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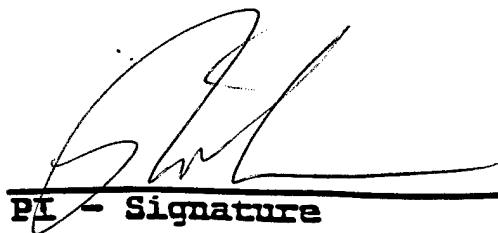
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THE ROLE OF BASIC FIBROBLAST GROWTH FACTOR IN HUMAN BREAST CANCERAnnual report, September 28, 1996**INTRODUCTION**

Basic fibroblast growth factor (bFGF, FGF-2) is a pleiotropic growth factor with multiple functions in multiple systems (1). It is one of the most important angiogenesis factors in breast cancer (2). Cancer cells acquire the capacity to secrete bFGF in a nonclassical manner as one of the last steps to malignant transformation (3). Secreted bFGF induces migration and proliferation of endothelial cells. We have determined that contrary to its documented role as a proliferative factor in endothelial cells and fibroblasts, bFGF inhibits the proliferation of a number of breast cancer cell lines, including MCF-7 cells, in which it causes an increase in cellular levels of the cyclin dependent kinase inhibitor p21^{WAF1/CIP1} and results in accumulation of cells in the G₁ phase of the cell cycle (Wang, et al., in preparation).

In light of its paradoxical effect on proliferation, we undertook an investigation to determine if the effects of bFGF on apoptosis in these cells was also paradoxical. Basic FGF is a survival factor in endothelial cells (4), fibroblasts (5) and CLL cells (6). We found that in fibroblasts, in which bFGF acts as a survival factor, overexpression of bFGF upregulates the cellular content of Bcl-2 (Wieder, et al., submitted). In this study, we investigated the effect of incubating MCF-7 cells with bFGF on the survival of these cells as assayed by colony formation, nuclear changes of apoptosis and DNA fragmentation after treatment with the chemotherapeutic agents etoposide, a topoisomerase II inhibitor, and 5-fluorouracil, an antimetabolite in vitro. We present evidence that bFGF acts as a death promoting factor in MCF-7 human breast cancer cells.

Programmed cell death or apoptosis is modulated by several families of gene products, including transcription modulators of the myc, max and mad family and members of the Bcl-2 family with variable opposing effects on cell survival. Bcl-2 and a related protein, BclX_L, promote cell survival while Bax, Bclx_s and Bak are death factors. Bad antagonizes protective effects of Bcl-2 and BclX_L, probably by displacing their heterodimeric partner Bax, the death factor (7). The Bcl-2 homolog Bax appears to act as a death factor when it is homodimerized, and heterodimerization with Bcl-2 promotes cell survival. In this study, we determined the effects of bFGF on Bcl-2 and Bax, two proteins with opposite effects on cell survival, and showed that bFGF causes an increase in cell death while concurrently downregulating Bcl-2 protein and mRNA levels in MCF-7 cells, allowing for decreased heterodimerization between Bcl-2 and Bax.

BODY**Effects of bFGF on colony forming efficiency**

Colony forming efficiency in tissue culture after 10 ± 2 days of incubation was used as an assay of survival following treatment with bFGF and the chemotherapeutic agents etoposide and 5-FU. Cell cultures were carried out in standard media consisting of DMEM, 10% heat-inactivated fetal calf serum (fcs), 2 mM glutamine, penicillin 50 units/ml/streptomycin 50 micrograms (μ g)/ml. Two thousand cells were incubated in triplicate 6 cm tissue culture plates and allowed to adhere for 24 hours. The media was then replaced by either fresh standard media or standard media supplemented with bFGF (R&D Systems, Minneapolis, MN) at various concentrations for the times indicated. The media was then replaced with standard media or media containing etoposide or 5-fluorouracil (5-FU) at the concentrations indicated for one hour. Adherent cells were washed once with PBS and incubated in fresh standard media for 10 ± 2 days. The plates were stained with methylene blue and colonies larger than or equal to 1 mm were manually counted.

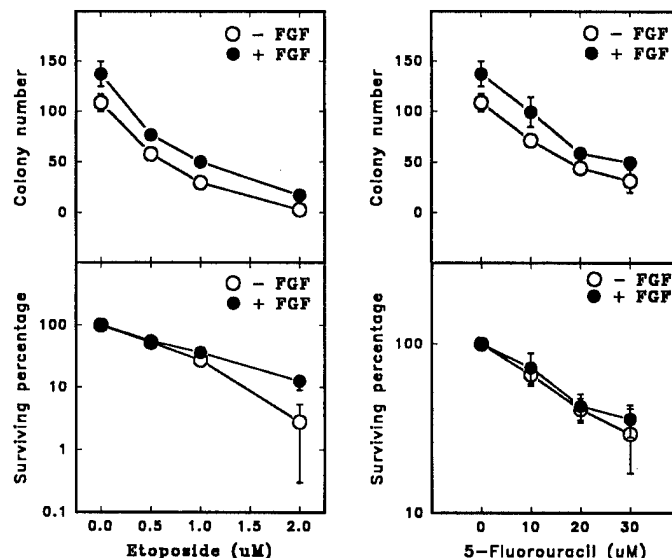


Figure 1. Effects of 24 hour preincubations with bFGF 1.0 ng/ml on the clonogenicity of NIH 3T3 cells treated with variable doses of etoposide and 5-FU for one hour. Lower panels demonstrate percentage decreases in colony formation.

Preincubation of NIH 3T3 cells with 1 ng/ml bFGF for 24 hours caused an approximately 30% increase in the clonogenic potential of NIH 3T3 cells (figure 1). Incubation of NIH 3T3 cells with bFGF prior to treatment with increasing concentrations of etoposide or 5-fluorouracil for one hour caused greater numbers of colonies to form compared to the drug treated cells alone (figure 1). These data support prior evidence that bFGF is a survival factor in fibroblasts.

In contrast, treatment of MCF-7 cells with bFGF 0.25 ng/ml for 24 hours caused decreases in their clonogenic potential (figure 2). Pretreatment with bFGF induced incremental decreases in colony formation due to variable doses of etoposide and 5-FU incubation for one hour or variable times of incubation with 1 μ M etoposide or 100 μ M 5-FU. The percentage decreases in colony formation of MCF-7 cells treated with chemotherapy drugs with or without pretreatment with bFGF were overlapping, indicating that the effect of bFGF was additive to that of etoposide or 5-FU-induced decreases in colony formation (figure 2).

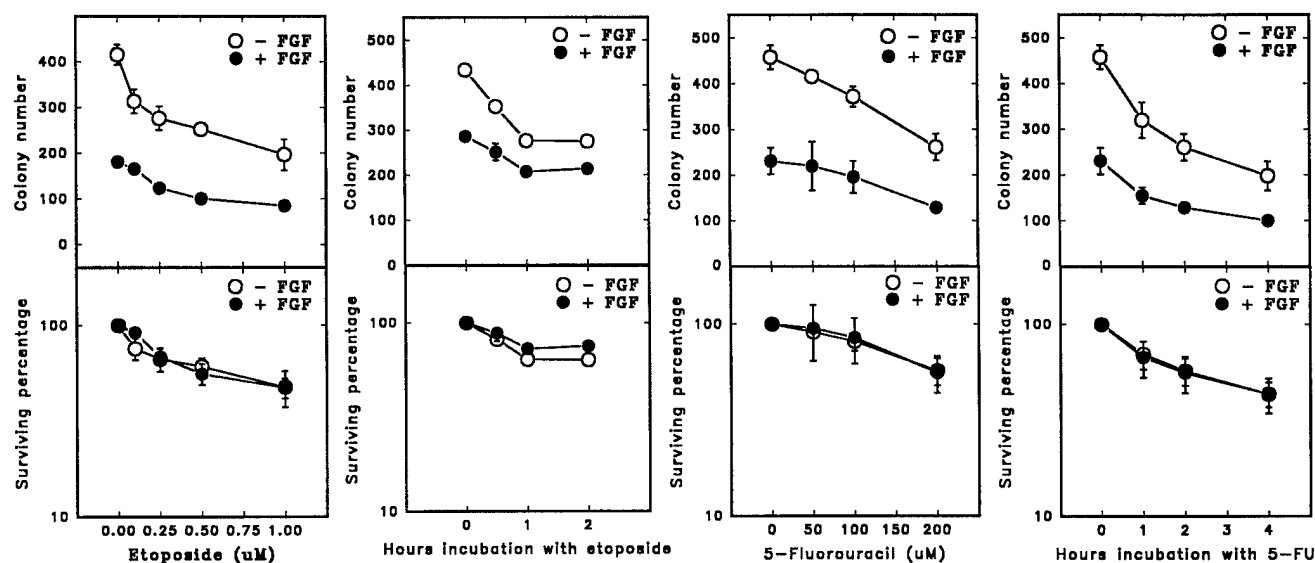


Figure 2. Effects of 24 hour preincubations with bFGF 0.25 ng/ml on the clonogenicity of MCF-7 cells treated with variable doses of etoposide and 5-FU for one hour or for variable times with 1 μ M etoposide and 100 μ M 5-FU. Lower panels demonstrate percentage decreases in colony formation. Overlapping curves denote additive effects.

The effects of bFGF on MCF-7 cell clonogenic potential were affected by dose intensity, or a product of dose and time. Figure 3A demonstrates that 1.0 ng/ml bFGF caused a consistent decrease in clonogenicity from six to 24 hours of incubation. When the data were re-plotted as a function of bFGF concentration, incubation for 24 hours decreased the clonogenic potential of MCF-7 cells at 0.25 and 0.5 ng/ml but did not have a statistically significant effect at 1.0 ng/ml. Data from multiple experiments suggested that the survival inhibitory effects of bFGF alone or as a potentiator of chemotherapy

agents were only seen at low dose intensities. This point is further illustrated in figure 3B where preincubation with variable doses of bFGF for 24 hours is used as a potentiator of the effects of etoposide and 5-FU. At 0.25 and 0.5 ng/ml bFGF the effects with 1 μ M etoposide are additive, but at higher doses, the graph depicting percent inhibition of colony formation demonstrates a less than additive effect of bFGF and etoposide. These data suggest that more than one mechanism is responsible for the effects of bFGF on cell survival in MCF-7 cells. Similar less than additive effects were also observed at higher doses of etoposide with low dose intensities of bFGF (not shown). The effects with 5-FU were, however, additive over a wider range of dose intensities of both bFGF and 5-FU.

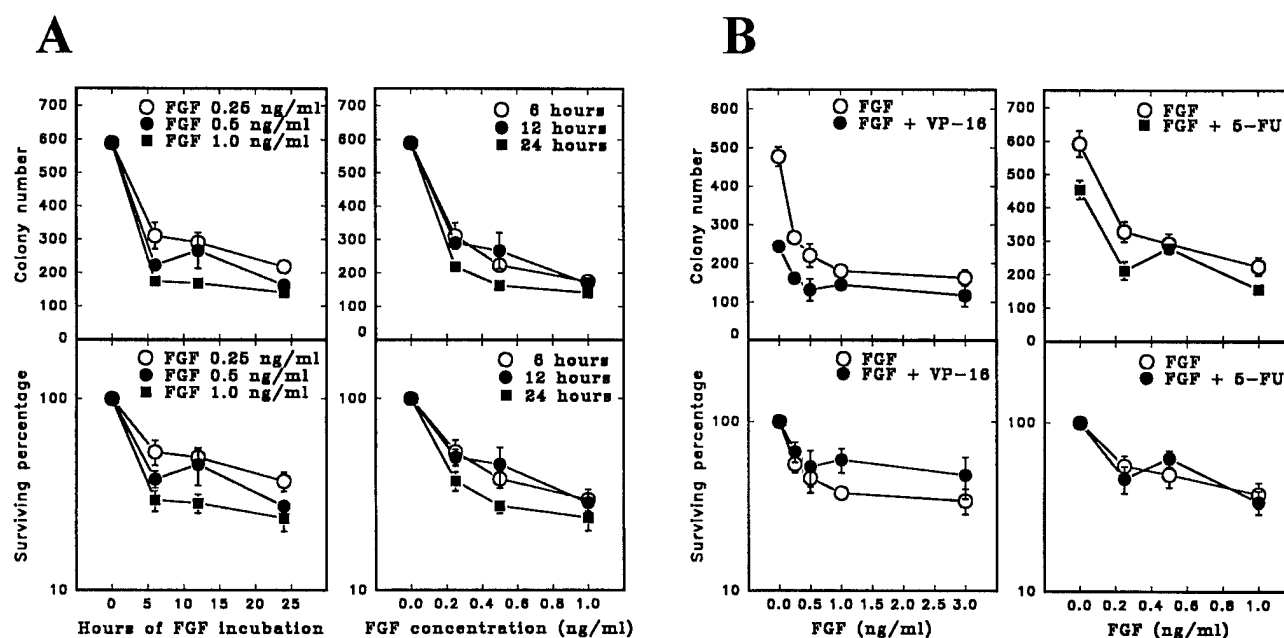


Figure 3. A. Effects of variable concentrations of bFGF incubated for variable times plotted separately as functions of time and dose. B. Effects of 24 hour preincubations with variable bFGF concentrations on the clonogenicity of MCF-7 cells treated for one hour with 1 μ M etoposide and 100 μ M 5-FU. Lower panels demonstrate percentage decreases in colony formation. Overlapping curves denote additive effects.

The effects of preincubating with bFGF were observed after only six hours and did not appear to increase substantially with longer preincubations of up to 24 hours (figure 4). We investigated the effects of bFGF treatment after incubating cells for one hour with chemotherapeutic agents. Figure 4 demonstrates that post-drug exposure to bFGF from 6 to 24 hours also decreased the clonogenic potential of MCF-7 cells by itself, as a control, and with treatment of cells with etoposide 1 μ M or 5-FU 100 μ M for one hour in an additive manner, as demonstrated by the overlapping percent inhibition curves (figure 4).

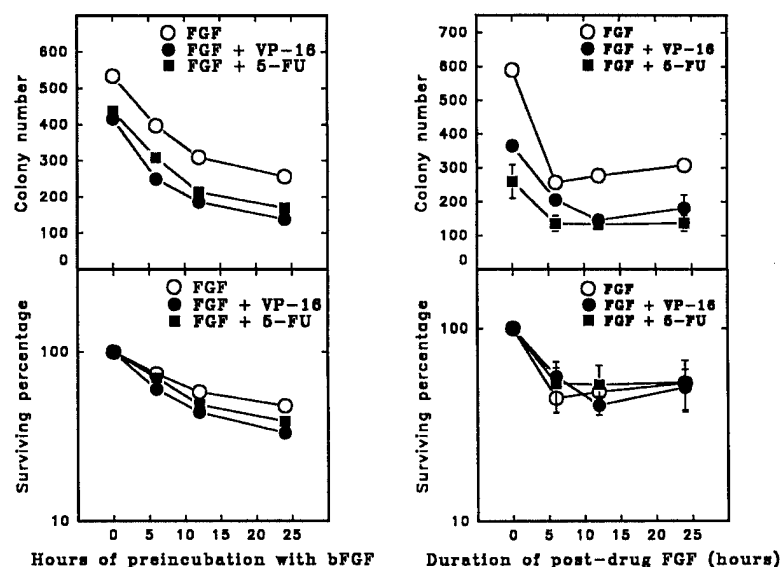


Figure 4. (Left) Effects of preincubations with bFGF 0.25 ng/ml for variable times on the clonogenicity of MCF-7 cells treated for one hour with 1 μ M etoposide or 100 μ M 5-FU. (Right) Effects of incubations with bFGF 0.25 ng/ml for variable times after treatment for one hour with 1 μ M etoposide or 100 μ M 5-FU on the clonogenicity of MCF-7 cells. Lower panels demonstrate percentage decreases in colony formation. Overlapping curves denote additive effects.

Effects of bFGF on programmed cell death

Morphologic assessment

To demonstrate that the effects of bFGF on decreasing survival as measured by clonogenic studies were due to an effect on programmed cell death, morphologic studies on cellular apoptosis were undertaken. A number of techniques, including Hoechst staining, propidium iodide staining for determination of subdiploid DNA content, ethidium bromide agarose gel electrophoresis were attempted but did not yield satisfactory data able to accurately and reproducibly quantify 10-50% changes in the rate of apoptotic cells necessary to determine the effects of bFGF in this system. Finally, a relatively simple and highly reproducible system of counting cells with morphologic changes was successfully undertaken to carry out these studies. Cells were cultured on glass microscope slide cover slips placed in 6 cm tissue culture dishes in standard media. Incubations with bFGF and etoposide were done as described above. Cover slips were removed three days after incubation with etoposide and Wright-Giemsa stained using a LeukoStat Stain Kit (Fisher Diagnostics, Pittsburgh, PA). Four hundred cells were manually counted in a blinded manner in triplicate under oil immersion at 1,000x magnification using an Olympus BX40 light microscope and the percentage of cells demonstrating nuclear chromatin condensation, intracytoplasmic apoptotic bodies, cytoplasmic vacuolation and membrane blebbing were calculated.

Figure 5A demonstrates the morphologic appearance of the cells under the various treatment conditions. We have previously demonstrated that incubation of MCF-7 cells with bFGF inhibits proliferation and restricts cells to the G₁ phase of the cell cycle (8). MCF-7 cells treated with bFGF appear larger than control cells, with larger nuclei and more prominent nucleoli. Approximately 10% of the cells exhibit characteristic changes of chromatin condensation and fragmentation of the nucleus, with fragmented bodies visible in the cytoplasm. Treatment of MCF-7 cells with etoposide 3 μ M or 5-FU 100 μ M also induces the same changes as seen with bFGF alone at about the same approximate rate. However, pretreatment of cells with bFGF and subsequent exposure to these chemotherapeutic agents accentuates the frequency and intensity of these changes, as shown in the photographs. Figures 5B and 5C demonstrate numerically the increases in apoptosis due to bFGF, to the two chemotherapeutic agents and to the combination of bFGF with etoposide or 5-FU. Pretreating with bFGF 0.25 ng/ml for 24 hours increased the rates of apoptosis seen with etoposide concentrations of 1 and 3 μ M and with 5-FU 50 and 100 μ M for 1 hour in a statistically significant manner in triplicate experiments carried out twice and counted blindly. Figure 5D demonstrates in a control experiment that arresting cells in G₁ with 5 μ g/ml aphidicolin, a reversible DNA polymerase inhibitor, did not significantly increase the rate of apoptosis above that achieved by treatment with etoposide (VP-16) 1 μ M or 5-FU 100 μ M, while preincubation with 0.25 ng/ml bFGF did. Arresting MCF-7 cells with aphidicolin did have the same approximately 10% effect on increasing apoptosis over baseline as did bFGF, however. These experiments demonstrate that bFGF by itself or as a pretreatment prior to chemotherapy exposure increases the rates of apoptosis in MCF-7 cells as determined by morphologic changes.

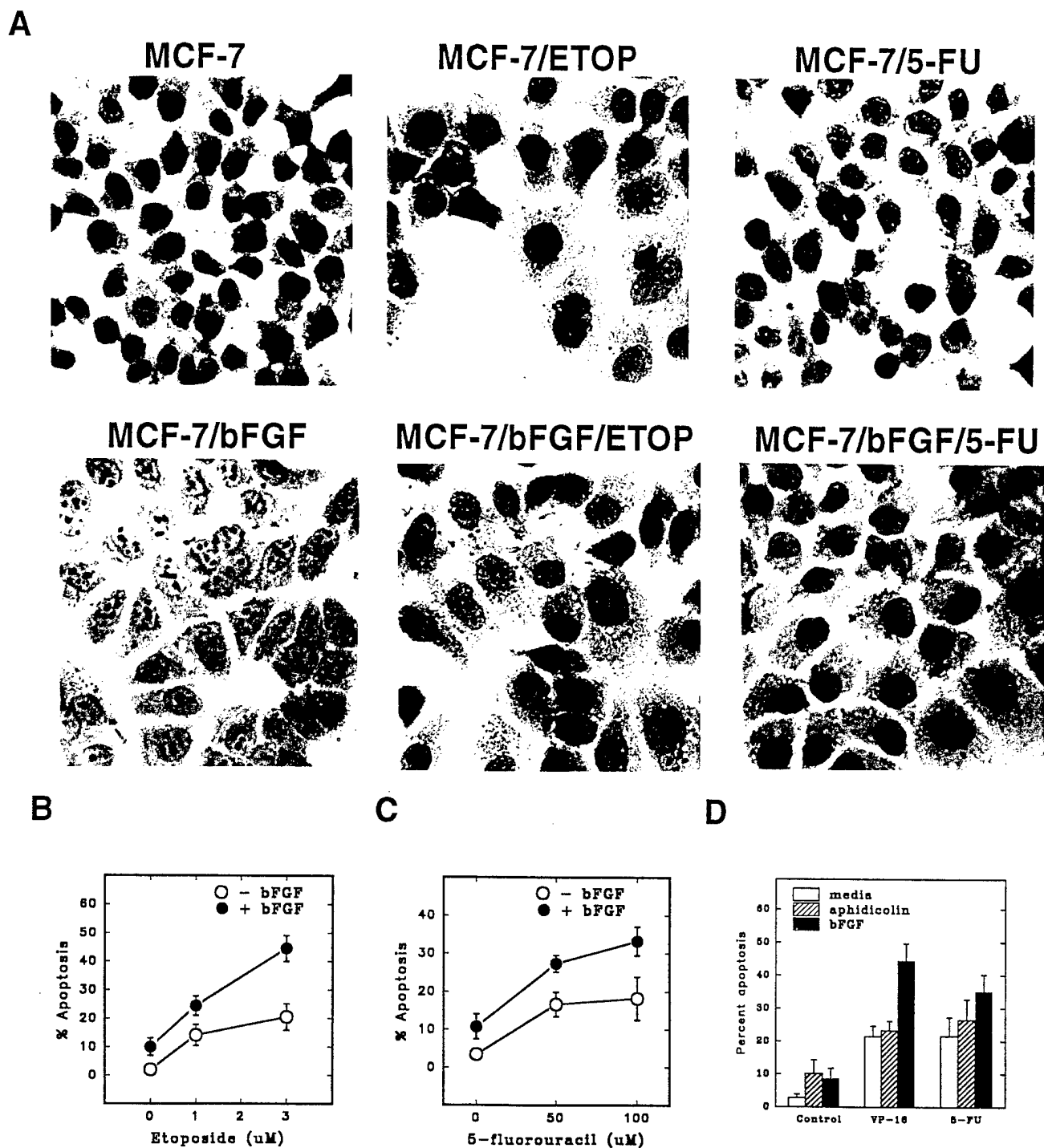


Figure 5. A. Morphologic appearance of MCF-7 cells, cells treated with bFGF, with etoposide, with 5-FU or with bFGF and etoposide and bFGF and 5-FU. Effect of bFGF 0.25 ng/ml incubation for 24 hours on etoposide-induced (B) or 5-FU-induced (C) apoptosis. D. effects of bFGF 0.25 ng/ml or aphidicolin 5 μ g/ml preincubation for 24 hours on one hour etoposide (VP-16) 1 μ M or 5-FU 100 μ M- induced apoptosis three days after treatment.

Measurement of DNA fragmentation

To confirm the data demonstrating an increase in cellular apoptosis induced by preincubation with bFGF, studies to measure DNA fragmentation were undertaken. The method of 3'-OH DNA end-labeling with dUTP-FITC and flow cytometric analysis was used. One to 5×10^5 cells were incubated in 10 cm diameter tissue culture dishes for 2 to 3 days in standard media. Media was removed and replaced with either fresh media or with bFGF at variable concentrations for 24 hours. Cells were then incubated with etoposide at the concentrations indicated for one hour. Apoptosis was measured two, three or four days later. Adherent cells were detached by treatment with trypsin and combined with cells which had become detached and were suspended in the supernatant. One million cells were centrifuged at 1200 rpm in a Beckman TJ-6 high speed centrifuge for 10 minutes at 4°C, washed with PBS, 2% FCS and fixed in 1% PBS-buffered paraformaldehyde on ice for 10 minutes. The cells were then washed with PBS, 2% FCS and permeabilized in a 0.1% Triton X-100, 0.1% sodium citrate solution for 10 minutes on ice. After an additional PBS, 2% FCS wash, cell pellets were resuspended in 50 μ l buffer containing 1 mM potassium cacodylate, 6.25 mM Tris-HCl, BSA 62.5 μ g/ml pH 6.6 (0.5X TdT reaction buffer, Boehringer Mannheim, Indianapolis IN) with 2.5 mM CoCl_2 and incubated with 3 nmol dATP, 2.5 U TdT, and 250 pmol FITC-dUTP in a 50 μ l volume at 37°C for 1 hour. The cells were then washed twice in PBS, 2% FCS and analyzed in a FCSTAR cytofluorimeter using a CELLQuest Version 1.0 program (Becton Dickinson, San Jose, CA).

Figure 6A demonstrates an incremental shift in the mean FITC-dUTP fluorescence in cell populations four days after treatment with increasing doses of etoposide or 5-FU. Pretreatment of cells with 0.25 ng/ml bFGF prior to exposure to chemotherapeutic agents caused incremental increases in fluorescence at all doses of drug attempted. Figure 6B demonstrates in graphic form the increased shifts in the fluorescence curve means in cells pretreated with bFGF. Figure 6C demonstrates that the observed increase in DNA fragmentation four days after a 24 hour bFGF treatment reaches an approximately 25% level at 0.25 ng/ml bFGF and remains stable. These values do vary from experiment to experiment, punctuating the technical difficulties in accurately quantitating percentages of apoptotic cells, however. As seen in the panel on the right, a maximal level of a 20% curve shifting did occur at 3 ng/ml bFGF. Nevertheless, all doses of bFGF used in the preincubations induced greater increases in DNA fragmentation of cells treated with etoposide 1 μ M or 5-FU 100 μ M than observed without bFGF pretreatment. This is illustrated by the graphic depiction of the data in the histograms in figure 6D.

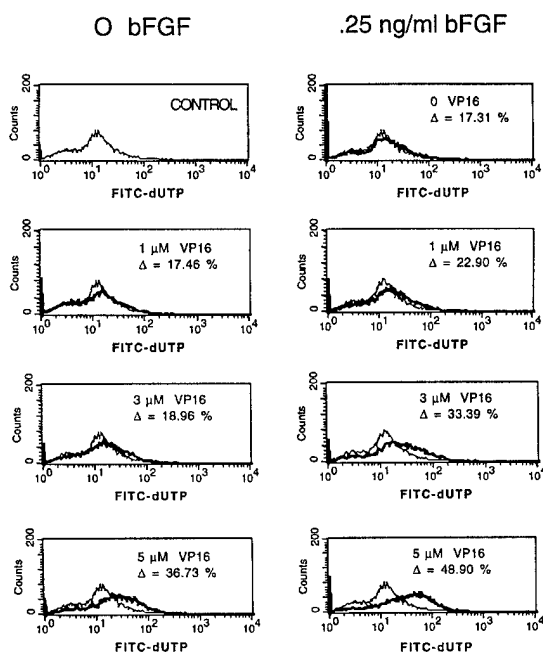
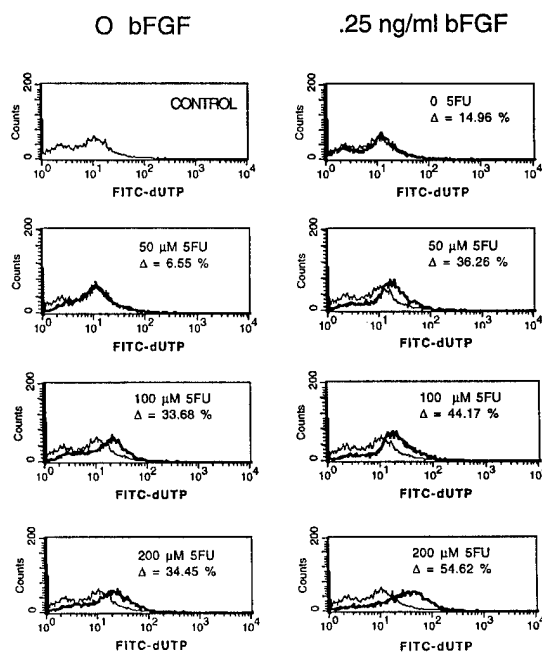
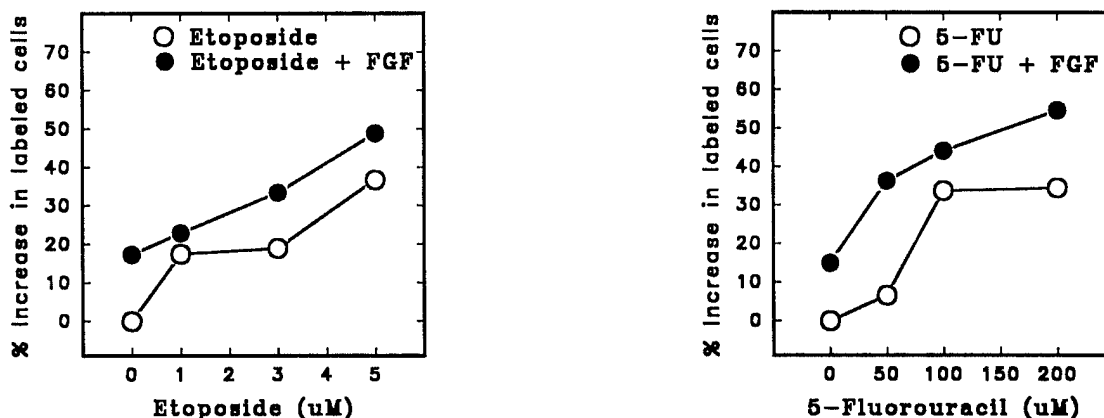
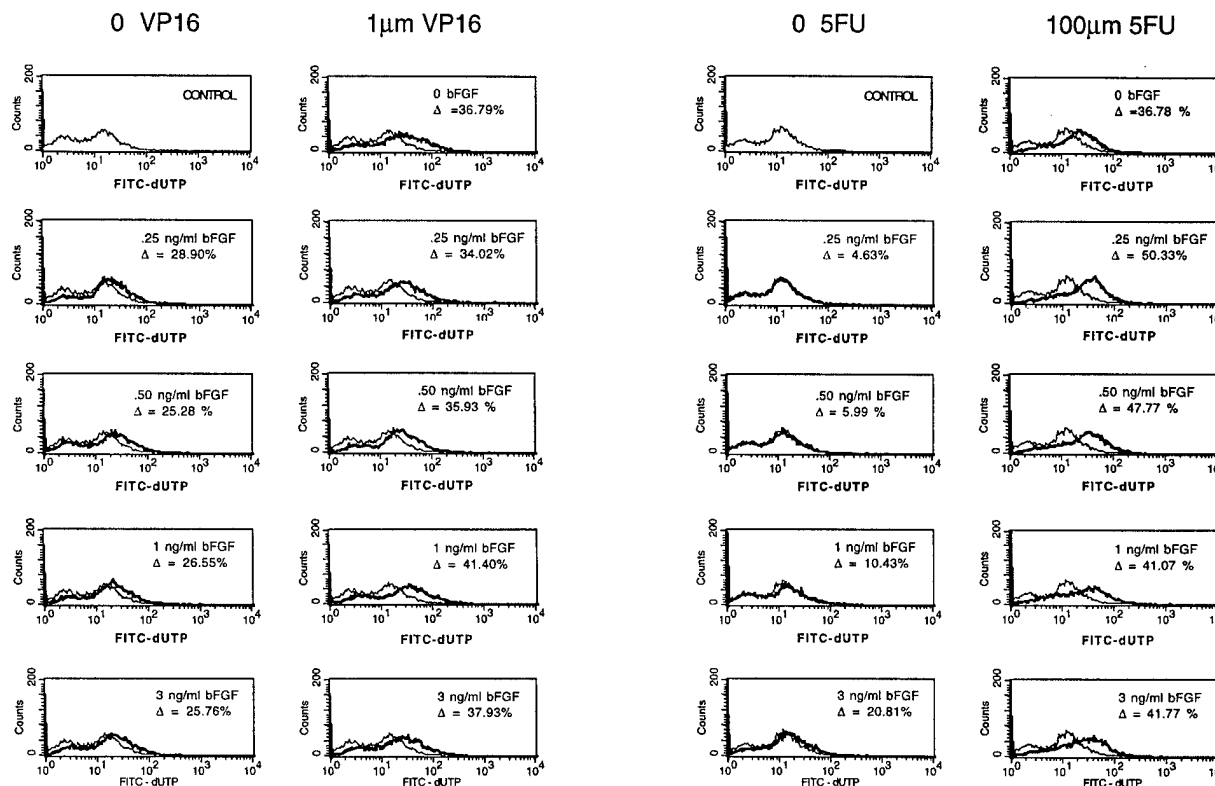
A**EFFECT OF bFGF ON VP16-INDUCED APOPTOSIS****EFFECT OF bFGF ON 5FU-INDUCED APOPTOSIS****B**

Figure 6. A. Flow cytometric analysis of MCF-7 cells treated for 1 hour with increasing doses of etoposide or 5-FU for one hour with or without a 24 hour pretreatment with bFGF 0.25 ng/ml that were labeled with FITC-dUTP by 3'-OH DNA terminal transferase four days after drug treatment. B. Graphic depiction of the percent shifts in the geometric means of the fluorescence curves as functions of bFGF pretreatment and drug dose.

C

EFFECT OF bFGF ON VP16-INDUCED APOPTOSIS

EFFECT OF bFGF ON 5FU-INDUCED APOPTOSIS



D

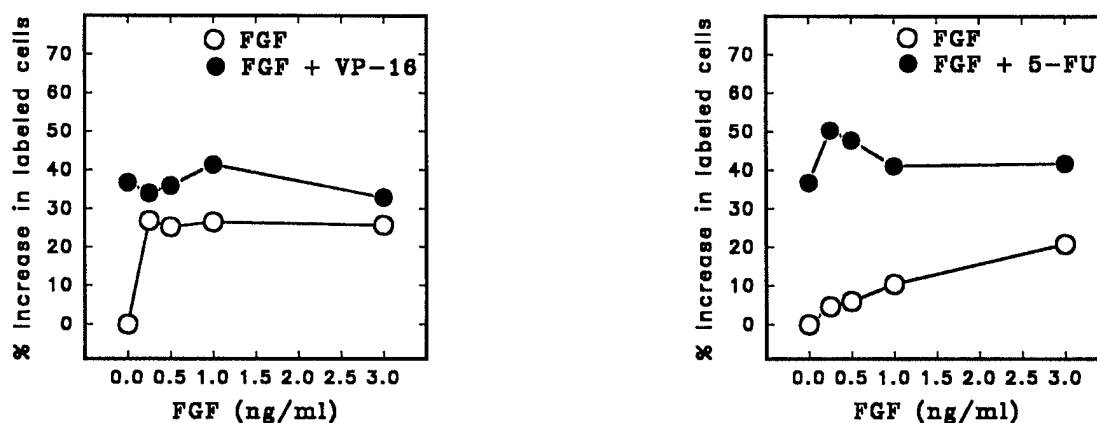


Figure 6. C. Flow cytometric analysis of MCF-7 cells treated for 24 hours with increasing doses of bFGF with or without subsequent treatment with etoposide 1 μ M or 5-FU 100 μ M for one hour, that were labeled with FITC-dUTP by 3'-OH DNA terminal transferase four days after drug treatment. D. Graphic depiction of the percent shifts in the geometric means of the fluorescence curves as functions of bFGF dose and drug treatment.

Immunoprecipitations and Western blots

In light of our observations that bFGF causes an increase in Bcl-2 protein levels in NIH 3T3 cells in which it is a survival factor, we measured the levels of Bcl-2 protein and mRNA using Western and Northern blotting in MCF-7 cells exposed to bFGF. Cells were grown to confluence and rendered quiescent by a 24 hour incubation in serum-free medium, consisting of DME, 2 mM glutamine, 0.5% endotoxin-free BSA fraction V (Calbiochem, La Jolla, CA). Cells were scraped from the plates in lysis buffer containing 20 mM Tris HCl pH 7.4, 1 mM Ethylene Glycol-bis(β -aminoethyl Ether) N,N,N',N'-Tetraacetic Acid (EGTA), 50 μ M NaVO₄, 50 mM NaF, 0.01 U/ml aprotinin, 1 μ g/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO), disrupted by sonication using a 4710 series Ultrasonics Homogenizer (Cole Parmer Instruments, Chicago, IL), centrifuged at 12,000 RPM at 4° C for 30 seconds in a microfuge, and the protein content of the lysates determined using a BioRad Protein Assay Kit I (Melville, NY) at 595 nm with a BSA standard. Lysates were mixed with 4x sample buffer to contain a final concentration of 0.25% Tris base, 8.4% SDS, 20% glycerol, 20% 2-mercaptoethanol, 0.4 ng/ml Pyronin Y and 0.6 ng/ml bromophenol blue, heat denatured at 100°C and 100 μ g of protein was electrophoresed in a 12.5% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) system. The gels were electroblotted onto Immobilon P membranes (Millipore Corp. Marlborough, MA), immunoblotted with the desired primary antibody and detected using the ECL chemiluminescence detection system (Amersham Corp., Arlington Heights, IL) (9). Total cellular RNA was prepared by the guanidinium thiocyanate extraction procedure, electrophoresed in 1% agarose gels, transferred to nylon filters by capillary transfer techniques and hybridized with ³²P-labeled 1 kbp hbFGF cDNA Eco R1 fragment used for construction of the bFGF vectors (10). Vector-specific sequences were identified by exposure to Kodak XAR-5 film at -80°C.

Figure 7A demonstrates that Bcl-2 levels decreased dramatically in a dose-dependent manner with addition of bFGF for 24 hours to MCF-7 cells. Figure 7B shows that bFGF also decreases the levels of Bcl-2 mRNA in a dose-dependent manner, as well, although the substantial increases occur only at 1.0 ng/ml or higher concentrations, and they appear far less than the decreases in Bcl-2 protein concentrations. Equal loading was determined by hybridization with an 18 S probe (not shown). Expression of bFGF may be modulated at both the mRNA levels as well as at the proteins levels. There is no data indicating whether the effects are on the synthetic or stability ends of the steady state levels of protein and mRNA observed. No effects of bFGF on Bax protein levels were observed in MCF-7 cells.

Co-immunoprecipitations of Bcl-2 and Bax were carried out, as described (11), to determine the effects of the decreased levels of Bcl-2 protein on the heterodimer formation between Bcl-2 and Bax. Briefly, cells were scraped from plates in 500 μ l KLB, containing 1% Triton X-100, 0.05% SDS, in PBS pH 7.0 containing 5 mM EDTA, 5 mM PMSF, 100 μ M NaVO₄ and 1 μ g/ml leupeptin (Sigma) (12) and lysed by a 5 second sonication using the Ultrasonics Homogenizer with a microtip at 4°C. 400 μ g protein were heat inactivated at 100°C for 10 minutes and incubated in 0.5 ml KLB with 5 μ g anti-Bcl-2 or anti-Bax monoclonal antibody overnight at 4°C. The antibody/protein complexes were immunoprecipitated with 15 μ l Protein G Plus/Protein A-Agarose (Oncogene Science, Uniondale, NY) and after a 2 hour 4°C incubation were centrifuged

for 15 minutes at 2000 rpm, washed twice with 1 ml KLB without SDS and twice with 50 mM Tris HCL pH 7.5 and electrophoresed and immunoblotted, as above. Figure 7C demonstrates that complexes immunoprecipitated with anti-Bax antibody contain less associated Bcl-2 if the cells were pre-treated with bFGF 10 ng/ml for 24 hours. The immunoprecipitates with anti-Bcl-2 antibody confirm the decreased cellular Bcl-2 in cells treated with bFGF.

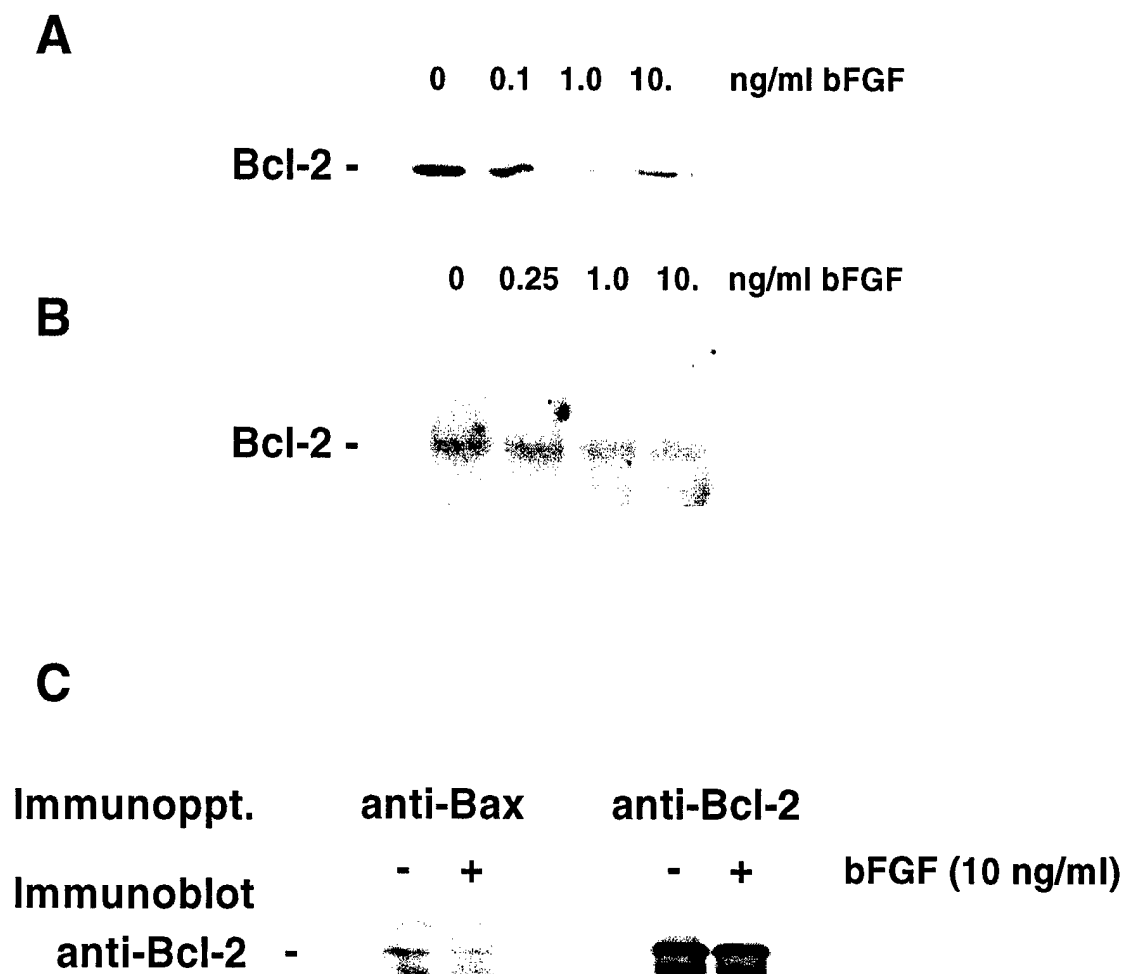


Figure 7. A. Effect of bFGF dose on cellular Bcl-2 protein content by Western blot after a 24 hour bFGF incubation. **B.** Effect of bFGF dose on Bcl-2 mRNA levels on Northern blot after 24 hour bFGF incubation. 18S RNA was used as a loading control. **C.** Effect of 24 hour bFGF incubation on co-immunoprecipitation of Bcl-2 with Bax in immunoprecipitation/Western blots.

CONCLUSIONS AND SIGNIFICANCE

Our data demonstrate that although bFGF is a survival factor in NIH 3T3 cells, it decreases survival in MCF-7 cells, as depicted by colony formation. The effects of bFGF on colony formation and survival from chemotherapy are additive with those of chemotherapeutic agents etoposide and 5-FU, and vary with the time and dose of incubation with bFGF. The effects are restricted to low dose densities of dose and time of exposure, suggesting different mechanisms responsible for cellular behavior at different bFGF concentrations. Programmed cell death was promoted by incubation of MCF-7 cells with bFGF alone and additively with the two chemotherapeutic agents. The death promoting effects of bFGF were evident when apoptosis was determined by morphologic criteria as well as by measurement of DNA fragmentation. The increased susceptibility to programmed cell death can be correlated with a decrease in cellular Bcl-2 protein levels that occur in a bFGF dose-dependent manner. Although there is no effect on Bax levels, the decreased cellular Bcl-2 protein results in a decreased association with Bax, allowing the latter to act as a death factor. The levels of Bcl-2 protein are modulated primarily at the protein level, although there is moderate downregulation of Bcl-2 mRNA as well, although to a much lesser extent. It is not clear whether modulation of mRNA or protein occurs at the synthetic or stability levels.

The significance of these data pertain to the effects of bFGF on breast cancers. If bFGF, which is a differentiation-associated factor in malignant and pre-malignant mammary epithelial tissue (13-15), acts as a death factor in mammary epithelial cells, loss of bFGF with dedifferentiation would render cells less responsive to the adverse effects of chemotherapeutic agents, thus establishing bFGF as an important prognostic factor in breast cancer. Further studies on the modulation of Bcl-2 by bFGF will gain further insight into the mechanisms used by mammary epithelial cells to modulate their survival in response to growth factors.

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