Basic Fibroblast Growth Factor Sensitizes NIH 3T3 Cells to Apoptosis Induced by Cisplatin

AARON B. COLEMAN, JAMIL MOMAND,[1] and SUSAN E. KANE

Department of Cell and Tumor Biology, City of Hope National Medical Center, Duarte, California

This paper is available online at http://www.molpharm.org

ABSTRACT

One mechanism by which chemotherapeutic agents kill tumor cells is by induction of apoptosis. Basic fibroblast growth factor (bFGF/FGF-2) has been reported to inhibit apoptosis in NIH 3T3 cells treated with chemotherapy drugs. We have investigated how bFGF modulates apoptosis induced by cisplatin in NIH 3T3 cells. Treatment with 10 μg/ml cisplatin for 12 h induced apoptosis in 2 to 13% of the cells at 24 h post-treatment. Preincubation with 10 ng/ml bFGF for 24 h led to cisplatin-induced apoptosis in 20% to 50% of the cells. Preincubation with lower concentrations of bFGF (0.1–1 ng/ml) or simultaneous addition of bFGF and cisplatin had no effect on the amount of apoptosis. Pretreatment with bFGF also significantly decreased the dose-dependent survival of NIH 3T3 cells exposed to cisplatin, as determined by colony formation. Cells treated with 10 ng/ml bFGF showed a distinct morphology, appearing smaller and more refractile, before cisplatin exposure. The enhancement of cisplatin-induced apoptosis and the morphology shift demonstrated the same dose response to bFGF, and both effects were reversible if bFGF was removed from the medium for 24 h before cisplatin treatment. Mitogenic response to bFGF by NIH 3T3 cells saturated at 0.5 ng/ml, as measured by ^{3}H-thymidine uptake, and this response was blocked by coaddition of suramin, an inhibitor of FGF ligand-receptor interactions. Suramin did not reverse the enhancement of cisplatin-induced apoptosis by bFGF. Therefore, bFGF sensitized NIH 3T3 cells to cisplatin, and this effect might be mediated through a pathway separate from that used for mitogenic signaling.

Chemotherapeutic agents can elicit a number of cellular responses including growth arrest and activation of apoptosis, or programmed cell death. Apoptosis is initiated through a complex signal transduction network that is only partially understood. Many cellular factors can alter the response of the cell to chemotherapeutic agents by modulating this response-signaling pathway. Signals from the extracellular environment may also be important in regulating an apoptotic response, as certain growth factors and cytokines can down-regulate the apoptotic response to chemotherapy drugs and decrease the sensitivity of cells to these agents (Borsellino et al., 1995; Grothey et al., 1999).

Basic fibroblast growth factor (bFGF/FGF-2) belongs to a family of pleiotropic cytokines that function in the normal physiology and pathology of many tissues (Szebenyi and Fallon, 1999). bFGF is also a potent angiogenic factor in that it acts as both a mitogen and chemoattractant for endothelial cells (Gospodarowicz et al., 1979). Extracellular bFGF binds to high-affinity and low-affinity sites on the cell surface. A family of four receptor-tyrosine kinases comprise the high-affinity binding sites for bFGF as well as other members of the FGF family (Johnson and Williams, 1993). Each of the four receptors has multiple splice variants, which have different extracellular domains and ligand binding specificities. bFGF also binds to heparan sulfate-containing proteoglycans with lower affinity, and interaction with these sites is thought to be necessary for binding and activation of the high-affinity receptors (Spivak-Kroizman et al., 1994). Binding of bFGF to high-affinity receptors leads to intracellular propagation of a mitogenic signal through activation of phospholipase C and the *ras-raf*-MAP kinase pathway (Mohammadi et al., 1991; Kouhara et al., 1997). For neural cells and smooth muscle cells, bFGF is a survival factor and it is necessary for the maintenance of these cells in culture. bFGF mediates this effect by inhibiting apoptosis (Fox and Shanley, 1996; Ohgoh et al., 1998).

Because of its ability to act as a survival factor in some differentiated cells, bFGF recently has been investigated as a factor that might rescue cells from apoptosis induced by chemotherapy drugs and DNA-damaging agents. Elevated levels of intracellular bFGF correlate with resistance to flu-

[1] Current address: Department of Chemistry and Biochemistry, California State University, Los Angeles.

ABBREVIATIONS: bFGF, basic fibroblast growth factor; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling; PI, propidium iodide; HSPG, heparan sulfate proteoglycan.

darabine in chronic lymphocytic leukemia (Menzel et al., 1996). Furthermore, overexpression of a bFGF cDNA in immortal mouse embryo fibroblasts (NIH 3T3) can result in resistance to a variety of agents, including etoposide, 5-fluorouracil, and N-(phosphonacetyl)-L-aspartate (Huang et al., 1994; Wieder et al., 1997). Expression in NIH 3T3 cells of a chimeric bFGF containing a signal peptide for secretion results in constitutive activation of FGF receptors through an autocrine loop and blocks apoptosis induced by treatment with cisplatin (Shaulian et al., 1997). Overexpression of bFGF is also associated with resistance to cisplatin in a human bladder cancer cell line (Miyake et al., 1998). Moreover, the addition of exogenous bFGF to endothelial cells inhibits apoptosis induced by DNA damage from ionizing radiation, both in vitro and in mice (Fuks et al., 1994). On the other hand, recent evidence suggests that both overexpressed and exogenous bFGF can enhance apoptosis in MCF7 breast tumor cells exposed to cisplatin, etoposide, or 5-fluorouracil (Wang et al., 1998; Fenig et al., 1999).

To understand how bFGF might affect the response to chemotherapeutic agents, it is necessary to elucidate the signal transduction pathways involved. We have begun this analysis in NIH 3T3 cells by studying the effect of exogenous bFGF on induction of apoptosis by cisplatin, a DNA cross-linking agent used in the treatment of many cancers. The cellular factors that render cells more sensitive or resistant to cisplatin are not entirely known, but probably include p53 status (Hawkins et al., 1996) and nucleotide excision repair machinery (Fan et al., 1995), and might also include survival factors such as bFGF (Shaulian et al., 1997). However, we found that cells pretreated with bFGF were more sensitive to cisplatin-induced apoptosis compared with cells not treated with bFGF. This effect was time-dependent and reversible, and required higher concentrations of bFGF than those needed to stimulate DNA synthesis in NIH 3T3 cells. Finally, the effect of bFGF on apoptosis was not reversed by suramin, a relatively nonspecific inhibitor of FGF receptor-mediated signaling. Therefore, bFGF appeared to enhance cisplatin-induced apoptosis in NIH 3T3 cells by a mechanism that might not be mediated by classical high-affinity FGF receptor pathways.

Materials and Methods

Cell Culture and Cell Proliferation Assays. NIH 3T3 cells were obtained from Dr. M. Gottesman (National Cancer Institute, Bethesda, MD) and were routinely carried in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Recombinant human bFGF was purchased from Promega (Madison, WI), and stock solutions were prepared in PBS with 0.5% BSA. Proliferation assays were initiated by seeding 2.5×10^4 cells into 6-cm plates in standard growth medium. The next day (day 0), the plates were refed with medium containing bFGF (1 or 10 ng/ml) or the PBS carrier only. The number of cells in triplicate plates was determined on days 0, 2, 3, and 4 by trypsinization and counting on a Coulter Counter.

For measurements of ^{3}H-thymidine incorporation, 1×10^4 NIH 3T3 cells were plated into each well of 24-well plates in standard growth medium and allowed to attach overnight.

The cells then were rinsed once with PBS, and rendered quiescent by incubation in DMEM with 0.1% CS for 24 h. The medium was replaced with DMEM + 0.1% CS with or without varying concentrations of bFGF, and the cells were incubated for another 24 h. ^{3}H-thymidine (5 μCi/ml, 70–90 Ci/mmol; Amersham, Buckinghamshire, England) was added during the last 4 h. The cells then were trypsinized and harvested onto glass fiber filters (#30; Schleicher and Schuell, Keene, NH), followed by washing with deionized water and drying with ethanol. The amount of radioactivity retained on each filter was measured by scintillation counting. In experiments including suramin, 100 μM suramin (Sigma Chemical Co., St. Louis, MO) was added to quiescent cells simultaneously with bFGF.

Apoptosis Assays. Cisplatin (Sigma) stock solutions were prepared in saline at 1 mg/ml and were stored at room temperature protected from light. Cells (6×10^5) were seeded in 10-cm plates and allowed to attach overnight. The next day, the cells were refed with standard growth medium containing bFGF or PBS carrier only and incubated for 24 h. The cells then were exposed to 10 μg/ml cisplatin in standard growth medium ($\pm$bFGF) for 12 h, after which the cisplatin medium was removed and replaced with fresh medium without cisplatin, maintaining the presence or absence of bFGF. When suramin was used, it was added simultaneously with bFGF and was maintained whenever bFGF was present. Cells were harvested either 18 or 24 h after the end of the cisplatin treatment. Floating and adherent cells were collected from duplicate plates for each treatment (adherent cells were trypsinized) and pelleted together. The cell pellet was resuspended in 0.5 ml of growth medium and added dropwise to 5 ml of cold 1% paraformaldehyde and incubated for 15 min on ice. The cells then were washed once with PBS, resuspended in 5 ml of 70% ethanol, and stored at −20°C for 3 to 5 days before TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling) analysis. A single plate of control cells not treated with cisplatin was run in parallel with cisplatin-treated cells in every experiment and was harvested on the second day after plating, before the cells became confluent.

For the TUNEL assay, fixed cells were pelleted from the ethanol, washed twice with Hanks balanced salt solution containing 0.1% BSA (HBSS-BSA), and transferred to a 1.5-ml microfuge tube. The cells were resuspended in a reaction mix containing 20 units terminal transferase (Boehringer Mannheim, Indianapolis, IN) and 20 μM biotin-16-dUTP (Boehringer Mannheim) in a final volume of 50 μl, and incubated at 37°C for 30 min. After washing once with HBSS-BSA, the cells were resuspended with 100 μl of 2.5 μg/ml avidin-fluorescein isothiocyanate (Sigma), in 4× SSC (0.6 M sodium chloride, 60 mM sodium citrate) containing 0.1% triton X-100 and 5% nonfat dried milk, and incubated at room temperature for 30 min. The cells then were washed once with HBSS containing 0.1% triton X-100 and resuspended in 0.5 to 1 ml of 5 μg/ml propidium iodide (PI) and 50 units of ribonuclease A (DNase free, Sigma) in PBS.

The samples were run and analyzed on a MoFlo (Cytomation, Fort Collins, CO) flow cytometer. Data was acquired by using dual laser excitation. Scatter signals were acquired with a HeNe laser (Spectra-Physics, Mountain View, CA). All fluorescence excitation was done at 488 nm from an Innova-90 Argon laser (Coherent, Santa Clara, CA) at 500

TABLE 1

Preincubation with bFGF increases the percentage of cells undergoing cisplatin-induced apoptosis

Apoptosis was determined by flow cytometric analysis of cells stained by TUNEL assay and PI incorporation as shown in Fig. 1. Values are the percentage of cells that stained TUNEL positive or with a sub-G_1 DNA content. The sum of these two populations was used to determine the final percentage of apoptotic cells.

Treatment[a]	TUNEL$^+$	Sub-G_1	Apoptosis
Experiment 1[b]			
No treatment	0.8	0.6	1.4
Cisplatin	1.7	2.9	4.5
Cisplatin + bFGF	15.7	27.0	42.7
Experiment 2[b]			
No treatment	0.5	0.3	0.7
bFGF	0.1	0.3	0.4
Cisplatin	1.3	0.8	2.0
Cisplatin + bFGF	8.2	13.2	21.4
Cisplatin + bFGF Simultaneous	1.6	0.7	2.2

[a] Cells were treated with standard growth medium alone (no treatment) or were exposed to 10 μg/ml cisplatin $\pm$ pretreatment with bFGF, as described in *Materials and Methods*.
[b] Experiment 1 is the quantitation of apoptotic cells from the experiment illustrated in Fig. 1. Experiment 2 shows data from a separate experiment in which cells were pretreated with 20 ng/ml bFGF. In the bottom row, cells were not pretreated with bFGF, but bFGF was added simultaneously with cisplatin and maintained until the cells were harvested.

mW. Fluorescein isothiocyanate emission was measured through a 530DF30 filter (Omega Optical, Brattleboro, VT). Cell cycle was determined by using PI, with emission mea-

sured through a 640EFLP filter (Omega Optical, Brattleboro, VT). The two fluorescent signals were separated with a 580DRLP dichoric filter. Data was acquired and analyzed with the Summit program (Cytomation). Raw signal data was gated from the PI fluorescence versus integrated-PI fluorescence scatter plot to exclude doublets and small debris.

Colony Formation. A total of 1×10^5 cells were seeded in 10-cm plates in standard growth medium and were allowed to attach overnight. The cells were pretreated with 10 ng/ml bFGF or PBS carrier only for 24 h and then were exposed to different concentrations of cisplatin ($\pm$bFGF) for 12 h. The cisplatin was washed away and the cells from each treatment were trypsinized and replated into triplicate 6-cm plates at 450 cells per plate. The plates were incubated for 7 days in the maintained presence or absence of bFGF and then were stained with 0.5% methylene blue in 50% ethanol. Prism Graph Pad software (version 2.0) was used to determine IC_{50} values.

Results

Pretreatment with bFGF Enhances Cisplatin-Induced Apoptosis. We investigated whether bFGF might affect cisplatin-induced apoptosis in NIH 3T3 cells. Cells were incubated for 24 h in the presence or absence of bFGF, then treated with 10 μg/ml cisplatin for 12 h, and finally
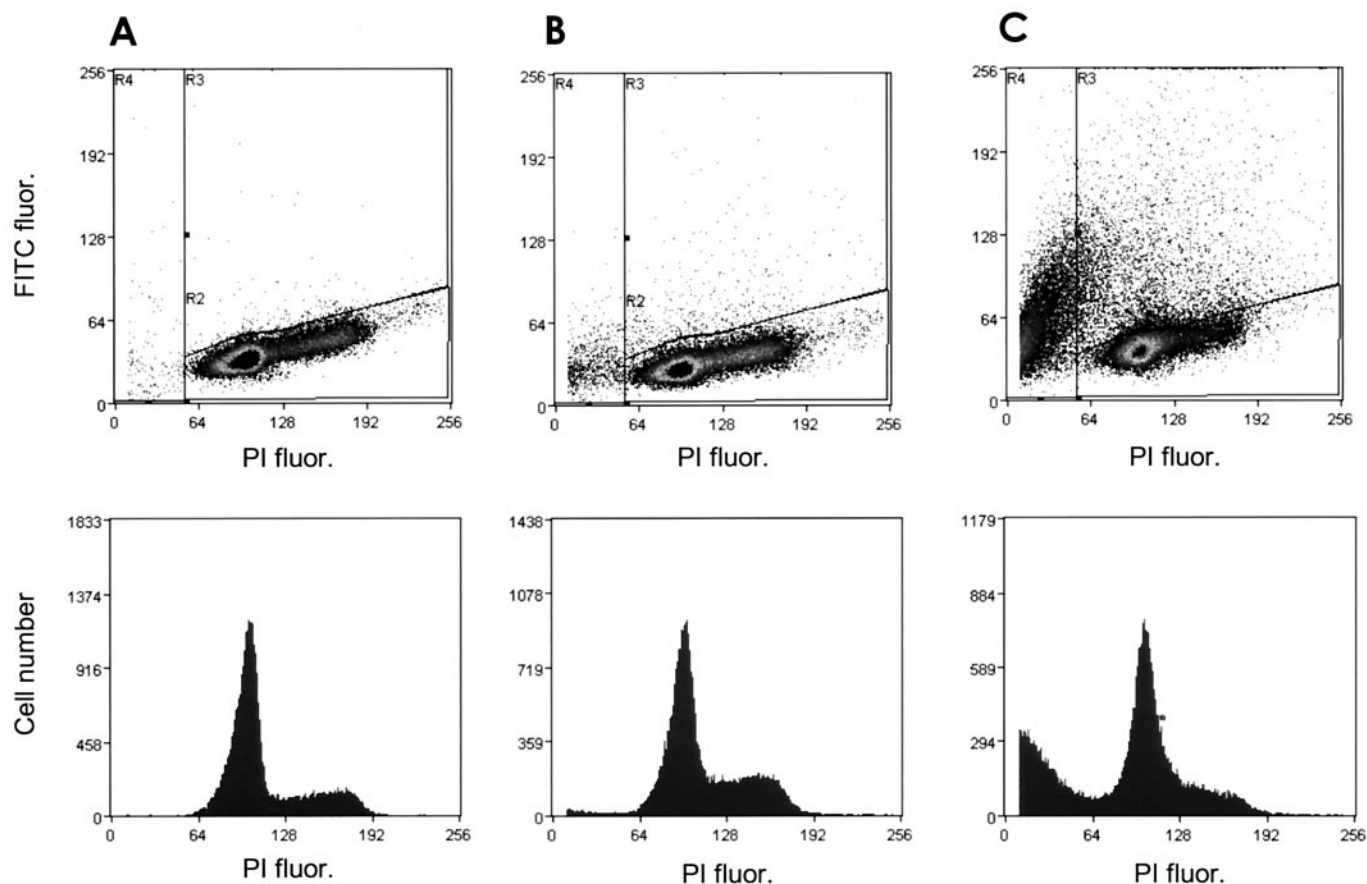

Fig. 1. Cisplatin-induced apoptosis in NIH 3T3 cells is enhanced by pretreatment with bFGF. The amount of apoptosis was measured by TUNEL assay in cells not exposed to cisplatin or bFGF (A), or in cells pretreated for 24 h with carrier only (B) or 10 ng/ml bFGF (C) and subsequently exposed to 10 μg/ml cisplatin for 12 h. Cisplatin-treated cells were harvested and fixed at 24 h post-treatment. TUNEL positivity is shown on the *y*-axis, and PI staining on the *x*-axis. For quantitation purposes, cells in the R3 (upper right) region were taken as TUNEL positive and cells in the R4 (left) region were taken as having sub-G_1 DNA content. Shown below are the PI histograms illustrating the cell cycle distribution in each sample.

refed with growth medium ($\pm$bFGF) lacking cisplatin for 24 h. Floating and attached cells were harvested and analyzed by flow cytometry for DNA content by PI staining and for DNA fragmentation by TUNEL assay. Apoptosis is a rapid process, and cells may quickly progress from a normal

DNA content that stains TUNEL-positive to degraded DNA of sub-G_1 content. Therefore, the total percentage of apoptotic cells was quantified as the percentage of cells with sub-G_1 DNA content plus the percentage of TUNEL-positive cells in the G_1-G_2/M range (Table 1).

Two representative experiments showing the effect of bFGF on cisplatin-induced apoptosis are shown in Table 1 and Fig. 1. In these experiments, cisplatin alone for 12 h caused apoptosis in a total of 2 to 5% of the cells by 24 h post-treatment, over a background of about 1% in cells that were not treated with cisplatin. Preincubation with 10 ng/ml bFGF for 24 h led to apoptosis in 20% to 40% of the cells after cisplatin treatment, with an increase in both the TUNEL-positive and sub-G_1 components (Table 1 and Fig. 1). Similar results were obtained in each of 12 independent experiments. Although there was some variability between experiments in the amount of apoptosis induced by cisplatin alone, preincubation of cells with bFGF enhanced cisplatin-induced apoptosis an average of 5-fold ($\pm$0.9-fold) for all 12 experiments combined. There was an overall significant difference in apoptosis in the presence and absence of bFGF ($P < .001$). Simultaneous incubation with bFGF and cisplatin for 12 h did not enhance apoptosis over cisplatin alone (Table 1), indicating that preincubation with bFGF was required for the enhancement effect. Incubation with bFGF alone for 24 h did not induce apoptosis in control cells not treated with cisplatin (Table 1).

bFGF is a mitogen for NIH 3T3 cells and stimulates maximal DNA synthesis at a concentration of 0.5 ng/ml (Florkiewicz et al., 1995; also see Fig. 5A). We determined whether enhancement of apoptosis correlated with bFGF's mitogenic activity. As shown in Fig. 2A, no enhancement of cisplatin-induced apoptosis occurred at 0.5 ng/ml bFGF. Instead, maximal enhancement of apoptosis was observed at a bFGF concentration of 10 ng/ml, with no increase in the effect at
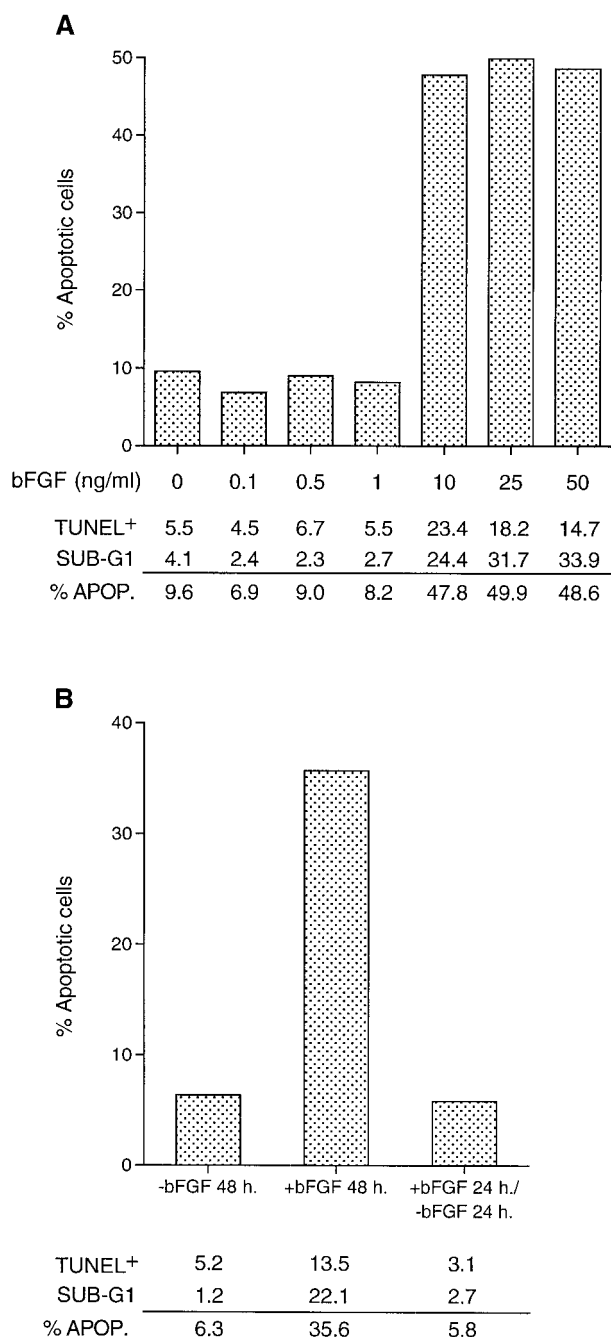
A

bFGF (ng/ml)	0	0.1	0.5	1	10	25	50
TUNEL+	5.5	4.5	6.7	5.5	23.4	18.2	14.7
SUB-G1	4.1	2.4	2.3	2.7	24.4	31.7	33.9
% APOP.	9.6	6.9	9.0	8.2	47.8	49.9	48.6

B

	-bFGF 48 h.	+bFGF 48 h.	+bFGF 24 h./ -bFGF 24 h.
TUNEL+	5.2	13.5	3.1
SUB-G1	1.2	22.1	2.7
% APOP.	6.3	35.6	5.8

Fig. 2. Dose response and reversibility of the enhancement of cisplatin-induced apoptosis by bFGF. NIH 3T3 cells were pretreated as indicated and then exposed to 10 μg/ml cisplatin for 12 h, and analyzed for apoptosis as in Fig. 1. A, cells were pretreated with increasing concentrations of bFGF for 24 h before exposure to cisplatin. In this experiment, the cells were harvested 18 h after cisplatin exposure. The bars represent the total amount of apoptosis (TUNEL-positive plus sub-G_1) detected with each treatment. The percentages for each population are given below the bars. B, cells were pretreated with or without 10 ng/ml bFGF for 48 h, or were pretreated with 10 ng/ml bFGF for 24 h and then rinsed with PBS and incubated without bFGF for another 24 h. Apoptosis was determined as in A.
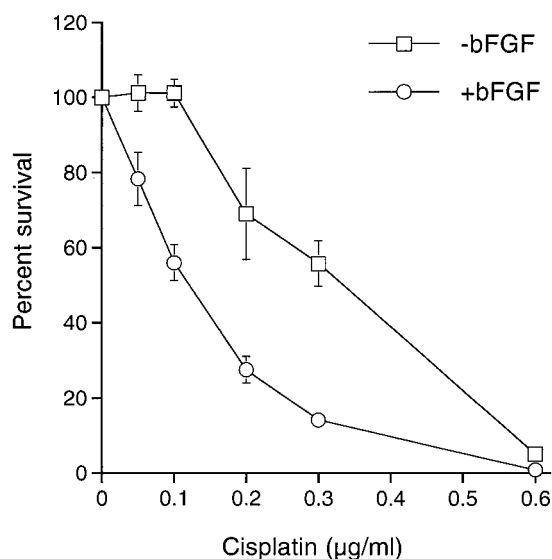
Fig. 3. Dose response to cisplatin. Subconfluent plates of cells were pretreated with or without 10 ng/ml bFGF for 24 h, and then were exposed to various concentrations of cisplatin for 12 h. The cells then were replated at low density and allowed to form colonies in the continued presence or absence of bFGF. Data points represent the average number of colonies formed from triplicate plates, expressed as the percent survival relative to 100% in the absence of cisplatin (with and without bFGF). Error bars indicate S.E.M.

higher concentrations (Fig. 2A). This experiment shows that enhancement of apoptosis does not correlate with mitogenic activity. Furthermore, it shows that bFGF enhancement of apoptosis is a saturable response.

To determine whether the effect of bFGF on cisplatin-induced apoptosis was reversible, we incubated NIH 3T3 cells with 10 ng/ml bFGF for 24 h and then either removed the bFGF or continued incubating in bFGF for an additional 24 h before adding cisplatin. Control cells were incubated without bFGF for 48 h before addition of cisplatin. Quantification of the subsequent apoptosis assays is shown in Fig. 2B. Incubation with bFGF for 48 h produced significantly enhanced apoptosis by cisplatin (35.6%). However, the amount of apoptosis was similar in bFGF-treated cells from which the bFGF was subsequently removed and in cells never exposed to bFGF (6.3% and 5.8%, respectively). Therefore, preincubation with 10 ng/ml bFGF enhanced cisplatin-induced apoptosis in a manner that was both dose-dependent and reversible.

Pretreatment with bFGF Sensitizes NIH 3T3 Cells to the Cytotoxic Effects of Cisplatin. The dose of cisplatin used to measure the effect of bFGF on apoptosis was lethal for both bFGF-treated and untreated cells. We used this high dose to assess changes in the apoptotic response to a high level of DNA damage. To determine whether bFGF also enhanced cytotoxicity to lower concentrations of cisplatin, we performed a colony-forming assay on NIH 3T3 cells after exposure to varying cisplatin concentrations, with or without prior incubation in bFGF. As shown in Fig. 3, pretreatment with bFGF for 24 h resulted in increased sensitivity to cisplatin. The IC_{50} for a 12-h exposure to cisplatin was 0.1 μg/ml in bFGF-treated cells, and 0.3 μg/ml in cells not treated with bFGF. This assay was also performed twice using a 1-h exposure to higher doses of cisplatin; in these experiments, pretreatment with bFGF led to a 2.1- and 2.6-fold decrease in the IC_{50} of cisplatin, relative to cells not treated with bFGF (data not shown).
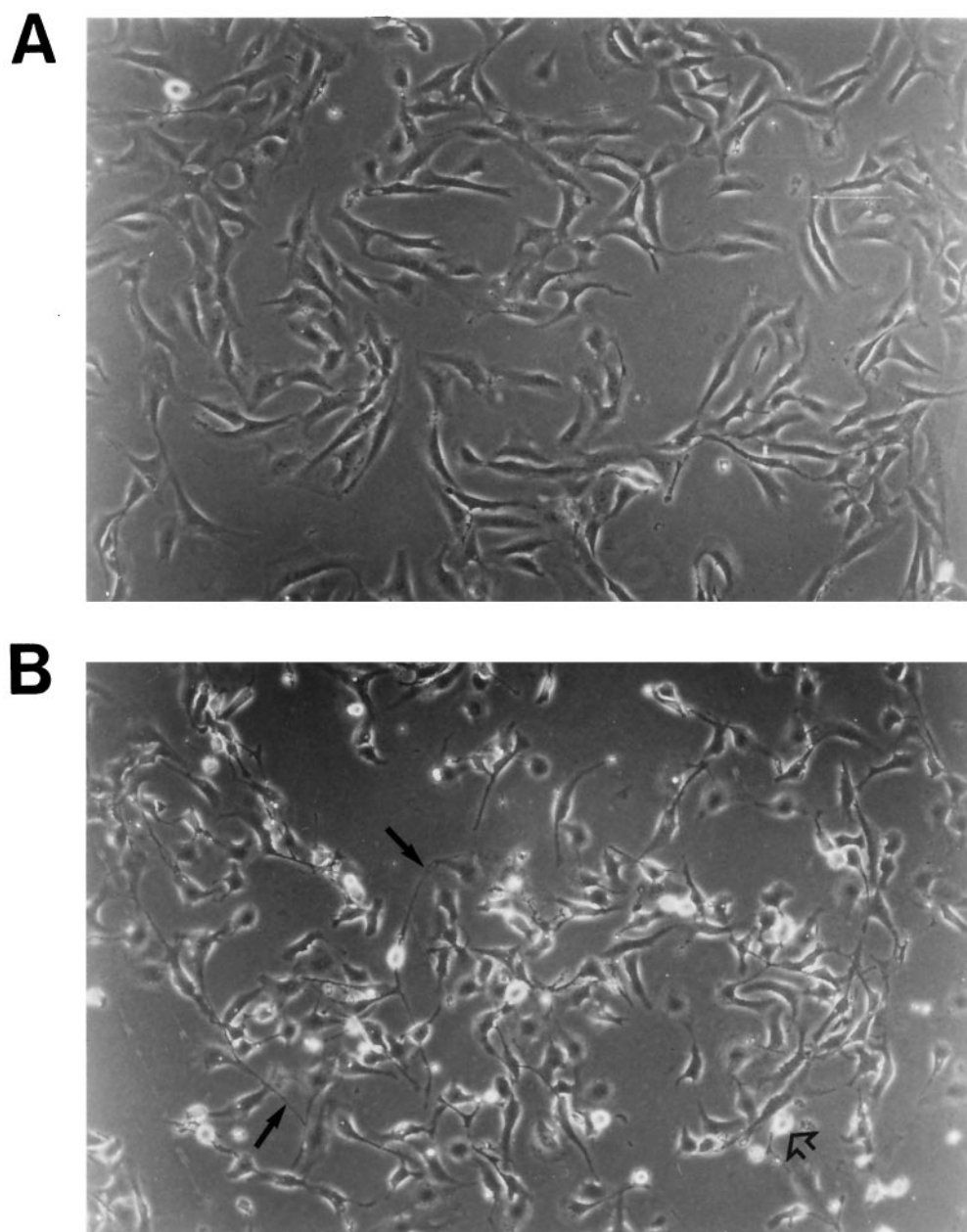

Fig. 4. Morphology of NIH 3T3 cells treated with 10 ng/ml bFGF. Cells (3×10^5) were seeded in 10-cm plates and allowed to attach overnight before treating without (A) or with (B) bFGF for 24 h. Photographs were taken by using phase contrast microscopy at an original magnification of $100\times$. The open arrow indicates a cell that is considered to be refractile, and the solid arrows indicate dendritic processes.

bFGF at 10 ng/ml Induces Specific Cellular Changes Not Associated with Lower Concentrations of bFGF. Previous studies have shown that bFGF can alter the morphology of certain cell types (Kato and Gospodarowicz, 1985; Kalman et al., 1999). We also noticed that NIH 3T3 cells acquired a distinct morphology after a 24-h incubation in the presence of bFGF, before cisplatin exposure. Analysis by light microscopy revealed that bFGF-treated cells tended to be smaller and rounder than the typical flat morphology of NIH 3T3 cells (Fig. 4). bFGF-treated cells also had an increased number of highly refractile cells and produced more dendritic processes than untreated cells. Induction of the altered morphology occurred with the same dose response to bFGF as the enhancement of cisplatin-induced apoptosis (Table 2). Very little change in morphology relative to untreated cells was observed at 1 ng/ml bFGF, whereas the change saturated at 10 ng/ml. These changes in cellular morphology were also reversible within 24 h, following the same trend as the enhancement of cisplatin-induced apoptosis (data not shown).

As discussed above, the concentrations of bFGF necessary to enhance cisplatin-induced apoptosis were significantly higher than those required for mitogenic activity as measured by ^{3}H-thymidine incorporation. These data are shown in Fig. 5A. As reported previously (Florkiewicz et al., 1995), 0.5 ng/ml bFGF stimulated maximal ^{3}H-thymidine incorporation, 4-fold over incorporation levels in the absence of bFGF. Concentrations of 5 and 10 ng/ml bFGF only produced 40% stimulation of ^{3}H-thymidine incorporation over the untreated control. Therefore, the bFGF concentrations that enhanced cisplatin-induced apoptosis (5 and 10 ng/ml) produced lower levels of mitogenic stimulation (^{3}H-thymidine incorporation) than did lower bFGF concentrations that did not enhance apoptosis. To confirm this result, we performed a cell proliferation assay in the presence or absence of bFGF. As shown in Fig. 5B, the addition of bFGF at both 1 ng/ml and 10 ng/ml stimulated a modest increase in the growth rate over untreated cells (At day 4, $P < .01$ for untreated versus 1 ng/ml, and $P < .001$ for untreated versus 10 ng/ml). Cells treated with 10 ng/ml bFGF grew to somewhat higher densities than cells treated with 1 ng/ml bFGF at 4 days, but this most likely reflected less contact inhibition due to the generally smaller size of cells treated with 10 ng/ml bFGF.

TABLE 2
Quantification of the bFGF-induced morphology shift in NIH 3T3 cells
Data above and below the space were obtained from different experiments. In the experiment with suramin, 10 ng/ml bFGF was used.

Treatment	Cells/Frame[a]	% Refractile[b]	Dendricity[c]
bFGF (ng/ml)			
0	281	5.7	6
1	313	15.3	5
2	245	20.4	9
5	318	24.8	24
10	337	50.4	50
20	318	42	55
No treatment	264	5.7	5
bFGF	209	23.9	37
Suramin	252	8.7	10
bFGF + suramin	247	22.7	32

[a] Total number of cells in a photograph taken of a random field of cells after incubation in the indicated conditions.
[b] Percentage of cells in the frame that are refractile, as indicated by the open arrow in Fig. 4B.
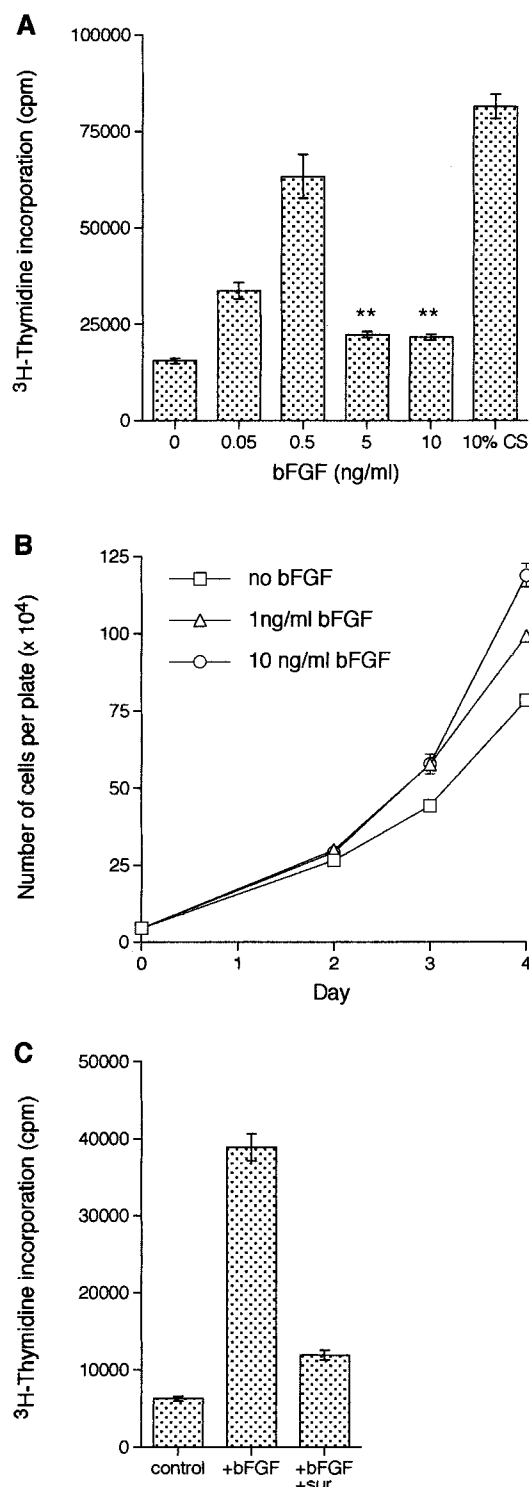[c] Total number of dendritic processes (solid arrows in Fig. 4B) in the frame.

Fig. 5. Cell proliferation and ^{3}H-thymidine incorporation in response to bFGF treatment. A, quiescent NIH 3T3 cells were treated with increasing concentrations of bFGF, or were placed back into 10% CS, and ^{3}H-thymidine incorporation was measured as described in *Materials and Methods*. Bars represent the average amount of radioactivity retained on filters from triplicate wells, ± S.E.M., expressed as cpm. B, 1×10^4 NIH 3T3 cells were plated in DMEM + 10% CS, treated with 0, 1, or 10 ng/ml bFGF, and then collected and counted over the course of 4 days. Values represent the average number of cells from triplicate plates ± S.E.M. C, quiescent NIH 3T3 cells were treated with 0.5 ng/ml bFGF, with or without 100 μM suramin (sur). Control cells received neither bFGF nor suramin. ^{3}H-thymidine incorporation was measured as in A. **$P < .01$ versus 0.5 ng/ml bFGF as determined by two-tailed t test.

It has been proposed that dividing cells are more susceptible to apoptotic stimuli than nondividing cells (Evan and Littlewood, 1998). However, both low and high bFGF concentrations stimulated a small increase in cell division in NIH 3T3 cells cultured in 10% calf serum, the conditions under which the apoptosis assays were performed, suggesting that overall proliferation rates could not account for the increase in cisplatin-induced apoptosis. It was still possible, however, that high concentrations of bFGF produced subtle effects on the timing of S-phase entry that could account for increased cisplatin sensitivity. To investigate this possibility, we examined the effect of high and low concentrations of bFGF on entry into S-phase of the cell cycle. NIH 3T3 cells were arrested by serum starvation and contact inhibition (Merrill, 1998) and then were released into standard growth medium with 0, 0.5, or 10 ng/ml bFGF. Curiously, both low and high concentrations of bFGF produced a delay in the entry of cells into S-phase relative to the control cells plated in growth medium alone (data not shown). Cells released into growth medium without bFGF began to enter S-phase by 16.5 h after release. Release of cells into growth medium containing 10 ng/ml bFGF caused a 3-h delay of entry into S-phase, whereas release into medium containing 0.5 ng/ml produced a somewhat shorter delay of 1.5 to 2 h. Although this does not rule out the possibility that effects of bFGF on cell cycle distribution could affect susceptibility to apoptosis, 0.5 ng/ml

bFGF did not enhance cisplatin-induced apoptosis, and therefore it is unlikely that alteration in the cell cycle is the only factor contributing to increased cisplatin sensitivity at higher bFGF concentrations.

Suramin Does Not Inhibit the Effect of bFGF on Apoptosis or Morphology. The disparity between the concentrations of bFGF required for mitogenesis and for enhancement of apoptosis suggested that these two effects may be initiated by different signaling pathways. Mitogenic signaling occurs through the well characterized high-affinity FGF receptors (Johnson and Williams, 1993). To establish whether activation of the high-affinity FGF receptors was necessary for the enhancement of cisplatin-induced apoptosis, we attempted to block the effect of bFGF with suramin. Suramin is a small, polyanionic compound that acts as a relatively nonspecific inhibitor of FGF receptor-ligand interactions (Yayon and Klagsbrun, 1990). At a concentration of 100 μM, suramin reduced by 83% the ^{3}H-thymidine incorporation stimulated by 0.5 ng/ml bFGF (Fig. 5C), suggesting that this concentration of suramin was able to block FGF receptor-mediated signaling.

To determine the effect of suramin on bFGF-enhanced apoptosis and morphology, we treated NIH 3T3 cells with or without 10 ng/ml bFGF for 24 h in the presence or absence of 100 μM suramin before exposure to cisplatin. Results are shown in Fig. 6. The addition of suramin with bFGF resulted

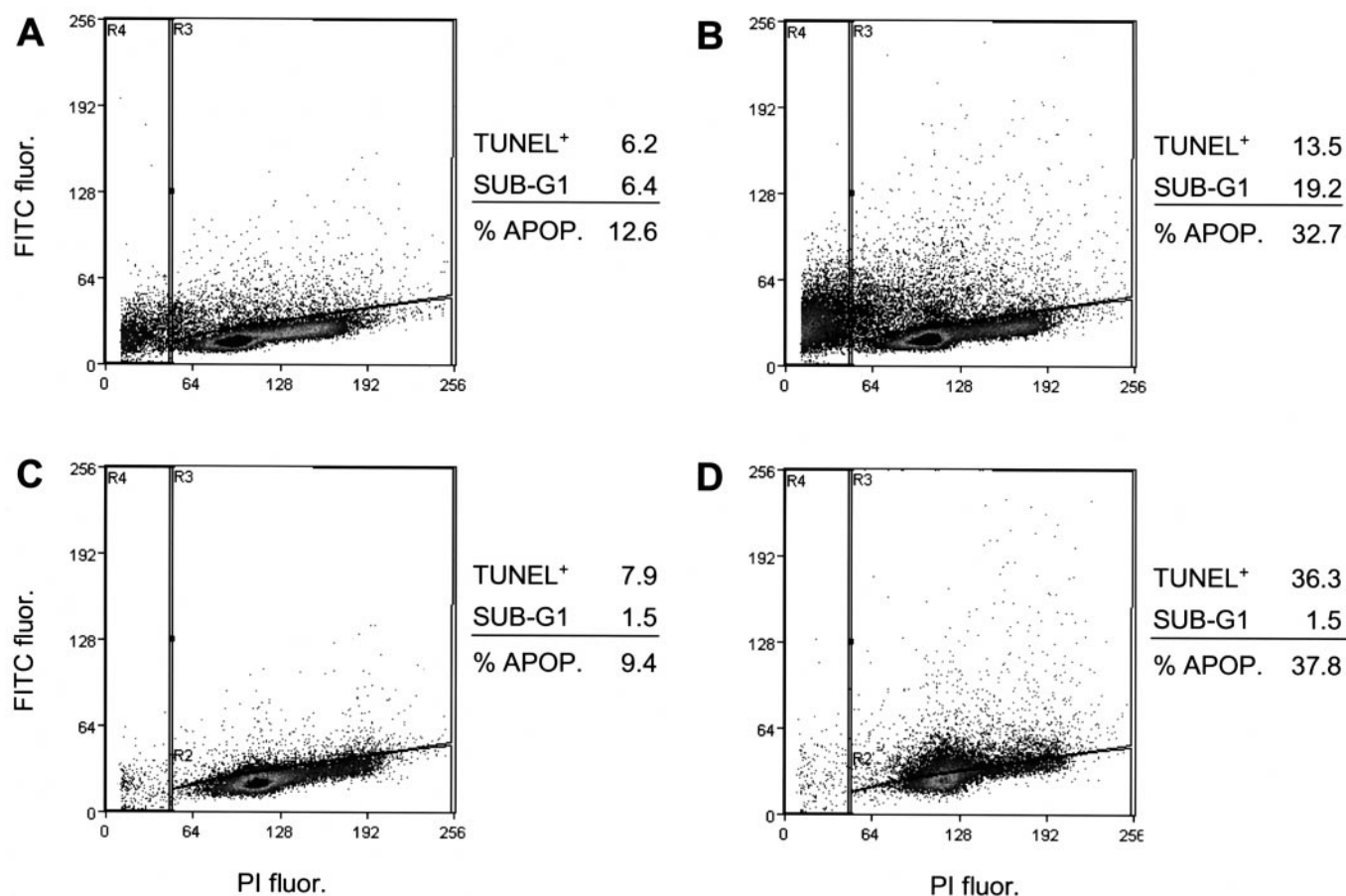|	TUNEL$^+$	SUB-G1	% APOP.
A	6.2	6.4	12.6
B	13.5	19.2	32.7
C	7.9	1.5	9.4
D	36.3	1.5	37.8

Fig. 6. The bFGF-enhanced accumulation of TUNEL-positive cells after cisplatin exposure is not reversed by suramin. NIH 3T3 cells were pretreated for 24 h with medium only (A), 10 ng/ml bFGF (B), 100 μM suramin (C), or both bFGF and suramin (D). The cells then were exposed to 10 μg/ml cisplatin for 12 h and harvested 24 h after the cisplatin was removed. The scatter plots were analyzed as in Fig. 1. TUNEL-positive (R3), sub-G$_1$ (R4), and the total percentage of apoptotic cells (% APOP) are given to the right of each plot.

in a sharp decrease in the percentage of sub-G_1 cells after exposure to cisplatin, relative to cells pretreated with bFGF alone (1.5% versus 19.2%, respectively), but increased the percentage of cells that stained TUNEL positive (from 13.5% to 36.3%). Pretreatment of cells with suramin alone also resulted in a shift toward fewer sub-G_1 cells, and somewhat more TUNEL-positive cells, compared with cells that received no pretreatment before cisplatin exposure (sub-G_1 shifted from 6.4% to 1.5%; TUNEL-positive shifted from 6.2% to 7.9%). Therefore, suramin appeared to alter cell death in such a way that nuclei with nicked DNA failed to fragment into sub-G_1 debris by 24 h, regardless of the presence of bFGF. The percentage of total apoptotic cells (TUNEL-positive plus sub-G_1) only shifted significantly with the presence or absence of bFGF. The percentages of total apoptosis in cells that were not given bFGF were 12.6% without suramin and 9.4% with suramin, whereas apoptosis in cells treated with bFGF was 32.7% without suramin and 37.8% with suramin. These data suggest that suramin did not affect the enhancement of cisplatin-induced apoptosis by bFGF.

We had observed a correlation between the enhancement of cisplatin-induced apoptosis and changes in cellular morphology at high bFGF concentrations. If these two effects are tightly linked, we would expect that the addition of suramin would not prevent this morphology shift. To test this, NIH 3T3 cells were incubated in 10 ng/ml bFGF plus 100 μM suramin for 24 h. Microscopic analysis showed that samples given bFGF and suramin had the same increase in the number of refractile cells and dendritic processes as cells treated with bFGF alone, relative to cells that received no bFGF (Table 2).

Discussion

The addition of exogenous bFGF to the growth medium of NIH 3T3 cells produced a strong and reproducible enhancement of cisplatin-induced apoptosis (Table 1 and Fig. 1). The addition of bFGF also significantly reduced the dose-dependent survival of cells exposed to increasing concentrations of cisplatin (Fig. 3), and therefore directly increased the sensitivity of NIH 3T3 cells to this chemotherapy drug. Exogenous bFGF did not produce any cytotoxic effects in the absence of cisplatin, and it had the expected stimulatory effect on cell growth at both low and high concentrations (Fig. 5B). Enhancement of cisplatin-induced apoptosis was observed at bFGF concentrations of 10 ng/ml and higher (Fig. 2A), whereas stimulation of ^{3}H-thymidine incorporation occurred at 0.5 ng/ml (Fig. 5A). The concentrations of bFGF that sensitized cells to cisplatin also produced a distinct shift in the cellular morphology of NIH 3T3 cells (Table 2 and Fig. 4), and both this morphology shift and the drug sensitization were reversible with similar kinetics (Fig. 2B and data not shown).

Other studies have examined the effects of bFGF on the sensitivity of cells to chemotherapy drugs, with varying results. Most of these studies have used expression of bFGF from a transfected cDNA. Overexpression of bFGF increases the survival of NIH 3T3 cells treated with etoposide or 5-fluorouracil (Wieder et al., 1997), and also provides resistance to the DNA damaging agent PALA (Huang et al., 1994). Shaulian et al. (1997) found that NIH 3T3 cells expressing a bFGF transgene encoding a signal peptide are resistant to cisplatin. In contrast, recent studies indicate that bFGF sensitizes MCF-7 breast tumor cells to apoptosis induced by cisplatin, etoposide, and 5-fluorouracil, whether bFGF is expressed from a transgene or added to the culture medium (Wang et al., 1998; Fenig et al., 1999; Maloof et al., 1999).

The ability of bFGF to sensitize or protect cells may depend on the cell type used, because bFGF sensitization previously has only been reported with MCF-7 cells. However, our current results suggest that bFGF can also sensitize NIH 3T3 cells to cisplatin. We similarly have observed sensitization to cisplatin cytotoxicity with exogenous bFGF added to MCF-7, SKOV3, and A2780 cell lines, and we have found bFGF-related sensitization of NIH 3T3 cells to doxorubicin and UV light (our unpublished results). Therefore, it does not appear that simple cell line differences or drug differences can account for the differential effects of bFGF. The recent reports that MCF-7 cells can be sensitized to chemotherapy with either transfected or exogenous bFGF suggest that it is not simply the mode of bFGF delivery that accounts for differential responses to bFGF (Wang et al., 1998; Fenig et al., 1999; Maloof et al., 1999). However, we cannot rule out the possibility that some cell types (such as NIH 3T3 cells) are protected by transfected bFGF but sensitized by exogenous bFGF.

Indeed, the overexpression of bFGF may affect the cellular response to chemotherapeutic agents differently than does the stimulation of cells with exogenous bFGF. Expression of bFGF from a full-length cDNA leads to the production of multiple intracellular isoforms of bFGF derived from the initiation of translation at alternative start codons on the same mRNA (Florkiewicz and Sommer, 1989). The 18-kDa isoform, initiated from a primary AUG start codon, remains localized in the cytoplasm, whereas three high molecular weight isoforms (22, 23, and 24 kDa) initiated from alternative upstream CUG start codons translocate to the nucleus, where they are thought to elicit distinct biological activities (Bikfalvi et al., 1995). The overexpression of 24-kDa bFGF alone provides resistance to ionizing radiation in HeLa cells, whereas overexpression of 18-kDa bFGF alone provides no resistance (Cohen-Jonathan et al., 1997). Therefore, expression of the high-molecular-weight bFGF isoforms may affect signal transduction pathways other than those activated by extracellular 18-kDa bFGF, and these different pathways may have different effects on the response of cells to DNA damaging agents.

We found that bFGF sensitized NIH 3T3 cells to cisplatin-induced apoptosis only at concentrations of 10 ng/ml and higher (Fig. 2). The shift in the cellular morphology of NIH 3T3 cells followed a similar dose dependence (Table 2). These concentrations of bFGF were significantly higher than those needed to stimulate ^{3}H-thymidine incorporation and cell growth (Fig. 5). Also, suramin, which inhibits bFGF from binding to and activating the high-affinity FGF receptor, prevented bFGF stimulation of DNA synthesis but did not affect the enhancement of cisplatin-induced apoptosis (Figs. 5 and 6). These data suggest that the signal by which bFGF sensitizes NIH 3T3 cells to cisplatin might be received and propagated through a separate receptor or signal transduction pathway than that which stimulates mitogenesis.

It is not clear how high concentrations of bFGF might act independently of "classic" FGF signaling, and at this point,

we can only speculate as to how this signal is received. The heparan sulfate proteoglycans (HSPGs) constitute the low-affinity cell-surface receptors for bFGF, and it is conceivable that they might act as mediators of bFGF activity at higher concentrations. bFGF binds to HSPGs with a K_d of approximately 1 to 2 nM (Moscatelli, 1987), and we have observed enhancement of cisplatin-induced apoptosis and changes in cellular morphology at a bFGF concentration of 5 to 10 ng/ml, or about 0.3 to 0.6 nM.

The syndecans (syndecan 1–4) are one group of transmembrane HSPGs that function in adhesion to the extracellular matrix, and it has been demonstrated recently that these low-affinity receptors are capable of transmitting extracellular signals into the cytoplasm. Syndecan-4 is expressed in mouse fibroblasts and is necessary for the assembly of focal adhesions and cellular spreading in these cells (Saoncella et al., 1999). Interestingly, the addition of 10 to 30 ng/ml bFGF to NIH 3T3 cells causes loss of phosphorylation from serine-183 in the cytoplasmic tail of syndecan-4 (Horowitz and Simons, 1998). The cytoplasmic tail has been shown to interact with signaling factors such as protein kinase C and the GTP-binding protein Rho (Saoncella et al., 1999). Also of interest in light of our observed effect of bFGF on NIH 3T3 cell morphology is the recent report that exogenous bFGF triggers distinct process outgrowth in astrocytes in culture; the effect is dependent on c-Ha-Ras and is blocked by Rac1 and RhoA, but the cell-surface mediators of this effect have not been studied (Kalman et al., 1999).

Another possibility is that at high concentrations bFGF competes for the binding of an unknown factor(s) to HSPGs at the cell surface or in the extracellular matrix. Many growth factors and cytokines bind to HSPGs, and such binding is necessary for high-affinity receptor binding and activation by some cytokines that are not members of the FGF family (Cook et al., 1995). There may be a factor(s) in serum or the extracellular matrix that has the potential to modulate the cell's susceptibility to apoptosis. If bFGF were to compete for the binding of an antiapoptotic factor to HSPGs, and thereby inhibit binding of this factor to its high-affinity receptor, then cells could be rendered more susceptible to apoptosis. We also cannot rule out an effect of high concentrations of bFGF on down-regulating high-affinity FGF receptors and/or its downstream signaling components that would otherwise confer a protective effect against cisplatin and other apoptotic signals.

Regardless of the mode of signal transduction, high concentrations of bFGF clearly sensitize NIH 3T3 cells to cisplatin, as demonstrated by an enhancement of cisplatin-induced apoptosis and a dose-dependent reduction in survival. A better understanding of how bFGF works to modulate apoptosis might yield answers as to why this factor protects cells under some conditions and sensitizes cells under other conditions. This knowledge could provide valuable insights into what role bFGF might play in the response of tumors to chemotherapy.

References

Bikfalvi A, Klein S, Pintucci G, Quarto N, Mignatti P and Rifkin DB (1995) Differential modulation of cell phenotype by different molecular weight forms of basic fibroblast growth factor: Possible intracellular signaling by the high molecular weight forms. *J Cell Biol* **129**:233–243.

Borsellino N, Belldegrun A and Bonavida B (1995) Endogenous interleukin 6 is a resistance factor for *cis*-diaminedichloroplatinum and etoposide-mediated cytotoxicity of human prostate carcinoma cell lines. *Cancer Res* **55**:4633–4639.

Cohen-Jonathan E, Toulas C, Monteil S, Couderc B, Maret A, Bard J, Prats H, Daly-Schveitzer N and Favre G (1997) Radioresistance induced by the high molecular forms of basic fibroblast growth factor is associated with an increased G_2 delay and a hyperphosphorylation of p34^{CDC2} in HeLa cells. *Cancer Res* **57**:1364–1370.

Cook PW, Ashton NM, Karkaria CE, Siess DC and Shipley GD (1995) Differential effects of a heparin antagonist (hexadimethrine) or chlorate on amphiregulin, basic fibroblast growth factor, and heparin-binding EGF-like growth factor activity. *J Cell Phys* **163**:418–429.

Evan G and Littlewood T (1998) A matter of life and cell death. *Science* **281**:1317–1322.

Fan S, Smith ML, Rivet DJ, Duba D, Zhan Q, Kohn KW, Fornace AJ and O'Connor PM (1995) Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. *Cancer Res* **55**:1649–1654.

Fenig E, Livnat T, Sharkon-Polak S, Wasserman L, Beery E, Lilling G, Yahalom J, Wieder R and Nordenberg J (1999) Basic fibroblast growth factor potentiates cisplatin-induced cytotoxicity in MCF-7 human breast cancer cells. *J Cancer Res Clin Oncol* **125**:556–562.

Florkiewicz RZ, Majack RA, Buechler RD and Florkiewicz E (1995) Quantitative export of FGF-2 occurs through an alternative, energy-dependent, non-ER/golgi pathway. *J Cell Phys* **162**:388–399.

Florkiewicz RZ and Sommer A (1989) Human basic fibroblast growth factor gene encodes four polypeptides: Three initiate translation from non-AUG codons. *Proc Natl Acad Sci USA* **86**:3978–3981.

Fox JC and Shanley JR (1996) Antisense inhibition of basic fibroblast growth factor induces apoptosis in smooth muscle cells. *J Biol Chem* **271**:12578–12584.

Fuks Z, Persaud RS, Alfieri A, McLoughlin M, Ehleiter D, Schwartz JL, Seddon AP, Cordon-Cardo C and Haimovitz-Friedman A (1994) Basic fibroblast growth factor protects endothelial cells against radiation-induced programmed cell death *in vitro* and *in vivo*. *Cancer Res* **54**:2582–2590.

Gospodarowicz D, Bialecki H and Thakral TK (1979) The angiogenic activity of the fibroblast and epidermal growth factor. *Exp Eye Res* **28**:501–514.

Grothey A, Voigt W, Schober C, Muller T, Dempke W and Schmoll HJ (1999) The role of insulin-like growth factor I and its receptor in cell growth, transformation, apoptosis, and chemoresistance in solid tumors. *J Cancer Res Clin Oncol* **125**:166–173.

Hawkins DS, Demers GW and Galloway DA (1996) Inactivation of p53 enhances sensitivity to multiple chemotherapeutic agents. *Cancer Res* **56**:892–898.

Horowitz A and Simons M (1998) Regulation of syndecan-4 phosphorylation *in vivo*. *J Biol Chem* **273**:10914–10918.

Huang A, Jin H and Wright JA (1994) Aberrant expression of basic fibroblast growth factor in NIH-3T3 cells alters drug resistance and gene amplification potential. *Exp Cell Res* **213**:335–339.

Johnson DE and Williams LT (1993) Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res* **60**:1–41.

Kalman D, Gomperts SN, Hardy S, Kitamura M and Bishop JM (1999) Ras family GTPases control growth of astrocyte processes. *Mol Biol Cell* **10**:1665–1683.

Kato Y and Gospodarowicz D (1985) Sulfated proteoglycan synthesis by confluent cultures of rabbit costal chondrocytes grown in the presence of fibroblast growth factor. *J Cell Biol* **100**:477–485.

Kouhara H, Hadari YR, Spivak-Kroizman T, Schilling J, Bar-Sagi D, Lax I and Schlessinger J (1997) A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell* **89**:693–702.

Maloof P, Wang Q, Wang H, Stein D, Denny TN, Yahalom J, Fenig E and Wieder R (1999) Overexpression of basic fibroblast growth factor (FGF-2) downregulates Bcl-2 and promotes apoptosis in MCF-7 human breast cancer cells. *Breast Cancer Res Treat* **56**:153–167.

Menzel T, Rahman Z, Calleja E, White K, Wilson EL, Wieder R and Gabrilove J (1996) Elevated intracellular level of basic fibroblast growth factor correlates with stage of chronic lymphocytic leukemia and is associated with resistance to fludarabine. *Blood* **87**:1056–1063.

Merrill GF (1998) Cell Synchronization. *Methods Cell Biol* **57**:229–249.

Miyake H, Hara I, Gohji K, Yoshimura K, Arakawa S and Kamidono S (1998) Expression of basic fibroblast growth factor is associated with resistance to cisplatin in a human bladder cancer cell line. *Cancer Lett* **123**:121–126.

Mohammadi M, Honegger AM, Rotin D, Fischer R, Bellot F, Li W, Dionne CA, Jaye M, Rubinstein M and Schlessinger J (1991) A tyrosine-phosphorylated carboxy-terminal peptide of the fibroblast growth factor receptor (Flg) is a binding site for the SH2 domain of phospholipase C-gamma 1. *Mol Cell Biol* **11**:5068–5078.

Moscatelli D (1987) High and low affinity binding sites for basic fibroblast growth factor on cultured cells: Absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. *J Cell Phys* **131**:123–130.

Ohgoh M, Kimura M, Ogura H, Katayama K and Nishizawa Y (1998) Apoptotic cell death of cultured cerebral cortical neurons induced by withdrawal of astroglial support. *Exp. Neurol* **149**:51–63.

Saoncella S, Echtermeyer F, Denhez F, Nowlen JK, Mosher DF, Robinson SD, Hynes RO and Goetinck PF (1999) Syndecan-4 signals cooperatively with integrins in a Rho-dependent manner in the assembly of focal adhesions and actin stress fibers. *Proc Natl Acad Sci USA* **96**:2805–2810.

Shaulian E, Resnitzky D, Shifman O, Blandino G, Amsterdam A, Yayon A and Oren M (1997) Induction of Mdm2 and enhancement of cell survival by bFGF. *Oncogene* **15:**2717–2725.

Spivak-Kroizman T, Lemmon MA, Dikic I, Ladbury JE, Pinchasi D, Huang J, Jaye M, Crumley G, Schlessinger J and Lax I (1994) Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation. *Cell* **79:**1015–1024.

Szebenyi G and Fallon JF (1999) Fibroblast growth factors as multifunctional signaling factors. *Int Rev Cytology* **185:**45–106.

Wang Q, Maloof P, Wang H, Fenig E, Stein D, Nichols G, Denny TN, Yahalom J and Wieder R (1998) Basic fibroblast growth factor downregulates Bcl-2 and promotes apoptosis in MCF-7 human breast cancer cells. *Exp Cell Res* **238:**177–187.

Wieder R, Wang H, Shirke S, Wang Q, Menzel T, Feirt N, Jakubowski AA and Gabrilove JL (1997) Low level expression of basic FGF upregulates Bcl-2 and delays apoptosis, but high intracellular levels are required to induce transformation in NIH 3T3 cells. *Growth Factors* **15:**41–60.

Yayon A and Klagsbrun M (1990) Autocrine transformation by chimeric signal peptide-basic fibroblast growth factor: Reversal by suramin. *Pro Natl Acad Sci USA* **87:**5346–5350.

Send reprint requests to: Susan E. Kane, Department of Cell and Tumor Biology, City of Hope National Medical Center, 1500 E. Duarte Rd., Duarte, CA 91010. E-mail: skane@coh.org