01\)) (Figure 1). Transient MCAO had no effect on VCAM-1 mRNA and 18S RNA levels (data not shown).

**Antisense Distribution in Brain**

Intracerebroventricular infusion of FITC-labeled antisense ODN for 1 day resulted in good tissue distribution of the ODN in brain parenchyma around lateral ventricles, septum, cortex, striatum, hippocampus, and blood vessels (Figure 2A to 2E).
Physiological Parameters in ICAM-1 Antisense ODN, Control ODN, and aCSF Infused Rats Subjected to Transient MCAO

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Brain Hb, g/dL</th>
<th>Body Hb, g/dL</th>
<th>Glucose, mg/dL</th>
<th>pH</th>
<th>PaO₂, mm Hg</th>
<th>PaCO₂, mm Hg</th>
<th>MAPB, mm Hg</th>
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<tbody>
<tr>
<td>Pre-ischemia (30 minutes before occlusion)</td>
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<tr>
<td>Antisense ODN</td>
<td>36.5±0.5</td>
<td>37.3±0.5</td>
<td>13±1.7</td>
<td>91±9</td>
<td>7.41±0.06</td>
<td>139±31</td>
<td>34.7±4.1</td>
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<tr>
<td>Control ODN</td>
<td>36.8±0.4</td>
<td>36.9±0.5</td>
<td>14±1.4</td>
<td>90±7</td>
<td>7.40±0.04</td>
<td>146±37</td>
<td>32.6±3.8</td>
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<td>aCSF</td>
<td>36.3±0.6</td>
<td>37.0±0.5</td>
<td>13±1.9</td>
<td>88±9</td>
<td>7.38±0.04</td>
<td>140±33</td>
<td>34.2±4.5</td>
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<td>During ischemia (30 minutes of occlusion)</td>
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<tr>
<td>Antisense ODN</td>
<td>36.7±0.6</td>
<td>36.9±0.6</td>
<td>14±1.7</td>
<td>90±6</td>
<td>7.39±0.05</td>
<td>141±35</td>
<td>33.3±4.2</td>
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<tr>
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<td>36.8±0.5</td>
<td>37.2±0.5</td>
<td>13±1.5</td>
<td>92±8</td>
<td>7.42±0.04</td>
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<td>Postischemia (30 minutes of reperfusion)</td>
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<tr>
<td>Antisense ODN</td>
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<td>7.40±0.06</td>
<td>138±32</td>
<td>35.1±5.3</td>
</tr>
</tbody>
</table>

Hb indicates hemoglobin; Gl, Glucose; MAPB, mean arterial blood pressure.

Values are mean±SD. There were no statistically significant differences in any of the parameters between the groups at a specific period or within a group at different periods (pre-ischemia, during ischemia, and postischemia).

ICAM-1 Antisense Had No Effect on Physiological Parameters

No significant differences were observed between the aCSF, antisense ODN, and control ODN groups in the rCBF monitored before, during, and after MCAO (Figure 2F). Previous studies from our laboratory showed that aCSF infusion has no significant effect on rCBF during MCAO compared with noninfused controls.

The physiological parameters (mean arterial blood pressure, pH, PaO₂, PaCO₂, hemoglobin, blood glucose, temporalis muscle and rectal temperatures) were not significantly different between the groups infused with aCSF, ICAM-1 control ODN, and ICAM-1 antisense ODN at a specific period between the groups or within a group at different periods (before, during, and after MCAO) (Table).

ICAM-1 Antisense Prevented ICAM-1 Protein Expression

In the aCSF- and control ODN–infused rats, transient MCAO resulted in a significant increase (by 5.4- to 6.7-fold; P<0.01) in ICAM-1 protein levels in the ipsilateral cortex compared with the respective contralateral cortex (Figure 2G). ICAM-1 antisense ODN infusion completely inhibited transient MCAO–induced ICAM-1 protein expression in the ipsilateral cortex (Figure 2G). In all 3 groups of rats (aCSF, control ODN, and antisense ODN), β-tubulin (housekeeping control) protein levels were not altered in the ipsilateral cortex compared with the contralateral cortex (Figure 2G). ICAM-1 immunoreactivity was observed in several large blood vessels and capillaries (shaded area in Figure 3A) in the ipsilateral side of the brain in ICAM-1 sense ODN–infused rats subjected to transient MCAO; ICAM-1 immunoreactivity was significantly less in both the cortex (Figure 3D) and striatum (Figure 3E) of the ICAM-1 antisense ODN–infused rats.

Effect of ICAM-1 Antisense on Infarct Volume and Neurological Deficits

In all groups of rats (no infusion, aCSF, control ODN, and antisense ODN), transient MCAO (1 hour) and reperfusion (24 hours) resulted in an infarct in the ipsilateral half of the
brain encompassing cerebral cortex and striatum from \(-5.2\) to \(+2.8\) from bregma. Figure 4 shows representative infarct areas in rats with control ODN and antisense ODN infusion. Total infarct volume was not significantly different between the noninfused \((214\pm36\, \text{mm}^3)\), aCSF \((206\pm41\, \text{mm}^3)\), and control ODN \((226\pm35\, \text{mm}^3)\) groups (Figure 5, top). The ICAM-1 antisense ODN group showed a significantly smaller infarct \((by\ 54\pm12\%\; P<0.05)\) than the other groups (Figure 5, top). Furthermore, the neurological deficits analyzed at 24-hour reperfusion were mild in the ICAM-1 antisense group, with a median neurological score of 1.25, compared with moderate/severe deficits in the noninfused, aCSF, and control ODN groups (median neurological scores were 2.4 to 2.75) (Figure 5, bottom).

**Discussion**

In brief, results of the present study show that prevention of ICAM-1 protein expression by antisense infusion significantly decreases transient focal ischemia–induced infarct size and neurological deficits, without interfering with rCBF. Previous studies from our laboratory established antisense knockdown as an effective strategy to evaluate the functional significance of specific proteins such as glutamate transporters and ornithine decarboxylase in ischemic brain damage.\(^{20,21}\) Using antisense, we recently showed that suppressor of cytokine signaling-3 induction after transient MCAO is an endogenous neuroprotective mechanism to control posts ischemic inflammation.\(^{4}\)

Transient focal ischemia leads to increased expression of several proinflammatory genes, and the resulting inflammation significantly contributes to stroke outcome.\(^{1,4,25-27}\) A recent study from our laboratory showed increased mRNA levels of ICAM-1, E-selectin, P-selectin, integrin α-M, IL-1β, IL-6, TNF-α, nuclear factor-κB (NF-κB), tPA, MIP-1α, MIP-2, MCP-1, and complement components C3 and C4 in rat brain after transient MCAO.\(^{4}\) Of these, the cytokines (IL-1β, IL-6, and TNF-α) and the chemokines (MIP-1, MIP-2, and MCP-1) stimulate NF-κB expression, which in turn upregulates the expression of adhesion molecules (E-selectin, P-selectin, and ICAM-1) and integrins. The selectins mediate the rolling of leukocytes on the endothelial surface, and ICAM-1 and integrins mediate the firm adhesion and transendothelial migration of leukocytes.\(^{10,28}\) In the posts ischemic brain, leukocytes exacerbate brain injury by physically obstructing capillaries to reduce blood flow during reperfusion and/or by releasing cytotoxic products once extravasated into the brain parenchyma.\(^{6,8-10}\)

Previous studies showed that attenuation of leukocyte infiltration by hypothermia and inhibition of adhesion molecules by specific antibodies reduce posts ischemic neuronal damage.\(^{29-32}\) It was demonstrated that anti–ICAM-1 antibody prevents ischemic brain damage in rat after transient, but not permanent, MCAO.\(^{30}\) This is understandable since leukocyte infiltration occurs much earlier after transient than permanent MCAO, and reperfusion predisposes the brain to an early inflammatory response. Reperfusion does not occur spontaneously in the majority of stroke patients, but recent studies showed promising results with thrombolytic protocols using prourokinase,\(^{14}\) recombinant tPA,\(^{15}\) UV laser,\(^{16}\) and ultrasound.\(^{17}\) Furthermore, posts ischemic hyperperfusion occurs in approximately 50% of patients by day 7 after intra-arterial thrombolysis-mediated recanalization.\(^{33}\) Hence, in stroke patients ICAM-1 inhibition may be beneficial if used in conjunction with a thrombolytic agent to open the occluded vessel. Thrombolytic treatment with recombinant tPA was
shown to be more effective if used together with anti–ICAM-1 antibodies.\textsuperscript{34} The choice of using antisense versus antibodies depends on the state of the patient. Since antisense binds to mRNA and inhibits ICAM-1 protein formation, it can be used as a preventive measure in patients at risk of having a stroke. Because antibodies bind to ICAM-1 protein that is already formed and stops its action, it may be more appropriate in poststroke patients. In conclusion, this is the first report to show the usefulness of antisense as a therapeutic possibility to prevent stroke-induced inflammatory neurological damage.

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References


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