Mice Expressing a Bovine Basic Fibroblast Growth Factor Transgene in the Brain Show Increased Resistance to Hypoxemic-Ischemic Cerebral Damage

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Background and Purpose: Cerebral intraventricular infusion of acidic or basic fibroblast growth factor has been shown to attenuate ischemic damage to hippocampal CA1 neurons in the gerbil. The purpose of the present study was to determine if the basic fibroblast growth factor transgenic mouse has an enhanced ability to resist the effects of severe cerebral hypoxemia-oligemia.

Methods: Mice that were transgenic for bovine basic fibroblast growth factor were exposed to right carotid artery ligation, hyperglycemia, and 20 minutes of 1% carbon monoxide. After 5 days' recovery, brains were examined for histological damage.

Results: Counts of CA1 neurons in the right hippocampus showed a significantly higher number of neurons per millimeter CA1 in hypoxic-ischemic transgenic mice compared with nontransgenic controls (transgenic, 260±33; nontransgenic, 151±37 neurons per millimeter CA1; P<.05).

Conclusions: The results indicate that basic fibroblast growth factor transgenic mice, as judged by CA1 hippocampal neuronal survival, have an enhanced ability to resist the effects of a complex hypoxic-ischemic cerebral insult. (Stroke. 1993;24:1735-1739.)

KEY WORDS • growth factors • hypoxia • neuroprotection • mice

Addition of fibroblast growth factor (FGF) to cultures of dissociated hippocampal neurons reduces glutamate-induced increases of intracellular calcium and as a result increases the threshold for glutamate neurotoxicity.1 Because increases of extracellular glutamate and intracellular calcium are believed to be major factors in the pathogenesis of hypoxic-ischemic brain damage,2-4 this observation suggests that FGF should have a protective effect on hypoxic-ischemic brain. This proposal is supported by observations that intraventricular infusions of acidic FGF5,6 and basic FGF (bFGF) protect hippocampal CA1 neurons in the ischemic gerbil brain. In addition, Yamada et al8 have shown that repeated intracisternal injections of bFGF prevent thalamic degeneration after cortical infarction in the rat.

The availability of an animal species that has enhanced cerebral expression of basic FGF has provided an opportunity to directly test the hypothesis that FGFs have a protective action in cerebral ischemia. The model is a transgenic (TG) mouse incorporating a transgene consisting of a Rous sarcoma virus enhancer fused to bovine bFGF cDNA.9 This mouse has been demonstrated to express the transgene mRNA in brain, heart, and skeletal muscle, and increased immunoreactive bFGF is present by Western analysis (see “Results”).

The objective of the present study was to determine if the bFGF expressing TG mouse has an enhanced ability to resist the effects of severe cerebral hypoxemia-oligemia.

Materials and Methods

Transgenic Mice

The plasmid used for pronuclear injection was made using the pBC12/RSV/CAT plasmid10 containing the RSV-LTR promoter/enhancer, rat pre-proinsulin intronic sequences, and the SV40 polyadenylation signal. The HindIII-BamHI fragment containing the CAT gene was removed, and a 1.4-kb cDNA sequence coding for bovine bFGF9 was cloned into the EcoRI site. A linear Aat II–Stu I fragment of the resulting plasmid was injected into the pronuclei of fertilized one-cell C57/BL6 × CD-1 eggs, which were transferred to a pseudopregnant female and allowed to develop to term. Founder mice and subsequent offspring were genotyped by Southern analysis.

Analysis of Transgene Expression

RNA was isolated by the method of Chirgwin et al11 and analyzed for expression of the transgene by RNase
exposure sexes that bands binding mL of 2 0.1% CHAPS. Eluates 275 bp were electrophoresed and antibody monoclonal Reilly, Thomas diaminobenzidine. 323 bp were concentrated in microconcentrator tubes (Amicon Corp, Danvers, Mass), electrophoresed on 12% polyacrylamide/sodium dodecyl sulfate gels, and transferred to a nylon membrane. Western analysis was performed using a monoclonal antibody to human bFGF (a gift from Dr. Thomas Reilly, Du Pont-Merck, Inc.). Immunoreactive bands were detected using secondary antibodies conjugated to horseradish peroxidase and developed with diaminobenzidine.

**Hypoxemic-Ischemic Exposure**

The experiments were carried out on mice of both sexes that were TG for bovine bFGF. NTG littersmates of both sexes were used as controls. Animals were allowed free access to water and commercial mouse pellets. Before hypoxemic-ischemic exposure the mice were anesthetized with 2.5% halothane in 30% O2/70% N2O, and the right carotid artery was surgically exposed and double ligated with 5-0 silk. Two hours later the animals were intraperitoneally injected with 0.25 mL 25% dextrose in H2O and 0.5 hours later were placed in a plastic bell jar (0.01 m3), which was flushed with 1% carbon monoxide in air at a rate of 5 L/min. After 20 minutes of exposure the animals were returned to room air. At 5 days of recovery the animals were anesthetized with pentobarbital (150 mg/kg IP), and the brain was fixed in situ by intracardiac perfusion of 100 to 125 mL of 10% formaldehyde, which was preceded by a brief 10 to 15 mL 0.9% saline washout. The fixed brain was subsequently prepared for paraffin sections (6 μm), stained with cresyl violet, and assessed for histological damage by counting of intact CA1 neurons and measurement of area of cerebral infarction using a video image processing program. Sections for analysis were taken at −1.0, −1.5, and −2.0 mm to the bregma. The experimenter performing the tissue analysis was blinded as to genotype until the completion of the study.

The results were analyzed using Wilcoxon's ranked sum test for unpaired samples, with P<.05 considered statistically significant.

**Results**

**Transgene Expression**

The results of RNase protection analysis of tissues from a TG mouse are shown in Fig 1. The bovine bFGF transgene transcript is expressed in skeletal muscle, heart, and brain but not in liver or lung. No transgene expression was detectable in kidney, parotid, pancreas, intestine, or testes (data not shown). Transgene expression was examined at the protein level by Western analysis. As can be seen in Fig 2, increased immunoreactive protein is detected in brain and skeletal muscle but not in kidney.
**Clinical and Pathological Effects**

As a preliminary baseline, counts of CA1 hippocampal neurons were carried out on normoxic NTG and TG mice. These counts indicated that the number of neurons per millimeter of CA1 length was equivalent in the two animal groups (NTG, 293 ± 12; TG, 303 ± 14 cells per millimeter CA1 [mean ± SEM]; P = NS; n = 6 animals in each group; Table).

Nineteen NTG and 17 TG mice were exposed to the sequence of right carotid occlusion, glucose injection, and 1% carbon monoxide. Four of the NTG and 5 of the TG mice experienced acute cardiovascular collapse, pulmonary edema, respiratory failure, and death either at the terminal stages of the carbon monoxide exposure or during the first few hours of reoxygenation. These deaths were judged to be due to the well-documented cardiac toxicity of acute carbon monoxide poisoning. In surviving animals, areas of right cerebral infarction were present in 11 (73%) of 15 NTG and 6 (50%) of 12 TG mice. The mean area of infarction in the TG group was only 55% that of the NTG group; however, because of data scatter, the differences were not statistically significant (Table).

Assessment of CA1 hippocampal neuronal density indicated severely decreased counts (less than 50% of control) in 11 (73%) of 15 NTG and in 3 (25%) of 12 TG mice. All NTG animals that showed cerebral infarction also showed severe CA1 damage, whereas only 3 of the 6 infarcted TG animals showed CA1 damage. The overall CA1 counts reflected the above differences, and as a group the TG animals had a statistically significant larger number of surviving CA1 neurons (Table; Fig 3). Analysis was also carried out on CA1 neuronal density in male and female subgroups within each experimental exposure, and this indicated no statistically intragroup differences based on sex (ischemic NTG males, 139 ± 54; NTG females, 161 ± 49; ischemic TG males, 252 ± 54; TG females, 270 ± 28 cells per millimeter CA1 [mean ± SEM]; P = NS).

**Discussion**

Models of experimental hypoxia-ischemia in the mouse have not been as well characterized or widely used as those developed in the rat and gerbil. The present carbon monoxide–carotid artery occlusion–hyperglycemia model was patterned after the hypoxemia-ischemia model of Salford et al and by the observation that addition of hyperglycemia leads to a high incidence of damage in this model. Because of the limited supply of TG mice it was decided to use this high-incidence-of-injury model with full awareness that the severity of the insult could possibly obscure small but potentially significant trends for enhanced resistance in the test group. In addition, it must be indicated that the model involves a number of potentially independent damaging factors, such as the direct toxic effect of carbon monoxide, ischemia, and tissue acidosis, which could complicate interpretation of the results.

With regard to the above limitations, the results of the present study indicate that the CA1 hippocampus of the bFGF TG mouse shows increased resistance to damage caused by a complex hypoxemic-ischemic insult. This conclusion is supported by data showing fewer damaged hippocampi, plus the larger number of surviving CA1 neurons in comparison to NTG littermates. In addition to the enhanced CA1 neuronal survival, TG mice showed a trend for a smaller area of cerebral infarction. These results are consistent with earlier studies that have shown that addition of FGF to hippocampal neuronal cultures or infusion of FGF into the cerebral ventricle of gerbils enhances hippocampal...
resistance to killing by in vitro glutamate addition or by in vivo global cerebral ischemia, respectively.

A number of mechanisms or factors must be considered in the interpretation of the results. The first is the possibility that the bFGF TG mouse has enhanced extracellular mechanisms for resisting hypoxemia-ischemia. These could include a superior ability of TG mice to maintain myocardial function and systemic blood pressure; as a consequence, the cerebral circulation could be enhanced. This possibility is not strongly supported by the present findings of equivalent numbers of acute cardiogenic deaths in the NTG and TG groups. Another extracerebral mechanism could include differences in body temperature, with TG mice having lower body and brain temperature during hypoxic-ischemic exposure. This question could not be tested in the present free-moving model of hypoxemia-ischemia and will require future study.

The second group of protective mechanisms could involve adaptation at the cerebral tissue level. The earlier work of Mattson et al1 has indicated that FGF reduces the increase in neuronal calcium that is induced by glutamate. This study has also shown that this action of FGF is blocked by addition of protein and RNA synthesis inhibitors, which suggests that FGF acts by induction of a protein product that protects the neuron from the actions of glutamate and/or calcium. The brain of bFGF TG mice may thus have increased contents or enhanced ability to produce putative neuroprotective proteins, such as those involved in the binding and transport of calcium,18 the heat shock response,19 or the protein ubiquitin cycle.20 In addition, because FGF is also known to be trophic to a variety of nonneuronal cells,21 it is possible that its neuroprotective action is mediated by glial or endothelial cells. This possibility has some support from the observations that the protective action of glia against glutamate excitotoxicity on cultured neurons is mediated in part by FGF.22 In addition, because FGF is a potent angiogenic factor,21 protection may result from increased brain perfusion, which limits ischemia.

The final possibility to consider is that the process of hypoxic-ischemia cerebral damage is only delayed in the bFGF TG mouse and that with longer survival times the end results in NTG and TG animals would be equivalent. The 5-day recovery period used in the present study may thus be within this “delay” period. Future studies will address this possibility.

In summary, the results indicate that TG mice that overexpress bFGF in the brain have an enhanced ability to resist the effects of a complex hypoxic-ischemic insult, as judged by hippocampal CA1 neuronal survival. These results are complementary to previous studies that have indicated that intraventricular infusions of FGF are neuroprotective in cerebral ischemia. The availability of this strain of TG mouse will allow for future studies aimed at defining the mechanisms responsible for this neuroprotective action.

References
8. Yamada K, Kinoshta A, Kohmura E, Sakaguchi T, Taguchi J, Kataoka K. Basic fibroblast growth factor prevents thalamic degen-
Basic fibroblast growth factor (bFGF), a potent endothelial cell mitogen that also exhibits both neurotrophic and gliotrophic activities, has been found to protect against excitatory amino acid–induced neurotoxicity in culture1–2 and to protect against N-methyl-D-aspartate neurotoxicity in neonatal rats.3 These observations, in conjunction with the increased levels of bFGF immunoreactivity at the site of focal brain injury,4 suggest that elevated levels of bFGF may play an important role in protecting against ischemic brain injury. With the successful construction of transgenic (TG) mice that express bovine bFGF mRNA and bFGF immunoreactivity in brain and in a few other organs, MacMillan and colleagues have now demonstrated that these TG mice show increased resistance to hypoxic-ischemic cerebral damage.

A couple of important findings are noted from this unique study: (1) TG mice are useful animal models for ascertaining the role of a particular factor in question in ischemic brain injury. The successful use of TG mice overexpressing human CuZn superoxide dismutase (SOD-1) in cerebral ischemia5 and in cold trauma6 clearly supports this notion. (2) Although the cellular origin and distribution (ie, neurons, glia, endothelia) of bFGF transgene expression have not been studied, this study nevertheless has shown that increased brain levels of bFGF provide some neuronal protection against hypoxic-ischemic damage. Future studies of pharmacological and therapeutic efficacy with bFGF in ischemic brain injury are warranted.

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