Fibroblast growth factor (FGF) has been shown to protect tissue damage in animal models of cerebral and myocardial ischemia. The cellular and molecular mechanisms of FGF effects have not been fully defined. In the present study, we have investigated the effect of FGF homologs on nitric oxide (NO)-mediated neuronal cell death. Addition of NO donor S-nitroso-N-acetylpenicillamine (SNAP) to cultures of human neuroblastoma SHSY-5Y cells resulted in a concentration-dependent cell death. TdT-mediated dUTP-X nick end labeling and oligonucleosome assays confirmed that NO-mediated cell death occurred through the apoptotic pathway. In the presence of 150 μM SNAP, about 40% of the cells in culture underwent apoptosis. Treatment with FGF-2 resulted in greater than 80% reduction in NO-induced cell death. FGF addition to cell cultures also enhanced cell survival without affecting cell proliferation. FGF-2 effectively inhibited NO-mediated apoptosis even when added 6 h after treatment with SNAP. Examination of other homologs of FGF on NO-mediated cell death showed that in SHSY-5Y cells, FGF-2 and FGF-4, but not other FGF homologs, inhibited NO-mediated apoptosis. These results show that FGF-2 was a potent cell survival factor and protected SHSY-5Y cells from NO-mediated apoptosis. These effects were limited to FGF-2 and FGF-4 homologs.

Nitric oxide (NO) plays an important role in neuronal cell death during cerebral ischemia. It has been demonstrated that cortical NO levels increase severalfold after middle cerebral artery occlusion (MCAO) (Malinski et al., 1993) and a significantly higher level of nitric-oxide synthase (NOS) activity is sustained over an extended period (Samdani et al., 1997). The initial burst of NO generation is apparently mediated by the constitutively expressed neuronal nitric-oxide synthase (nNOS) (Kader et al., 1993; Malinski et al., 1993). This calcium-dependent isoform of NOS is stimulated in response to N-methyl-D-aspartate (NMDA) receptor ion channel activation (Dawson et al., 1993). Subsequent and sustained NO production during ischemic injury is apparently attributed to an increased expression of nNOS (Gotth et al., 1996) and induction of inducible NOS gene (Iadecola et al., 1995). NO and its oxidative metabolites, e.g., peroxinitrite have been implicated in the initiation and promotion of neuronal cell death and ischemic brain injury (Eliasson et al., 1999). Inhibition of NO synthesis by NOS inhibitors, e.g., nitro-L-arginine methylster (Coert et al., 1999), l-nitroarginine, and 7-nitroindazole significantly reduces infarct size in various models of cerebral ischemia (Yoshida et al., 1994). In nNOS gene knockout mice wherein NOS activity was reduced to less than 5% of normal, the size of cerebral infarct in response to MCAO was significantly reduced (Hara et al., 1996). Similarly, the cerebral lesions in response to NMDA microinjection were 45% smaller in nNOS knockout mice compared with the wild type (Huang et al., 1994). Cortical cultures derived from the nNOS knockout mice were resistant to NMDA-mediated cytotoxicity (Dawson et al., 1996). These data strongly support the role of NO in neuronal cell damage in vitro and in vivo.

Basic fibroblast growth factor (bFGF) also designated as FGF-2 was initially discovered as a potent stimulator of fibroblast, smooth muscle, mesenchymal, and endothelial cell proliferation (Gospodarowicz et al., 1974). Subsequent studies have shown that FGF also acts as a trophic factor for neuronal cells, promoting cell survival (Finklestein et al., 1993), growth, and differentiation in vitro (Gurney et al., 1992). In human brain, FGF-2 receptors are predominantly expressed in the central nervous system neurons and in cerebellar Purkinje cells (Cordon-Cordo et al., 1990). Expression of FGF-2 is significantly increased during neuronal injury (Kiyota et al., 1991). Similarly, the expression of FGF
receptors is enhanced after cerebral ischemia (Kiyota et al., 1991). Recent studies have shown that FGF-2 reduces infarct size in experimental models of cerebral ischemia. Intravenous infusion of FGF-2, at the onset of reperfusion after a 3-h ischemia in rats, produced a significant reduction in infarct size and improvement in neurological performance (Koketsu et al., 1994; Fisher et al., 1995; Jiang et al., 1996; Ren and Finklestein, 1997). Similar reduction in infarction was obtained in a model of permanent MCAO in rats (Tanaka et al., 1995), cats (Bethel et al., 1997), and mice (Huang et al., 1997). FGF-2 was also found to reduce neuronal injury in response to microinjection of NMDA or ischemic insult in neonatal rats (Nozaki et al., 1993) and neuronal cell death in global model of ischemia in gerbil (Nakata et al., 1993). The acute effects of FGF in the models of cerebral ischemia suggest that FGF may directly or indirectly affect mechanisms involved in ischemic neuronal damage. The molecular mechanism of neuroprotective action of FGF-2, however, is not understood. Because NO plays an important role in neuronal damage after cerebral ischemia, we have studied the effect of FGF on NO-mediated cell death. Human neuroblastoma SH-SY-5Y cells were used for the investigation of the effect of FGF family of growth factors on NO-mediated cell death. These cells undergo apoptosis in response to NO and exhibit various activities of apoptosis pathway, including the expression of bcl-2, bax, caspases, and activation of poly(ADP-ribose) polymerase. Our results show that NO donors produce a dose-dependent death of SH-SY-5Y cells. Cell death induced by NO was apoptotic in nature. Treatment with FGF-2 offered significant protection from NO-mediated apoptosis. FGF-2-mediated protection was observed even when its addition was delayed for 6 h after treatment with SNAP. Among the members of FGF family tested, FGF-2 was the most effective protector of NO-mediated cell death.

**Materials and Methods**

**Cell Culture**

Human neuroblastoma SH-SY-5Y cells (American Type Culture Collection, Manassas, VA) were initiated at passage 21 and maintained in a 1:1 mixture of Eagle’s minimum essential medium and Ham’s F-12 medium (prepared in endotoxin-free water) containing 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO2. Cells were passaged by trypsinization at 60% confluence and used up to passage 31. No discernable changes were observed either in the morphology or response to FGF during the 10 passages.

**Fibroblast Growth Factors**

Human recombinant FGF-2 was purchased from Upstate Biotechnology (Lake Placid, NY) Other FGF homologs were obtained form R&D Systems (Minneapolis, MN).

**Measurement of Cell Viability**

Cell viability was measured using two different methods as follows.

**Cell Counting.** Cells were seeded in 48-well plates at a density of 120,000 cells/well in a 1:1 mixture of Eagle’s minimum essential medium and Ham’s F-12 medium containing 10% FBS and indicated concentrations of FGF. After 1 h, an indicated amount of SNAP was added and cells were further incubated for 22 h. Effect of SNAP on cell number was determined after removal of cells floating in the medium and then counting the attached cells after trypsinization.

**FGF-2**

![Fig. 1. Effect of FGF-2 on NO-mediated cell death. SHSY-5Y cells were plated in 48-well plates (120,000 cells/well) in the absence (○) or presence (■) of 10 ng/ml FGF-2. After 1 h, indicated concentrations of SNAP were added. The cells were then incubated for 24 h. Surviving cells were trypsinized and counted. Values are mean ± S.E.M. of triplicates. *P < .005.](image1)

![Fig. 2. Concentration-dependent inhibition of SHSY-5Y cell death by FGF-2. Cells (20,000 cells/well) were plated in 96-well microtiter plate in the presence of indicated concentrations of FGF-2. SNAP (300 μM) was then added and the cells were further incubated for 22 h. Cell survival was determined using XTT assay. Values are mean ± S.E.M. of triplicates. *P < .005.](image2)
Determination of Apoptosis

Apoptotic cell death is distinguished from necrotic cell death by nuclear damage, resulting in DNA cleavage into oligonucleosomes by endogenous endonucleases. The characteristic fragmentation of chromatin into nucleosome of about 180 base pairs, produced during apoptosis, can be assayed by either enzymatic labeling of DNA strands (terminal deoxynucleotidyl transferase biotin-medi- ated dUTP nick end labeling, TUNEL) (Chapman et al., 1995) or by quantifying the oligonucleosomes generated using histone specific antibody (Allen et al., 1997). We have used both these methods to demonstrate NO-induced apoptosis in SHSY-5Y cells. For TUNEL assay, cells were cultured and treated with SNAP and FGF-2 as described in the XTT assay. After 24 h, cells were fixed with zinc- formaldehyde for 30 min and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, followed by a 60-min incubation with TUNEL labeling mixture (Boehringer Mannheim). Cells were washed with PBS and the labeled DNA fragments were observed by fluorescence microscopy.

For oligonucleosome measurement, cells were cultured as in the XTT assay. After 24-h incubation with 300 μM SNAP in the presence or absence of 10 ng/ml FGF-2, oligonucleosomes formed were measured using the Oligonucleosome assay kit from Boehringer Mannheim. Absorbance was measured at 450 nm.

Determination of DNA Synthesis

SHSY-5Y cells were cultured in 96-well plates at a density of 8000 cells/well. After 48 h, cells were treated with the indicated concentrations of FGF-2 and 1 μCi of [3H]thymidine. After 24 h, cells were fixed with methanol and counting radioactivity using a scintillation counter. The data are normalized for [3H]thymidine incorporation per 8000 cells.

**Statistical Analysis**

The results are expressed as the mean ± S.E.M. Significance level was evaluated by one-tailed Student’s t test and tested at P values specified in the figure legends.

**Results**

**Effect of NO and FGF on Cell Survival.** Effect of NO on the survival of SHSY-5Y neuroblastoma cells was determined by culturing cells in 48-well plates and then treating with varying concentrations of SNAP (0–600 μM) in the absence or presence of 10 ng/ml FGF-2. After 24 h, cell survival was determined by cell counting using a Coulter counter. Figure 1 shows that treatment of SHSY-5Y cells with SNAP produced a concentration-dependent decrease in cell number. In the presence of 10 ng/ml FGF-2, loss of cells was greatly reduced. For example, in the presence of 100 μM SNAP, cell number in culture was reduced by 28 ± 7.3%. Addition of FGF-2 produced complete protection from NO-mediated cell death. At higher concentrations of SNAP where cell death was more than 70%, protection by FGF-2 was less effective. Figure 1 also shows that the total number of cells in the FGF-2-treated cultures (0 μM SNAP) were significantly higher than the control cultures, suggesting that FGF-2 may have either increased cell proliferation or enhanced survival of SHSY-5Y cells.

The effect of FGF-2 on NO-mediated cell death was also determined by XTT assay that detected conversion of tetrazolium salts to formazan dye by metabolically active cells. Figure 2 shows that in the presence of 300 μM SNAP, cell survival in culture was reduced by 42 ± 4.2%. Addition of FGF-2 before SNAP treatment resulted in more than 90% reduction in cell death. Maximal effect of FGF was observed at 3 ng/ml. Taken together, the results in Figs. 1 and 2 demonstrate that FGF-2 reduced NO-mediated cytotoxicity in SHSY-5Y cells.

**Effect of FGF-2 on Survival and Proliferation of SHSY-5Y Cells.** An increase in cell number observed in the presence of FGF-2 (Fig. 1, 0 μM SNAP) could have been due
to a stimulation of cell proliferation and/or reduction in cell death. These effects of FGF-2 were further investigated by examining its effect on DNA synthesis in SHSY-5Y cells. Figure 3A shows that inclusion of FGF-2 in cell culture produced a concentration-dependent increase in cell number over a 72-h incubation period. In the presence of optimum concentration of FGF (10 ng/ml), the increase in cell number was about 2-fold over the control cultures. To determine whether the increase in cell number was due to a mitogenic effect of FGF-2, the effect of FGF on DNA synthesis was determined after the cells were plated for 48 h. Figure 3B shows that treatment of cells with FGF-2 did not significantly alter DNA synthesis. Under similar experimental conditions, FGF-2 produced a 3- to 4-fold increase in DNA synthesis in endothelial cells (data not shown). These data suggest that FGF-2 acts as a survival factor, and also protects SHSY-5Y cells from NO-mediated cell death.

NO Mediates Apoptosis in SHSY-5Y Cells. To determine whether the cell death observed in response to SNAP (as determined by XTT and cell number) was apoptotic in nature, a TUNEL assay on SNAP and FGF-2-treated cells was performed. Figure 4 shows that in control cultures only a few cells were TUNEL positive. Treatment with 300 μM SNAP resulted in 58 TUNEL-positive cells per field. In the presence of 10 ng/ml FGF-2, the TUNEL-positive cells in SNAP-treated cultures were reduced to six per field. These results show that NO-mediated cell death was predominantly due to apoptosis. The TUNEL assay further confirmed the protective effect of FGF-2.

That NO-mediated cell death was, predominantly, due to apoptosis was also confirmed by DNA fragmentation and oligosome generation as measured by an enzyme-linked immunosorbent assay. Treatment of SHSY-5Y cells with 150 μM SNAP produced a 3.5-fold increase in oligosome generation. Figure 5 shows that FGF-2 produced a concentration-dependent reduction in oligosome generation. These data further support that NO-mediated cell death was predominantly due to apoptosis and that FGF-2 was a potent protector of NO-mediated apoptosis in neuronal SHSY-5Y cells.

Delayed Addition of FGF Induces Cytoprotection. In the above-mentioned studies, FGF-2 was added to the cells 1 h before the treatment with SNAP. To determine whether FGF-2 affected early or late events of NO-mediated apoptosis, FGF was added at various times after treatment with SNAP. Cell survival was then determined using XTT assay. As shown in Fig. 6, FGF-2 inhibited NO-mediated cell death

Fig. 4. Effect of FGF-2 on NO-mediated apoptosis. Apoptosis in SHSY-5Y cells in the absence (A) or presence (B) of 300 μM SNAP, or SNAP + 10 ng/ml FGF-2 (C) was determined using TUNEL assay. Cell nuclei exhibiting fluorescence in the field of view shown here are counted as apoptotic cells. A phase contrast image of control cells (D) indicates that the lack of fluorescence is not simply due to the absence of cells in the plates. The phase contrast images of other cultures (A–C) were similar to that shown in D.
even when its addition was delayed up to 6 h. These results suggest that the site of FGF-2 action may reside at events that occur 6 h after NO treatment.

Effect of FGF Homologs on NO-Mediated Cell Death. Figure 7 shows the effect of different homologs of FGF on NO-mediated cytotoxicity in SHSY-5Y cells. Cells were plated in the absence or presence of 10 ng/ml of the indicated growth factor. SNAP (300 μM) was then added and the cultures were incubated for 24 h. Cell survival was determined by XTT assay. As shown here, FGF-2 produced the highest effect on NO-mediated cell death. Significant effect was also observed with FGF-4. Other homologs of FGF neither enhanced cell survival nor protected from NO-mediated cytotoxicity.

Discussion

In experimental models of myocardial and cerebral ischemia, treatment with FGF family of polypeptides significantly reduces infarct size (Koketsu et al., 1994; Fisher et al., 1995; Jiang et al., 1996; Ren and Finklestein, 1997). FGF induces hypotension and increases cerebral blood flow (Regli et al., 1994). A part of the acute effect of FGF on infarction could be attributed to its vasodilatory activity. FGF treatment also supports the survival of neurons in central nervous system (Cuevas et al., 1991). Other studies suggest that FGF protects neuronal cell survival in vitro (Finklestein et al., 1993). Thus, the mechanism of the neuroprotective action of FGF is not fully understood. A variety of studies have suggested that NMDA receptor activation and production of nitric oxide play an important role in ischemia-induced cerebral neuronal cell death (Garthwaite et al., 1989). The aim of the present study was to determine whether FGF-2 treatment offered protection of neuronal cells from NO-mediated apoptosis. Our results show that in cultures of human neuroblastoma SHSY-5Y cells, NO donor SNAP produced a dose-dependent reduction in viable cells. Addition of FGF-2 to these cultures produced a dramatic reversal of cell death. Protection from NO-mediated cell death was demonstrated by counting actual number of viable cells as well as by XTT assay. Using TUNEL and oligosome assays, we have demonstrated that at lower concentrations of SNAP (50–150 μM) NO-induced cell death predominantly occurred via the apoptotic pathway. At these concentrations of SNAP, FGF treatment effectively reduced cell death. At high concentrations of SNAP, where cell death probably occurs through a combination of apoptosis and necrosis, the protective action of FGF-2 was significantly reduced. These results suggest that the effect of FGF-2 may be on the mechanisms of apoptosis and not simply on the generation or degradation of NO. We also found that in steady-state cultures of SHSY-5Y cells, addition of FGF-2 produced a significant increase in cell number over time. FGF-2 treatment did not significantly enhance DNA synthesis in SHSY-5Y cells, suggesting that the increase in cell number was due to an increased cell survival and not due to an increase in cell proliferation.

Our results show that FGF-2 was effective in antagonizing NO-mediated apoptosis even when added 6 h after NO treatment. These data concur with the in vivo studies wherein the infarct size was significantly reduced when FGF treatment was initiated 2 h after the onset of ischemia. These data suggest that FGF-2 affects late-stage events in the apoptosis pathway. The in vivo (Fisher et al., 1995; Jiang et al., 1996; Ren and Finklestein, 1997) and in vitro data suggest that FGF-2 therapy may offer a wider time window for the treatment of stroke patients.

The FGF gene family consists of at least 19 different genes with varying sequence homology (Ohyabashi et al., 1998; Xie et al., 1999). FGF genes are expressed in a tissue-selective...
manner (Cordon-Corde et al., 1990). To determine whether, neuroprotection was a common feature of all members of FGF gene family or a selective property of FGF-2, we tested the effect of homologs on NO-mediated cell death. Our results suggest that a significant protection was achieved only by FGF-2 and FGF-4. The selective effect of FGF-2 and FGF-4 on cell survival observed here is likely due to the nature of the FGF receptors expressed by SH-SY5Y cells. FGF receptors are encoded by four different genes (Johnson and Williams, 1993). The different FGF gene products exhibit varying degree of affinity for these receptors. Furthermore, the existence of splice variants of FGF receptors with varying ligand-binding properties could lead a cell selective action of FGF homologs (Werner et al., 1992). Based on the known receptor binding profile of FGF homologs, our data suggest that the effect of FGF-2 in SH-SY5Y cells is mediated through FGF receptor 1.

There has been a significant interest in understanding the mechanism of neuroprotection by peptide growth factors. In neuronal and non-neuronal cells, NO has been shown to affect several key steps of the apoptosis pathway. For example, NO has been shown to increase caspase 3 (Uehara et al., 1991), down-regulate bcl-2 (Tamataki et al., 1998), and increase cellular accumulation of p53 (Kitamura et al., 1998; Glockzin et al., 1999). Recent studies have also shown that NO inhibits proteosome activity that may play role in the inhibition of p53 and bax degradation (Glockzin et al., 1999). Because FGF has been shown to induce bcl-2 expression in hippocampal neurons (Tamataki et al., 1998), it may be that the expression of bcl-2 is an important step in protecting against NO-induced death in SH-SY5Y cells. Overexpression of bcl-2 gene has been shown to reduce apoptosis in rat sympathetic neuronal cultures deprived of neurotrophic factors (Garcia et al., 1992). In bcl-2 transgenic mice, neuronal cells are protected from ischemia-induced injury (Martinou et al., 1994). Our data indicating that neuroprotection by FGF is observed even up to 6 h after SNAP treatment are consistent with the involvement of late event such as bcl-2 expression. In hippocampal neurons, a significant decline in NO-induced bcl-2 occurs at 6 to 8 h after NO treatment (Tamataki et al., 1998). Thus, although the molecular target for FGF action in NO-induced neuroprotection remains to be understood, our studies suggest that SH-SY5Y cells could serve a useful model for delineating the FGF-mediated signaling pathway for inhibition of apoptosis.

References


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