1999-02-05

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Synergistic Effects of a Combination of Low-Dose Basic Fibroblast Growth Factor and Citicoline After Temporary Experimental Focal Ischemia

Wolf R. Schäbitz, MD; Fuhai Li, MD; Katsumi Irie, MD; Bobby W. Sandage, Jr, PhD;
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Background and Purpose—Basic fibroblast growth factor (bFGF) and citicoline (cytidine 5′-diphosphate choline, an endogenous compound that stabilizes membrane function) have demonstrated neuroprotective effects after focal cerebral ischemia. Both agents are candidates for future stroke therapy in humans. For evaluation of synergistic effects of bFGF and citicoline, a low-dose combination of both compounds was tested against each compound alone and placebo.

Methods—Four groups of Sprague-Dawley rats (n=12 per group) underwent 90 minutes of focal cerebral ischemia with the use of the suture model of middle cerebral artery occlusion. Animals were randomly and blindly assigned to one of the following treatment groups: placebo, low-dose citicoline (250 mg/kg IP daily for 4 days), low-dose bFGF (10 μg/kg per hour IV for 3 hours), and the combination of both (250 mg/kg citicoline and 10 μg/kg per hour bFGF). Triphenyltetrazolium chloride staining was used after 4 days to determine postmortem infarction. Neurological scores were assessed on a daily basis.

Results—The premature mortality rate was 41.7% in the placebo and citicoline groups, 33.3% in the bFGF group, and 25% (*P*=NS) in the combination group. The mean neurological score on day 4 was 3.1±1.6 (placebo), 3.1±1.6 (citicoline), 2.9±1.5 (bFGF), and 2.4±1.4 (combination) (*P*=NS). The mean volume of infarction was significantly reduced in the combination group (136.5±25.4 mm^3) versus placebo (172.6±48.9 mm^3; *P*=0.036, Fisher test), versus citicoline alone (186.0±35.7 mm^3; *P*=0.005, Fisher test), and versus bFGF alone (176.0±49.2 mm^3; *P*=0.023, Fisher test).

Conclusions—These results demonstrate synergistic effects of a low-dose combination of the growth factor bFGF and citicoline after temporary experimental focal cerebral ischemia and furthermore support the effectiveness of a combination treatment regimen for the management of acute stroke. (***Stroke***. 1999;30:427-432.)

Key Words: cerebral ischemia, focal ■ citicoline ■ growth factors ■ rats

Neuroprotective therapy is effective in animal stroke models but remains unproven in clinical stroke. Neuroprotective agents such as *N*-methyl-D-aspartate antagonists are well tolerated in animals but may cause major side effects in humans (eg, psychotic events). Reducing doses may reduce side effects but may also lower the neuroprotective capability of the agent. One way to reduce the dose of drugs and to maintain, or even increase, neuroprotective effects is to use a combination of agents that act on different steps in the ischemic cascade. Recently, combination studies for the treatment of experimental focal cerebral ischemia were reported with encouraging results for the combination of thrombolytic and neuroprotective therapy[1–3] and 2 different neuroprotective agents.[4]

An intriguing idea for the treatment of acute stroke is the use of growth factors that have not only neuroprotective but also regenerative and proliferative capacities. A promising

growth factor for the treatment of acute focal cerebral ischemia in stroke models is basic fibroblast growth factor (bFGF), a 18-kDa polypeptide that mediates the differentiation, survival, and regeneration of neuronal cells through specific receptors.[5] It has been shown in several in vivo experiments in different species that bFGF has neuroprotective and regenerative capabilities when administered after focal cerebral ischemia.[6–13] Possible mechanisms of action for bFGF include a direct neuroprotective effect and an effect on cerebral blood flow (CBF) and cerebrovascular tone.[8,14,15]

Citicoline (cytidine 5′-diphosphate choline), a naturally occurring endogenous compound that serves as an intermediate in the synthesis of the membrane phosphatidylcholine, is thought to have membrane-stabilizing functions and to reduce free fatty acid formation during stroke.[16,17] Citicoline

reduces the size of infarction and improves the neurological outcome in experimental models of focal cerebral ischemia.[1,4,18,19] In clinical studies, treatment with citicoline improved cognitive and behavioral function in patients with memory deficits.[20,21] After human stroke, treatment with citicoline improved neurological outcome[22–24] and reduced ischemic lesion volume as demonstrated by diffusion-weighted MRI.[25]

To study the effect of a combination of bFGF and citicoline on focal cerebral ischemia seems rationale since both agents act through different mechanisms and may therefore have a synergistic effect. Furthermore, bFGF and citicoline are currently under clinical investigation as candidates for future stroke therapy. For the combination of both agents, we purposely chose dose levels for each compound that were unlikely to be effective alone after cerebral ischemia.[4,12,18,26] In this study we investigated the treatment effect of citicoline (250 mg/kg), bFGF (10 μg/kg per hour), and the combination of both versus placebo using the suture occlusion model of the middle cerebral artery (MCA). The aim of the study was to determine whether the combination treatment had salutatory effects on mortality, on neurological outcome, and, in particular, on infarction.

Materials and Methods

In 48 male Sprague-Dawley rats, weighing 300 to 350 g, 90 minutes of temporary focal cerebral ischemia was induced with the use of the intraluminal suture occlusion method. Our procedures were approved by the Animal Research Committee of the University of Massachusetts Medical School (Animal Research Committee Protocol No. 643). All experiments were performed in a blinded manner. Nonfasted animals were randomly assigned before surgery to one of the following groups: 12 animals treated with low-dose citicoline (citicoline group), 12 animals treated with low-dose bFGF (bFGF group), 12 animals treated with a combination of low-dose citicoline and low-dose bFGF (combination group), and 12 animals treated with placebo.

The animals were anesthetized intraperitoneally with 400 mg/kg of chloral hydrate. The left femoral artery was cannulated with PE-50 polyethylene tubing for continuous monitoring of arterial blood pressure and blood sampling for analysis of blood gases. Measurements were performed before surgery and 15, 105, and 195 minutes after ischemic onset. Rectal temperature was maintained during surgery at 37°C with a feedback-regulated heating lamp. The right MCA was occluded by the transvascular suture method as previously described.[18,27] For MCA occlusion, the right common carotid artery and the right external carotid artery were exposed through a midline neck incision. The distal common carotid artery and the external carotid artery were first ligated with a 3-0 silk suture. A 4-0 monofilament nylon suture (40 mm in length), whose tip had been rounded by heating near a flame and then coated with silicon (Bayer), was inserted through an arteriectomy of the common carotid artery and gently advanced into the internal carotid artery. Positioned ≈17 mm from the bifurcation, the tip of the suture occluded unilaterally the proximal anterior cerebral artery, the origins of the MCA, and the posterior communicating artery. To prevent bleeding, the common carotid artery was loosely ligated with a 3-0 silk suture just distal to the arteriotomy.

Intravenous infusion of the growth factor or vehicle was started 30 minutes after vessel occlusion and maintained for 3 hours; citicoline- or placebo-treated animals received vehicle (1% BSA in physiological saline), and bFGF-treated animals (bFGF and combination groups) received 10 μg bFGF per kilogram per hour in 1% BSA in physiological saline. After reperfusion, animals were treated with an intraperitoneal bolus of citicoline 250 mg/kg in physiological saline (citicoline and combinations groups) or with the vehicle alone (bFGF

and placebo groups). At the conclusion of the intravenous infusion, the animals were allowed to recover from the anesthesia and were scored neurologically according to a 6-point scale: 0=no neurological deficit, 1=failure to extend left forepaw fully, 2=circling to the left, 3=falling to the left, 4=no spontaneous walking with a depressed level of consciousness, and 5=death.[27] Animals that died within 12 hours of vessel occlusion were not included in the study.

During the next 3 days the animals had free access to food and water. The neurological examination and the intraperitoneal treatment with citicoline or vehicle were performed in all animals on a daily basis. On the fourth day the animals were killed with an overdose of chloral hydrate (administered intraperitoneally) and decapitated. The brains were removed and inspected for subarachnoid hemorrhage. Animals with subarachnoid hemorrhage were excluded from the study. The brains then were frozen at −20°C and coronally sectioned into six 2-mm-thick slices. The brain slices were incubated for 30 minutes in a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C and then fixed by immersion in a 10% buffered formalin solution. TTC stains normal brain tissue red, while ischemic tissue turns white.[28,29] For quantification of infarct volumes, the brain slices were photographed with a charge-coupled device camera (EDC-1000HR Computer Camera, Electrim Corporation), and image analysis was performed on a personal computer with a commercially available image analysis program (Bio Scan, Optomas). To compensate for the effect of brain edema, the corrected infarct volume was calculated by the following formula: corrected infarct area equals left hemisphere area minus (right hemisphere area minus infarct area).[30,31] The infarcted area was calculated separately for cortical and subcortical regions. The corrected mean total infarct volume and cortical and subcortical infarct volumes then were calculated by multiplying the respective corrected infarct areas by slice thickness. The brains of animals that died prematurely after ≥12 hours after occlusion were harvested within 8 hours and evaluated in a manner similar to that used for those who were electively killed.[29]

Values presented in this study are expressed as mean±SD. After acquisition of all data, the randomization code was broken. ANOVA and subsequent post hoc Fisher protected least significant difference test were used to determine the statistical significance of differences in physiological variables and volumes of infarction among all 4 groups. The Kruskal-Wallis test was performed for nonparametric variables such as mortality rate and neurological score. A P value <0.05 was considered statistically significant.

Results

Physiological variables, presented in Table 1, showed no significant differences when all 4 groups were compared. The percent body weight decline for the surviving animals in the placebo, citicoline, bFGF, and combination groups was 17%, 16%, 18.5%, and 16.8%, respectively (P=NS). In the placebo and citicoline groups, 5 of 12 animals died between 24 and 96 hours after ischemia; thus, the premature mortality rate in both groups was 41.7%. Four of 12 animals died in the bFGF group, 24 to 96 hours after ischemia; the mortality rate was 33.3%. Premature death occurred in only 3 of 12 animals in the combination treatment group; the mortality rate in this group was 25% (P=NS). The mean neurological score for the daily examinations was not statistically significantly different among the 4 groups (Table 2).

The mean volume of infarction was 172.6±48.9 mm^3 in the placebo group, 186.0±35.7 mm^3 in the low-dose citicoline alone group, 176.0±49.2 mm^3 in the low-dose bFGF alone group, and 136.5±25.4 mm^3 in the combination treatment group (Figure 1). The difference of the mean values of infarct volume was significant for the combination group versus the controls (P=0.036, Fisher test), versus the

TABLE 1.   Physiological Parameters for All Groups at Baseline and 15, 105, and 195 Minutes After MCA Occlusion

	Group			
	Placebo	Citicoline	bFGF	Combination
Baseline				
pH	7.41±0.05	7.41±0.07	7.42±0.05	7.38±0.08
P_{CO_2}, mm Hg	38.2±4.2	36.7±3.0	32.6±7.6	39.3±5.8
P_{O_2}, mm Hg	102.4±13.7	90.3±9.7	92.5±5.4	88.8±8.2
MABP, mm Hg	93.5±8.5	90.5±9.5	94.4±6.9	102.5±11.3
Body temperature, °C	37.0±0.2	36.9±0.2	37.1±0.1	37.0±0.1
15 min after MCAO				
pH	7.39±0.05	7.34±0.09	7.42±0.10	7.37±0.08
P_{CO_2}, mm Hg	33.5±6.7	43.0±9.2	34.2±5.4	42.4±4.9
P_{O_2}, mm Hg	94.8±10.1	80.4±13.8	91.7±8.1	87.8±9.2
MABP, mm Hg	84.5±4.1	83.5±5.5	88.3±2.9	90.5±7.3
Body temperature, °C	37.2±0.2	37.0±0.4	37.2±0.1	37.1±0.1
105 min after MCAO				
pH	7.36±0.03	7.33±0.05	7.35±0.03	7.37±0.07
P_{CO_2}, mm Hg	33.2±7.3	42.1±4.9	33.1±8.8	36.2±6.1
P_{O_2}, mm Hg	89.9±9.6	79.1±6.2	85.1±9.8	85.7±11
MABP, mm Hg	87.5±14.6	90.5±7.9	82.4±8.9	77±13.9
Body temperature, °C	36.9±0.1	37.0±0.3	37.0±0.1	37.0±0.1
195 min after MCAO				
pH	7.34±0.04	7.33±0.07	7.33±0.10	7.35±0.03
P_{CO_2}, mm Hg	34.3±10.4	42.2±5.2	35.0±7.6	40.1±4.2
P_{O_2}, mm Hg	91.9±4.6	82.0±10.9	84.3±7	87.5±8.7
MABP, mm Hg	98.5±6.6	101.5±8.8	89.7±9.5	87.5±14.6
Body temperature, °C	37.0±0.1	36.8±0.1	37.1±0.2	37.1±0.2

Values are mean±SD. MCAO indicates MCA occlusion. Differences in physiological parameters among groups were not statistically significant ($P>0.05$, ANOVA).

citicoline-treated group ($P=0.005$, Fisher test), and versus the bFGF-treated group ($P=0.023$, Fisher test). The subcortical infarct volume was 66.5±18.3 mm³ in the placebo group, 66.2±15.8 mm³ in the citicoline-treated group, 69.5±18.1 mm³ in the bFGF-treated group, and 48.6±11.2 mm³ in the combination-treated group (Figure 2); the difference was significant for the combination versus the placebo, citicoline, and bFGF groups ($P=0.009$, $P=0.01$, and $P=0.003$, respectively; Fisher test). The cortical infarct volume was 106.1±34.1 mm³ in the placebo group, 119.7±22.2 mm³ in the citicoline-treated group, 106.5±38.1 mm³ in the bFGF-treated group, and 87.4±24.9 mm³ in the combination-treated group ($P=NS$) (Figure 2).

TABLE 2.   Neurological Scores

Group	Day 2	Day 3	Day 4
Placebo	2.3±1.0	2.9±1.5	3.1±1.6
Citicoline	2.5±1.1	3.0±1.5	3.1±1.6
bFGF	2.4±1.2	2.8±1.4	2.9±1.5
Combination	1.8±1.1	2.3±1.3	2.4±1.4

Values are mean±SD. Differences among groups were not statistically significant ($P>0.05$, Kruskal-Wallis test).

Discussion

The results of this study demonstrate to our knowledge for the first time that a low-dose combination of a growth factor (bFGF) and a neuroprotectant (citicoline) significantly reduces infarct volume after temporary focal cerebral ischemia. The reduction in infarct volume produced by the combination therapy versus placebo was 22% (Figure 1). The reduction was more effective in subcortical than in cortical areas of infarction (Figure 2). There was no treatment effect for either of the 2 compounds alone, nor were there significant side effects at the low doses used in this study. In particular, no significant decrease of blood pressure occurred in the bFGF-treated groups, since the chosen dose was below the reported hypotensive dose of 60 μg/kg per hour.[12,32] No significant difference in weight loss, which is a known side effect after bFGF treatment, was observed among the groups during the experiment.[10]

The neuroprotective effects of bFGF and citicoline after experimental focal cerebral ischemia are well documented and include infarct reduction and improved neurological and behavioral outcome.[1,4,8,12,18,19] Some of these studies demonstrated a dramatic neuroprotective effect with infarct reductions up to 50%.[12,18] However, the doses used in these single
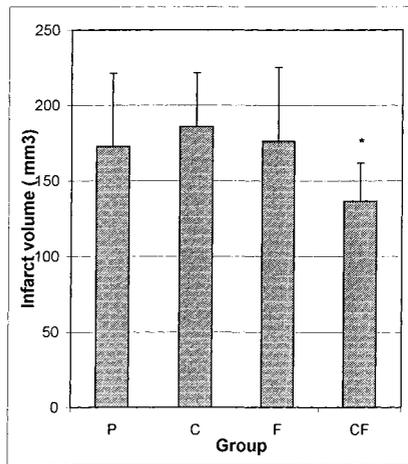
Figure 1. Mean infarct volumes. Data are mean±SD. P indicates placebo; C, citicoline group; F, bFGF group; and CF, combination group. *The difference of the mean infarct volume was statistically significant for CF versus P, C, and F ($P<0.05$, Fisher test).

compound studies with systemic administration were much higher than the ones we used for this experiment (500 mg/kg citicoline versus 250 mg/kg and 45 μg/kg per hour of bFGF versus 10 μg/kg per hour). To study possible synergy between citicoline and bFGF, the dosage level for each individual compound was purposely chosen on the basis of doses previously demonstrated not to be neuroprotective[1,4] or empirically chosen below their reported neuroprotective efficiency.[12,26] We confirm here that 250 mg/kg of citicoline and 10 μg/kg per hour of bFGF are not neuroprotective doses in this model of temporary focal cerebral ischemia. However, a combination of the same doses was able to achieve a significant neuroprotective effect versus placebo and each drug alone without producing any side effects. The exploration of the effectiveness of combination therapies for stroke seems rational since cerebral ischemia triggers a multitude of pathophysiological and biochemical events that affect the
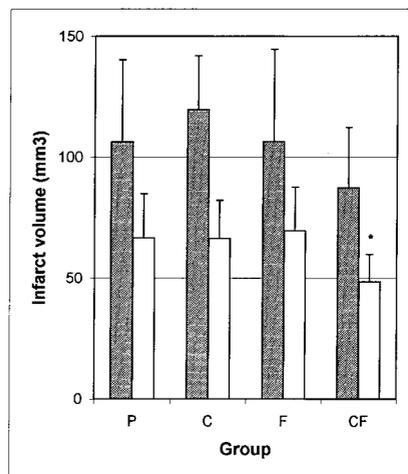

Figure 2. Cortical and subcortical infarct volumes. Data are mean±SD. P indicates placebo; C, citicoline group; F, bFGF group; and CF, combination group *The difference of the subcortical infarct volume was statistically significant for CF versus P, C, and F ($P<0.05$, Fisher test).

evolution of focal ischemic injury differently. Impeding different steps in the ischemic cascade with different therapeutic agents may not only synergistically enhance the neuroprotective effect but may also allow the use of lower doses of each drug and consecutively reduce side effects. This approach has been demonstrated to be promising in prior experimental studies[1–4,19] and may serve as a future strategy for stroke therapy in humans. The combination of citicoline and bFGF is of special interest because both agents are currently under investigation in clinical trials and may be available for human stroke therapy in the near future.

Potential bFGF-mediated mechanisms that contribute to the reduction in infarct size may be direct protective effects on neurons, glia, and cerebral endothelial cells and positive effects on cerebrovascular tone and CBF.[8,12–15,33–36] bFGF protects neurons in vitro against a number of insults that play crucial roles in the pathophysiology of ischemia such as anoxia, hypoglycemia, excitatory amino acids, Ca^{2+} ionophores, free radicals, and nitric oxide.[14,33–36] Moreover, the neuroprotective action of bFGF may depend on neuronal gene transcription and protein synthesis, particularly at the margins of focal infarcts.[12,33] Another possible mechanism of bFGF-induced reduction in infarct size is through the regulation of cerebrovascular tone and CBF. Topical application of bFGF dilates pial cerebral arteries in rats,[15] increases regional blood flow, and may increase the blood supply to the compromised penumbral regions of focal cerebral ischemia.[8] However, no effect on CBF was found in a cat model of focal cerebral ischemia.[13]

Mechanisms for citicoline-mediated neuroprotection include membrane stabilization and restoration. In the normal synthetic process, the enzyme cholinephosphotransferase catalyzes the reaction of citicoline with diacylglycerol to produce phosphatidylcholine and cytidine-5′-monophosphate (CMP).[16,17] After an ischemic event, ATP-dependent removal of CMP is impaired, and increased CMP concentrations reverse the normal cholinephosphotransferase reaction to yield diglycerides and cytidine 5′-diphosphate choline.[37] The very active diglyceride lipase rapidly degrades the accumulated diglycerides to free fatty acids.[38,39] Accumulated free fatty acids are converted to oxygenated metabolites through the cyclooxygenase and lipooxygenase pathways, and these products are involved in ischemia-associated edema and inflammation.[40] Restoration of the cholinephosphotransferase reaction by citicoline prevents the release of free fatty acids during ischemia and helps to remove diacylglycerol by channeling it into phosphatidylcholine.[16,17]

In conclusion, our present findings suggest that a low-dose combination of the growth factor bFGF and citicoline is safe and reduces infarct volume after temporary experimental focal cerebral ischemia. This result may encourage further studies combining the unique capabilities of growth factors with other neuroprotectants or thrombolysis. Since bFGF and citicoline are already under clinical investigation, it may be rational to explore combination therapy with both agents in human stroke.

Acknowledgments

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Editorial Comment

As many readers are well aware, rarely does a single therapeutic agent provide truly impressive neuroprotection in experimental stroke. Because the list of cellular and molecular actors in ischemic injury is voluminous, there is an increasing need for solid investigations of mechanistically driven, combination therapy. The accompanying article uses just such an approach and offers the important observation that low doses of bFGF and citicoline can synergistically

improve tissue injury in a standard rodent model of focal cerebral ischemia. Abundant literature demonstrates that many members of the growth factor families are neuroprotective and regenerative in experiental brain injury, although efficacy in human stroke and cerebral ischemia remains unclear. Citicoline, in contrast, has been shown to be useful in cognitively impaired patients and to improve functional outcomes in acute stroke.[1,2] The compound's mechanism(s) is not straightforward and is often placed under the broad umbrella of "membrane stabilization."

The practical rationale for combination therapy includes (1) the ability to target an outcome level that is greater than can be achieved with either agent alone, whether additive or synergistic, or (2) the reduction of individual agent dosages with consequent reduction of undesirable side effects or toxicity. In the study of Schäbitz et al, the authors deliberately chose doses of each agent for that were lower relative to their previous studies, and therefore potentially suboptimal, with the goal of avoiding complications such as hypotension. This strategy did allow the demonstration of a positive experimental result. Furthermore, it underscores the need for a thoughtful study design with clear rationale and knowledge of each compound's dose-response relationships if a combined therapy investigation is to be successful. It should be noted that CBF and brain temperature were not measured in the various treatment groups. The apparent rationale was that these variables had been previously examined in studies using higher doses of a single agent, and no flow or temperature effects were noted. Nevertheless, it can be difficult to predict the effect of combined therapy on key physiological parameters from historical data based on a single agent.

It should also be noted that histological protection was limited to subcortical brain regions. Presumably citicoline acts on all cells, and bFGF is not known for cell selectivity in neuronal rescue. Because infarction was determined by TTC staining, the authors appropriately do not speculate on regional or cellular heterogenity in the synergism of citicoline and bFGF. However, further work will be important to differentiate the target cells or even potential gray versus white matter sensitivity to the combined therapy.

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