Inhibition of Integrin αβ3 Ameliorates Focal Cerebral Ischemic Damage in the Rat Middle Cerebral Artery Occlusion Model

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Background and Purpose—Recent studies have shown that selective inhibition of specific subsets of intercellular adhesion molecules protects the brain during ischemia. We studied selective inhibition of integrin αβ3 with cyclo [Arg-Gly-Asp-D-Phe-Val] (cRGDfV) in the rat middle cerebral artery occlusion model (MCAO).

Methods—Rats were treated before and after MCAO with cRGDfV. Physiological parameters, expression of integrin αβ3, infarction volume, brain water content, Evans Blue exudation, IgG exudation, histology, immunohistochemistry, and western blotting were studied in 4 groups of animals: sham operation (n=13), untreated (n=18), nonfunctioning peptide treatment (n=19), and cRGDfV treatment (n=27).

Results—Treatment with cRGDfV reduced infarction, reduced brain edema, reduced exudation of Evans blue and IgG, and prevented fibrinogen deposition. Western blotting showed reduction of phosphorylated Flk-1 (a vascular endothelial growth factor [VEGF] receptor), reduction of phosphorylated FAK (an intracellular kinase phosphorylated in the presence of VEGF), reduction of VEGF, and reduction of fibrinogen in the cRGDfV treatment group.

Conclusions—The selective integrin αβ3 inhibitor cRGDfV improves outcomes in the MCAO model by preserving the blood-brain barrier, which mechanistically may occur in a VEGF- and VEGF-receptor–dependent manner. (Stroke. 2006;37:1902-1909.)

Key Words: cerebral ischemia • fibrinogen • integrin αβ3 • VEGF

Recent attention has turned to inhibition of intercellular adhesion molecules for treatment of ischemic stroke.1 With ischemic injury to the brain, integrin αβ3 is selectively upregulated whereas many others are downregulated.2,3,4,5 Selective inhibitors of αβ3 have been incompletely studied in animal models of brain ischemia. Blockade of other subgroups of intercellular adhesion molecules, including selectins, is neuroprotective in ischemic injury.1

Integrin αβ3’s receptor domain interacts with the peptide sequence arginine-glycine-aspartic acid (RGD),3 and cyclo [Arg-Gly-Asp-D-Phe-Val] (cRGDfIV) inhibits this (IC50=6.41 nM).7 Cyclo-RGDfIV is a potent inhibitor of endothelial cell invasion and differentiation in vitro, and this effect occurs through an inhibition of vascular endothelial growth factor (VEGF)- and fibroblast growth factor–mediated angiogenesis.6 VEGF stimulates integrin αβ3 expression in vitro, and is highly correlated to integrin αβ3 expression in vivo.4 Cyclo-RGfIV has no effect on normal vessels,8 nor any hematological toxicity.9

We hypothesize that (1) inhibition of integrin αβ3 preserves the blood-brain barrier (BBB) in focal cerebral ischemia, possibly by inhibition of VEGF-mediated BBB breakdown, and that (2) inhibition of integrin αβ3 preserves microcirculation by inhibition of deposition of fibrinogen.

Our study is the first to describe the effects of selective inhibition of αβ3 with cRGDfV in the middle cerebral artery occlusion (MCAO) model.

Materials and Methods
All experiments were approved by the Institutional Animal Care and Use Committee at Loma Linda University. Seventy-seven male Sprague-Dawley rats (280 to 320 g; Harlan; Indianapolis, Ind) were housed under identical conditions. Rats were anesthetized with α-chloralose (Fisher Scientific; 60 mg/kg IP) and urethane (Acros Organics; 600 mg/kg IP). Rats were randomly divided into 4 groups: (1) Group N, MCAO, no treatment (n=18), (2) Group C, MCAO, nonfunctioning control peptide (n=19), (3) Group R, MCAO, cRGDfIV treatment (n=27), and (4) Group S, sham-operation group, no treatment (n=13).

Treatment Methods
Drug treatment was administered IV via left femoral vein. Group N was injected with 0.5 mL phosphate buffered saline (PBS; Sigma-Aldrich) 1 hour before and 3 hours after MCAO. Group C was injected with a nonfunctioning peptide (cyclo[Arg-Ala-Asp-D-Phe-Val]), 100

Received March 11, 2006; accepted April 11, 2006.
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Stroke is available at http://www.strokeaha.org DOI: 10.1161/01.STR.0000226991.27540.f2

1902
μg/kg body weight, 1 hour before and 3 hours after MCAO (Peptide International). Group R was injected IV with cRGDfV (cyclo[Arg-Gly-Asp-d-Phe-Val]), 100 μg/kg body weight, 1 hour before and 3 hours after MCAO (Peptide International). Both peptides were diluted with distilled water to a concentration of 150 μg/mL. The doses of peptides are similar to doses used in previous reports.10

MCAO Model
After induction of general anesthesia the rats were intubated and ventilated with an animal ventilator (Harvard Apparatus). Rectal temperature was maintained at 37±1°C with a heating pad. Mean arterial blood pressure was monitored via the left femoral artery. The MCAO model used in this study has been described elsewhere in detail.11,12 A 0.008 silicon-coated monofilament suture was used for the MCAO (Doccol, Co). After 2 hours of occlusion the suture was withdrawn and cerebral blood flow was allowed to recover. All rats were euthanized 24 hours after reperfusion. The first author performed all surgeries, and pilot studies confirmed an average cerebral blood flow drop of 88±7% by laser doppler. The success rate of MCAO was 92%, with 4% failure of occlusion and 4% subarachnoid hemorrhage, both of which were excluded from further analysis.

Measurement of Infarct Size
Standard 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) infarct measurement techniques were used to measure infarct size. Brains were sectioned coronally into 2-mm slices (Harvard Apparatus; South Natick, Mass) and immersed in 2% TTC solution at 37°C for 5 minutes, followed by 10% formaldehyde solution. Infarction area was traced and quantified with Image J 1.33u, n=4 for Groups S, N, R, and n=6 for Group R.

Brain-Water Content
Using established dissection techniques,13 rat brains were separated into anterior cerebral artery (ACA) perfusion cortices, MCA perfusion cortices, posterior cerebral artery (PCA) perfusion cortices, basal ganglia, hippocampi, cerebellum and brain stem. The brain samples were weighed immediately after dissection (wet weight) and then dried at 105°C for 24 hours. The percent of water content was calculated with the formula ([wet weight−dry weight]/wet weight)×100%, n=4 for Groups S, N, and C, and n=6 for Group R.

Evans Blue Extravasation
BBB permeability was assessed with Evans Blue extravasation studies.14,15 Under general anesthesia, Evans blue dye (4%; 2.5 mL/kg) was injected IV and allowed to circulate for 60 minutes followed by 120 mL PBS perfusion via aorta. The brains were removed and divided into the right and left hemispheres and other areas. Brain samples were homogenized, and protein was precipitated and quantified spectrophotometrically (Genesis 10uv; Thermo Electron Corp), n=4 for Groups S, N, and C, and n=6 for Group R.

Western Blotting
Western blotting followed standard techniques. Primary antibodies included rabbit anti-phosphorylated FAK (p-FAK; sc11765), rabbit anti–phosphorylated-Flk-1 (p-Flk-1, sc16628), mouse anti-VEGF (sc7269), goat anti-fibrinogen β (sc18029), goat anti-actin (sc1615), rabbit anti-FAK (sc315; Santa Cruz Biotechnology; Santa Cruz, Calif). The membranes were incubated with the appropriate Cruz Marker compatible secondary antibody. Bands were detected with chemiluminescence detection kit (ECL plus; Amersham Bioscience). Blot bands were quantified using the densitometry method (Image J 1.33u), n=4 for Groups S, N, and C, and n=6 for Group R. The value of the sham is expressed as 100%, and other groups are expressed as a percentage of the sham group.

Figure 1. Photomicrographs. Immunofluorescent evaluation of integrin receptors. Triple staining for integrin αv, β3, and NeuN (a marker for neurons) demonstrates that neurons in Groups N and C highly express αvβ3 after MCAO. This expression is absent in Group S, and attenuated in Group R. Arrowheads indicate positive cells. Scale bar=10 μm.
Histological Analysis
Rat brains were fixed with PBS, 4% formalin, and 30% sucrose. Coronal brain sections were embedded in TBS Tissue Freezing Medium (Triangle Biomedical Science), sliced into 10-μm-thick slices by cryostat (CM3050S; Leica Microsystems), and placed on slides.

Nissl staining and Hematoxylin-Eosin (H&E) staining followed standard protocols. Immunohistochemical staining followed the protocol of the commercial ABC staining kit (Santa Cruz Biotechnology). We used rabbit anti–p-FAK (sc11765), rabbit anti–p-Flk-1 (sc16628), rabbit anti-VEGF (sc507), goat anti-fibrinogen β (sc18029), rabbit anti-CD68 (sc9139), rabbit anti–lysosome-associated membrane glycoprotein (LAMP)-3 (sc15363) and goat anti-myeloperoxidase (MPO; sc16128; Santa Cruz Biotechnology; Santa Cruz, Calif). For detection of BBB breakdown, goat anti-rat IgG-biotin conjugated (sc2041; Santa Cruz Biotechnology) was used. Immunofluorescence studies required goat anti-integrin αv (sc6617), rabbit anti-integrin β3 (sc7311) (Santa Cruz Biotechnology; Santa Cruz, Calif), mouse anti-NeuN (MAB377; Chemicon, Temecula, Calif), as well as the appropriate secondary antibodies (Jackson Immuno Research).

Statistical Analysis
Data are expressed as mean±SEM. Differences between individual groups were analyzed using standard post hoc t tests supported by Sigma Stat 3.0.1. P<0.05 was considered statistically significant. χ² test was done for analysis of mortality.

Results
Administration of PBS, control peptide, or cRGDFV did not affect mean arterial blood pressure, which was 70±10 mm Hg in each group after reperfusion. The mortality 24 hours after MCAO was 11% (1 of 18 rats) in Group N, 16% (3 of 19 rats) in Group C, and 4% (1 of 27 rats) in Group R. No rats died in the sham group. There was no statistically significant difference in mortality between the 4 groups on χ² analysis.

Immunofluorescent staining of the ischemic core of the MCAO lesion revealed that integrin αvβ3 was present in Groups N and C but absent in Group S and suppressed in Group R (Figure 1). Tissue was stained for αv and β3, the individual components of the integrin αvβ3, as well as NeuN (a neuronal marker). On TTC stain, treatment with cRGDFV resulted in significantly smaller total infarct area (Figure 2A and 2B) as well as significantly smaller infarct areas in the cortex and basal ganglia when analyzed separately (data not shown).

Figure 2. Photomicrographs and graphs. (A) Total infarction volume is less in Group R than in Groups N and C. (B) TTC stain of infarct areas. (C) Evans-Blue dye extravasation is less in Group R than in Groups N, C, or S. (D) IgG extravasation is less in Group R than in Groups N, C, or S. # indicates P<0.05 compared with S, * indicates P<0.05 compared with R.
Group R had significantly less extravasation of Evans Blue dye than Groups N and C (Figure 2C). Whole-brain IgG immunohistochemistry demonstrated significantly smaller lesions in Group R than in Groups N and C (Figure 2D). This was confirmed quantitatively with computer image analysis (data not shown). The IgG-positive lesions in Groups N and C correlate well with the results from the TTC staining (Figure 2B). The cRGDFV treatment group (Group R) had significantly less BBB breakdown on both Evans Blue and IgG extravasation studies.

Brain-water content of the MCA perfusion cortex, PCA perfusion cortex and basal ganglia was smaller in Group R than in Groups N and C (Figure 3A through 3E). Brain-water content of the ACA perfusion cortex and hippocampus was not significantly different between Groups R, N, and C, although the trend is consistent with the other brain regions.

Nissl stain of the cortex demonstrated ischemic damage in Groups N and C (Figure 4A). This damage is absent in Group S and considerably attenuated in Group R. Hematoxylin-Eosin stain of the basal ganglia showed large numbers of fibrinogen-fibrin collections and occluded vessels in Groups N and C, fewer in Group R, and none in Group S (Figure 4A). Immunohistochemical staining for nonspecific markers of inflammatory cells (CD68, LAMP 3, and MPO) demonstrated more inflammatory cells in the penumbra of Groups N and C than Groups R and S (Figure 4B).

Expression of p-FAK is significantly reduced in Group R on Western Blot Analysis (Figure 5A), and this is supported qualitatively by immunohistochemical staining (Figure 5B). Normal interaction between VEGF and integrin αβ3 leads to phosphorylation of FAK among other effects, and suppression of p-FAK indicates that cRGDFV successfully inhibited integrin αβ3. We did not study the downstream cell signaling cascades of FAK, although many are known to exist. Western Blot Analysis of p-Flk-1, one of the receptors of VEGF, showed that expression of p-Flk-1 is significantly reduced in Group R (Figure 5C). This is supported qualitatively with the immunohistochemical staining that shows that p-Flk-1 is reduced in Group R compared with Groups N and C (Figure 5D). Both VEGF and fibrinogen are significantly reduced on Western Blot Analysis in the cRGDFV treatment group (Group R; Figure 6A and 6B). Immunohistochemical stain supports this qualitatively (Figure 6C and 6D), as does H&E stain in the case of fibrinogen-fibrin (Figure 4A).

**Discussion**

Treatment with cRGDFV was associated with decreased infarction area, decreased brain edema, decreased inflammation, decreased deposition of fibrinogen, and decreased breakdown of the BBB. The mechanism of these beneficial effects involves the inhibition of integrin αβ3 and its downstream signaling pathways.
events in the cRGDfV treatment group may involve VEGF and VEGF receptors. Flk-1 is one of the receptors of VEGF,18 and it is active in its phosphorylated form. P-Flk-1 was reduced with cRGDfV treatment (Figure 5C and 5D). Integrin $\alpha v \beta 3$ activates VEGF receptors, and inhibition of integrin $\beta 3$ has been shown to reduce phosphorylation of VEGF receptors, thereby limiting the biological effects of VEGF.19 Figure 5A and 5B shows diminished p-FAK in Group R, indicating that VEGF-mediated intracellular signaling was also diminished. Because VEGF has deleterious effects in the initial time period after MCAO,20,21 inhibition of VEGF receptors by cRGDfV was likely beneficial to the animals.

CRGDfV therapy was associated with decreased VEGF production (Figure 6A and 6B). This may be the result of inhibitory action on the VEGF receptor, or possibly a consequence of improved microcirculation that itself is the result of cRGDfV-driven reduction of platelet-fibrinogen/fibrin aggregation (Figure 4). VEGF is known to have a myriad deleterious effects early in ischemic stroke, including increased BBB leakage, increased risk of hemorrhagic transformation, increased infarction zone,20,21 and increased platelet adhesion.22 VEGF is elaborated during ischemic stroke23 (Figure 4), and cRGDfV-associated reduction of VEGF was likely beneficial to the animals in this study.

CRGDfV therapy was associated with decreased fibrinogen/fibrin elaboration (Figure 6C and 6D) and deposition (Figure 4A). Fibrinogen is one of ligands of integrin $\alpha v \beta 3$.24 Integrin $\alpha v \beta 3$ is upregulated in arterioles in the ischemic core in the primate cerebral ischemic model, and expression of integrin $\alpha v \beta 3$ correlates with fibrin deposition.3 Fibrinogen deposition occurs in the MCAO model,25 and blockade of fibrinogen prevents platelet adhesion to the vessel wall.26 Thrombin has also been shown to interact with integrin $\alpha v \beta 3$,27 although we did not study this interaction.

Others have demonstrated that VEGF induces $\alpha v \beta 3$ expression in vitro, and that VEGF and integrin $\alpha v \beta 3$ expression are highly correlated in vivo.4 Integrin $\alpha v \beta 3$ expression may have been suppressed in a VEGF-dependent manner by treatment with cRGDfV, or possibly by direct, receptor-driven downregulation induced by cRGDfV (Figure 1).

We observed a significant increase in brain edema in the PCA perfusion cortex in Groups C and N, and this was ameliorated with cRGDfV treatment (Figure 3C). The PCA perfusion cortex became edematous secondarily in the MCAO procedure, likely as a consequence of proximity to the much-larger MCA perfusion cortex. For reasons that are unclear, this phenomenon was not observed in the ACA perfusion cortex.

CRGDfV therapy was associated with a decrease of inflammatory cells in the penumbra (Figure 4B), which may be a consequence of reduced infarction volume (Figure 2A), inhibition of integrin $\alpha v \beta 3$ expression (Figure 1) or reduction of VEGF (Figure 6A). Previous work
demonstrated that αvβ3 blockade reduces monocyte or macrophage movement through vessel walls in vivo and in vitro. VEGF is known to enhance macrophage activity.

One of the limitations of the present study is that all animals were euthanized at 24 hours, whereas ischemic stroke maturation occurs over several days. Our study emphasized early brain injury, and the possible long-term...
effects of cRGDFV including vascular endothelial apoptosis were not studied. We used combined pre- and postoperative treatment in this study to provide proof-of-concept. Future studies will evaluate exclusive postischemic treatment, the long-term durability of treatment, and the possible use of cRGDFV as an adjunctive treatment to established therapeutic methods such as recombinant tissue plasminogen activator.

Figure 6. Western blots and photomicrographs. (A) VEGF expression on Western blots. (B) VEGF expression on immunohistochemical stain is reduced in Group R. Arrowheads indicate positive cells in the basal ganglia. Scale bar=50 µm. (C) Fibrinogen expression on Western blots. (D) Fibrinogen expression on immunohistochemical stain is reduced in Group R. Circles indicate fibrinogen deposits. Scale bars=50 µm. # indicates P<0.05 compared with S, * indicates P<0.05 compared with R.
Conclusions
The integrin αvβ3 inhibitor cRGDfV improves outcomes in the MCAO model and is associated with improved BBB integrity and decreased elaboration and activity of VEGF.

Sources of Funding
This study was partially supported by National Institutes of Health (NIH) grants NS45694, HD43120, and NS43338 to J.H.Z.

Disclosures
None.

References
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Stroke. 2006;37:1902-1909; originally published online June 1, 2006;
doi: 10.1161/01.STR.0000226991.27540.f2
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/37/7/1902

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