Transforming Growth Factor-β1 Reduces Infarct Size After Experimental Cerebral Ischemia in a Rabbit Model

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Background and Purpose: The aim of this study was to examine the effect of transforming growth factor-β1, a cytokine shown to ameliorate cardiac ischemia, in a rabbit model of thromboembolic stroke.

Methods: An autologous clot embolus was introduced intracranially through the right internal carotid artery in 21 New Zealand White rabbits, with seven in each group receiving either vehicle control (albumin) or 10 or 50 μg transforming growth factor-β1 administered as an intracarotid bolus immediately before autologous clot embolization. Multiple physiological parameters were monitored, including regional cerebral blood flow, arterial blood gases, hematocrit, glucose, core temperature, and mean arterial pressure. The brain was harvested 4 hours after embolization, and infarct size was determined planimetrically as a percentage of the entire hemisphere.

Results: Brain infarct size was reduced in both the 10-μg (16.7±4.0% [mean ±SEM], p< 0.05) and 50-μg (21.7±4.5%) transforming growth factor-β1-treated groups when compared with the control group (31.9 ±6.6%). Regional cerebral blood flow did not show any significant intergroup or intragroup variation over time, although the 10-μg transforming growth factor-β1 group experienced a greater return of cerebral blood flow in the first 2 hours after embolization.

Conclusions: Transforming growth factor-β1 reduced brain infarct size in a rabbit model of thromboembolic stroke. This effect was not related to a direct effect on blood flow. Studies are ongoing to determine the mechanism by which transforming growth factor-β1 salvages ischemic brain. (Stroke 1993;24:558–562)

KEY WORDS • cerebral ischemia • transforming growth factors • rabbits

The three transforming growth factor-β (TGF-β) isoforms are fully characterized homodimers that are present in most mammalian cells.1 Recent interest has focused on the positive role of these peptides in inflammation and repair.2 This is supported by studies demonstrating both the chemotactic and anabolic properties of TGF-β1 as well as a direct role in wound healing in experimental studies.1–4

Tissue ischemia, similar to inflammation, is characterized by the early arrival of neutrophils to the site of injury. Studies in the heart,5,6 brain,7–9 and other organs10,11 have suggested that the early arrival of leukocytes at an ischemic site may exacerbate injury. In the central nervous system, this hypothesis has been recently examined in a rabbit model of thromboembolic stroke in which an autologous clot embolus is delivered to the anterior circulation of the brain.7 In both this

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model and others, agents that reduce neutrophil function or activity have resulted in a reduction in ischemic brain injury. TGF-β1 has been demonstrated to reduce neutrophil adherence to endothelial cells12 and to suppress the release of potentially deleterious oxygen-derived13 and nitrogen-derived14 metabolites by macrophages in vitro.

Additionally, astrocyte dysfunction may also exacerbate ischemic brain injury. Indeed, suppression of astrocyte swelling has been proposed to reduce ischemic brain injury.15 Of interest, TGF-β1 has recently been shown to suppress some aspects of astrocyte function in vitro.16

The ability of TGF-β1 to afford cardioprotection in a model of myocardial ischemia17 as well as its demonstrated suppression of both leukocyte13,14 and astrocyte16 function suggests a potential beneficial role for this peptide in acute cerebral ischemia. In the present study, we examined the effect of the most abundant isoform, TGF-β1, in a rabbit model of thromboembolic stroke.

Materials and Methods

The rabbit model of thromboembolic stroke used in the current study has been described in detail else-
where. Briefly, New Zealand White rabbits (2.5-3.2 kg) of either sex were anesthetized by use of acepromazine (20 mg i.m.) and ketamine (50 mg/kg i.m.). Aortic and femoral vein catheters were placed via the femoral route. The aortic catheter was used for monitoring mean arterial pressure (MAP), for blood sampling, and for determining systemic hydrogen clearance used in the hydrogen clearance technique for regional cerebral blood flow (rCBF). Thirty-gauge platinum-iridium electrodes, polarized to -0.6 V, were placed 2 mm into the cortical mantle through trough craniectomies immediately posterior to the coronal suture bilaterally for the measurement of rCBF by the hydrogen clearance technique. Each site was hermetically sealed with fast-setting epoxy.

All rabbits were then tracheostomized and mechanically ventilated. An esophageal probe was placed to monitor core temperature. The region of the right common carotid artery bifurcation was then isolated, and the external carotid artery was ligated.

Baseline measurements of all parameters (MAP, rCBF, serum glucose, hematocrit, temperature, and arterial blood gases [ABG]) were obtained, recorded, and repeated to ensure reproducibility. After the demonstration of reproducible baseline values, TGF-β1 (10 or 50 μg; R&D Systems, Minneapolis, Minn.) or albumin control was administered via the right internal carotid artery to the intracranial circulation. TGF-β1 contained 0.1% bovine serum albumin and was dissolved using 50 μL of 4 M HCl. The final volume and pH were adjusted by adding 450 μL of phosphate-buffered saline, pH 7.4, to the sample. The dosages were based on the amounts used in previous studies of cardioprotection. The route of administration used was believed to maximize the availability of TGF-β1 to the intracranial circuit.

Immediately after delivery of the TGF-β1 the catheter was removed, and a second catheter containing an autologous clot embolus was introduced into the right internal carotid artery through the common carotid artery. The clot embolus was then delivered to the cerebral circulation, and a single film carotid arteriogram was performed. Any animal not demonstrating a reduction in rCBF to ≤15 mL/100 g per minute (two) was eliminated from further study. All animals were supported for a total of 4 hours after the embolic event. Repeat determination of all of the above physiological parameters was performed 10 minutes after embolization and at hourly intervals throughout the remainder of the experiment. At the end of the experimental protocol, the rabbits were killed with an overdose of sodium pentobarbital (150 mg/kg) in accordance with procedures outlined by the University of Vermont Institutional Animal Care and Utilization Committee. The brains were harvested and inspected grossly for verification of clot placement. All brains were then sectioned coronally into 2-mm slices and stained with the dye 2,3,5-triphenyltetrazolium chloride. This dye allows for the determination of infarct size by staining only viable tissue brick red. The volume of brain infarct, as determined planimetrically using an IBM image analyzer. A total of 24 rabbits were used in this study. Two rabbits that did not meet the rCBF criteria after embolization (≤15 mL/100 g per minute) were eliminated from the study. One additional rabbit did not survive secondary to ventilatory complications. A total of seven rabbits were ultimately used for analysis in each of the three groups.

Elicited peritoneal neutrophils were harvested from New Zealand White rabbits as follows: 200 mL of sterile saline containing 500 units heparin and 0.5% glycogen (Sigma Chemical Co., St. Louis, Mo.) was injected intraperitoneally under sterile conditions using a flexible 20-gauge angiocatheter (B.D. Co., Rutherford, N.J.). Four hours later, the peritoneal fluid was recovered into iced plastic tubes using a flexible 20-gauge angiocatheter. The recovered peritoneal fluid was centrifuged at 220g, and the pellet was subjected to a hypotonic lysis to destroy any contaminant red blood cells. The sample was then recentrifuged at 220g and the leukocyte pellet reconstituted in Hanks' balanced salt solution (HBSS) as previously described. The neutrophil population was noted to be >95% pure and viable, as assessed by Wright-Giemsa staining and trypan blue exclusion, respectively. All cells were used within 3 hours of their preparation.

Neutrophil aggregation was induced with submaximal concentrations of N-formyl-methionyl-leucyl-phenylalanine (fMLP, 10^{-9} M, Sigma; prepared in 0.001% dimethyl sulfoxide), as previously described, using 0% and 100% light transmission preset with the neutrophil-rich suspension (1.5 x 10^{6}/mL) and 80% of the neutrophil suspension, respectively. Neutrophils were preincubated with varying concentrations of TGF-β1 (10-1,000 pg/mL) for 10 minutes before the addition of fMLP. Results are expressed as a percentage of the control value obtained using a block design.

The generation of superoxide anions was measured spectrophotometrically as the reduction of cytochrome c. Neutrophils (1.5 x 10^{6}/mL) were preincubated with cytochalasin B (10 μg/mL, Sigma) for 15 minutes in the presence or absence of TGF-β1 (1-1,000 pg/mL), followed by the addition of ferricytochrome c (1.5 mg/mL, from horse heart, type III, Sigma). A duplicate set of samples contained 25 μg superoxide dismutase (SOD) to serve as the negative control. The samples were then incubated for 10 minutes at 37°C using fMLP (10^{-6}) as the stimulus, and the reaction was stopped with the addition of 25 μg SOD to all experimental samples. All samples receiving SOD previously received an equal volume of HBSS. The reduction of cytochrome c was measured spectrophotometrically at 550 nm (Beckman spectrophotometer, model 260). Results were compared with vehicle controls and expressed as a percentage of the control value.

The release of β-glucuronidase from the neutrophil primary granules was measured using Sigma kit No. 325. Neutrophils (1.5 x 10^{6}) were preincubated with cytochalasin B and varying concentrations of TGF-β1 as described for the superoxide anion assay. After the addition of the stimulus fMLP (10^{-6}), the samples were again incubated at 37°C for 10 minutes and the reaction then terminated by high-speed centrifugation (10,000g, 30 seconds). The supernate was then incubated with phenolphthalein mono-β-glucuronic acid. Any free β-glucuronidase will catalyze the liberation of phenolphthalein, which was then measured spectrophotometrically at 550 nm. Results were compared with control
samples and the values expressed as the percentage of the control value.

Hematocrit, ABG, serum glucose values, rCBF, and all in vitro neutrophil assays were analyzed through the use of repeated-measures analysis of variance (ANOVA). After a significant time effect, individual contrasts were used to compare the time at embolization to baseline and later time points. Measurements of infarct size in each experimental group were compared with the control value by applying Dunnett's test after a one-way ANOVA. All tests of hypotheses were performed at the α = 0.05 level of significance. Results reported are mean ± SEM.

Results

Baseline rCBF was very similar (F<sub>2,8</sub> = 0.27) in all three groups: 90.1 ± 8.1 in the control group and 90.1 ± 16.7 and 101.0 ± 9.5 mL/100 g per minute in the groups receiving 10 and 50 μg TGF-β1, respectively (Figure 1). Immediately after embolization, rCBF was significantly reduced (F<sub>2,8</sub> = 86.83, and 121 for control and 10 and 50 μg TGF-β1, respectively, all p < 0.0001) to <10 mL/100 g per minute in all three groups. Some recovery of rCBF was noted in all three groups, although only the group receiving 10 μg TGF-β1 demonstrated an rCBF above the ischemic threshold of >15 mL/100 g per minute (hours 1 and 2). No comparisons of the clot time versus the later time points reached significance. Although both groups receiving TGF-β1 demonstrated reductions in brain infarct size, this was only significant in the group receiving 10 μg TGF-β1 (16.7 ± 4.0% and 21.7 ± 4.5% for 10 and 50 μg TGF-β1, respectively, versus 31.9 ± 6.6% of the hemisphere in the control group; Figure 2; 10-μg group, Dunnett’s t statistic = 2.08, p < 0.05).

Serum glucose values were very similar in all groups (F<sub>2,18</sub> = 0.54, NS) throughout the protocol with initial glucose values of 11.4 ± 5.13 mmol/L in the control group versus 12.3 ± 4.02 and 9.9 ± 2.70 mmol/L in the TGF-β1 10- and 50-μg groups. These values slowly declined over time to reach final values of 8.39 ± 5.0 mmol/L in the control group versus 10.61 ± 3.82 and 9.01 ± 4.65 mmol/L in the TGF-β1 10- and 50-μg groups, respectively. Similarly, ABG (PCO<sub>2</sub>, 30–37 mm Hg, F = 1.28; PO<sub>2</sub>, 100–140 mm Hg, F = 0.62; and pH, 7.36–7.45, F = 0.36) and hematocrit (30–35%, F = 0.2) were kept within physiological range throughout the experimental protocol. The MAP was held within the range of 55–60 mm Hg.

TGF-β1 pretreatment failed to significantly affect neutrophil aggregation or the release of β-glucuronidase or superoxide anion after activation with the tripeptide fMLP (10<sup>-4</sup> to 10<sup>-3</sup> M) (Table 1).

Discussion

TGF-β1 significantly reduced brain infarct size in a rabbit model of thromboembolic stroke. The mechanism(s) by which TGF-β1 exerted this protective effect is unclear, although an effect on systemic blood pressure, hematocrit, or ABG is unlikely because these parameters were held constant throughout the experimental protocol.

The neuroprotective effects of TGF-β1 could be the result of either a direct neuronal effect or one or more factors arising from the systemic response to embolization. The exact mechanism(s) by which TGF-β1 exerted its protective effect is unclear. It may be that TGF-β1 had a direct effect on brain tissue or systemic parameters affecting the state of the cerebral vasculature.

Table 1. Effect of Transforming Growth Factor-β1 on Neutrophil Function

<table>
<thead>
<tr>
<th>Assay</th>
<th>TGF-β&lt;sub&gt;1&lt;/sub&gt; (pg/mL)</th>
<th>Percentage of control response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregation</td>
<td>10</td>
<td>95.3 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>90.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>90.3 ± 2.0</td>
</tr>
<tr>
<td>Superoxide release</td>
<td>1</td>
<td>99.4 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>102.8 ± 9.0</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>89.0 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>89.0 ± 12.3</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>1</td>
<td>103.6 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>97.7 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>114.4 ± 8.0</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>108.9 ± 5.6</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. TGF-β<sub>1</sub>, transforming growth factor-β1. Polymorphonuclear leukocyte aggregation (n = 5; F<sub>2,19</sub> = 1.07), superoxide anion release (n = 5; F<sub>4,20</sub> = 1.79), and the release of β-glucuronidase (n = 5; F<sub>4,20</sub> = 1.97) were not significantly affected after treatment with TGF-β1 (by repeated-measures analysis of variance).
indirect mechanisms, such as the regulation of rCBF. The direct effect of TGF-β1 on neuronal function awaits investigation. Support for more indirect mechanisms relates to the higher rCBF noted in the first 2 hours after embolization in the group receiving 10 μg TGF-β1. Although this increase in rCBF was not statistically significant, the difference may be physiologically important because the threshold for neuronal survivability is generally thought to be approximately 15 mL/100 g per minute.22 Thus, even the temporary maintenance of rCBF in the range of 20 mL/100 g per minute in the 10-μg TGF-β1 group may have contributed to this group demonstrating the smallest infarct size. It is unclear how TGF-β1 may affect rCBF; although TGF-β1 may encourage angiogenesis,23 it is doubtful that this mechanism would account for a significant change in rCBF because of the acute nature of the experiments. It is of interest that the administration of intracarotid TGF-β1 under baseline conditions did not alter rCBF (data not shown).

Alternatively, the action of TGF-β1 may be the result of effect(s) on other cell types. In previous studies, we found that agents that suppressed either neutrophil and/or astrocyte function reduce brain infarct size.7,15 Although TGF-β1 has been demonstrated to reduce neutrophil adherence to endothelial cells,12 there is also evidence that it may upregulate the expression of lymphocyte function-associated antigen-1, a cell surface receptor present on leukocytes that is responsible for cell adhesion.24 Our in vitro results failed to demonstrate an effect by TGF-β1 on FMLP-induced neutrophil aggregation, an in vitro correlate of chemotaxis,25 or the generation of superoxide anion and β-glucuronidase, although an examination of the effects of TGF-β1 on neutrophil function in vivo awaits further study.

TGF-β1 has also been demonstrated to inhibit astrocyte growth and differentiation.16 Astrocytes are capable of producing various cytokines such as tumor necrosis factor (TNF).26 Interestingly, both an exaggerated release and deleterious effect of TNF have been demonstrated in a model of cardiac ischemia.17 In these studies, TGF-β1 was capable of reducing both the release of TNF as well as the concomitant ischemic injury. Thus, although not directly examined in the present study, it is conceivable that similar mechanisms are operational in cerebral ischemia. Conversely, although TGF-β1 was administered intra-arterially in an attempt to optimize delivery to the region of ischemia, it is unclear whether TGF-β1 is able to cross either an intact blood–brain barrier or if the current paradigm sufficiently modifies the blood–brain barrier to result in the accumulation of TGF-β1 within the brain parenchyma.

The use of two different doses of TGF-β1 allowed us to begin to examine the dose–response effect of this cytokine in cerebral ischemia. The critical dependence on the dose of TGF-β1 to study outcome has been well documented previously.1 The doses of TGF-β1 used in the current study were chosen based on amounts used in previous reports on cardioprotection. Since the lower dose, 10 μg, demonstrated the greatest effect, it would be of interest to study even lower doses in future experiments. It is possible that the higher concentrations of TGF-β1 allow this cytokine to interact with additional cell types, producing deleterious effects that may serve to counteract the beneficial effects seen with a more selective action demonstrated at lower concentrations.

It is also conceivable that in examining infarct size acutely the effect of TGF-β1 is that of delaying infarct evolution rather than ultimate salvage. To clarify this issue, further time points would need to be examined. The ability of TGF-β1 to even delay infarct evolution, however, may ultimately allow for the initiation of other more definitive therapies to salvage ischemic brain.

In conclusion, the administration of TGF-β1 results in the significant salvage of ischemic brain in a rabbit model of thromboembolic stroke. The mechanism(s) responsible for this result remain to be elucidated but may include the indirect enhancement of rCBF and/or the suppression of deleterious cytokines, such as TNF, by astrocytes. This is the first in vivo evidence of a protective role for TGF-β1 in the central nervous system.

References

A growing body of evidence indicates that peptide growth factors can attenuate the effects of ischemia on the brain. The present article by Gross et al adds transforming growth factor-β1 to this list. The study of the mechanisms of action of trophic factors on ischemic brain, which include possible roles for nonneuronal cell actions, ion channel modulation, and messenger RNA and protein synthesis, promises to open up new concepts and directions for the study and management of cerebral ischemia.

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Transforming growth factor-beta 1 reduces infarct size after experimental cerebral ischemia in a rabbit model.

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*Stroke*. 1993;24:558-562
doi: 10.1161/01.STR.24.4.558

*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

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