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Chemosensitization by fibroblast growth factor-2 is not dependent upon proliferation, S-phase accumulation, or p53 status

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Abstract

Fibroblast growth factor-2 (bFGF/FGF-2) is a pleiotropic growth factor that functions as a survival factor and directs apoptosis during embryogenesis and development. As a survival factor, FGF-2 would be expected to protect cells against drug toxicities. Such protection has been reported in some cells treated with some chemotherapeutic drugs. However, we recently demonstrated that FGF-2 can sensitize NIH 3T3 mouse fibroblasts to the cytotoxic and apoptotic effects of cisplatin. Sensitization requires prolonged incubation of cells with FGF-2 before the addition of cisplatin, and it requires an FGF-2 concentration (5–10 ng/mL) that is higher than that needed for its mitogenic effects (0.5 ng/mL). We now report that FGF-2 can also sensitize MCF7 human breast cancer cells and A2780 human ovarian cancer cells, as well as NIH 3T3 cells, to cisplatin. FGF-2 did not affect the cisplatin sensitivity of SKOV3 ovarian cancer cells or a panel of seven pancreatic cancer cell lines. We have demonstrated that the sensitizing effect is not simply a function of the mitogenic activity of FGF-2 on cells, as we did not observe sensitization with other growth-stimulatory factors (FGF-1 and epidermal growth factor); the sensitizing effect of FGF-2 was observed even with cell lines that were not growth-stimulated by FGF-2; and sensitization was not restricted to cells in S-phase of the cell cycle. These results indicate that cell proliferation is neither necessary nor sufficient for sensitization by FGF-2. Moreover, sensitization to cisplatin appears to be p53-independent, as p53-null 3T3 10-1 cells were equally sensitized by FGF-2. Finally, FGF-2 also sensitized NIH 3T3 and MCF7 cells to carboplatin, and had smaller effects on the sensitivity of these cell lines to doxorubicin and docetaxel. FGF-2 had no effect on sensitivity to etoposide in any cell line tested. Therefore, sensitization by FGF-2 was most effective with the platinum compounds, suggesting that this activity may be specific to particular mechanisms of drug action.

Keywords: Basic fibroblast growth factor; Chemotherapy; Cisplatin; Signal transduction; Ovarian carcinoma; Breast cancer

1. Introduction

A variety of intracellular and extracellular factors can affect the response of tumor cells to chemotherapy drugs, and the development of drug resistance is a constant limitation to the treatment of cancer. FGF-2 is a pleiotropic growth factor that functions in diverse areas of cell physiology, such as angiogenesis, cell differentiation and development, motility, and control of cell proliferation [1]. There are five different molecular weight isoforms of the FGF-2 protein, derived from differential initiation of translation from the same mRNA, and these isoforms have distinct subcellular localization [2,3]. The smallest isoform (18 kDa) is found in the cytoplasm and can be exported, whereas four larger isoforms (22-, 23-, 24-kDa and a recently discovered 34-kDa isoform) localize to the nucleus. Extracellular FGF-2 binds with high affinity to a family of four receptor-tyrosine kinases (FGFR1-4) and with lower affinity to heparan sulfate-containing proteoglycans [4,5], leading to intracellular signal transduction through the activation of phospholipase C and the ras-raf-MAPK pathway [6,7].

FGF-2 plays a critical role in embryogenesis, largely through its ability to help direct the appropriate pattern of cell death in the developing embryo. To control cell survival, FGF-2 exerts both antiapoptotic [8,9] and proapoptotic

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Abbreviations: EGF, epidermal growth factor; FGF-1, fibroblast growth factor-1; FGF-2, fibroblast growth factor-2; MAPK, mitogen-activated protein kinase; TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling.

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[10] signals on embryonic and differentiated cells *in vivo*. Furthermore, FGF-2 is a survival factor for certain differentiated cells *in vitro*, preventing apoptosis in cultured smooth muscle cells and neural cells [11,12].

Because FGF-2 has the potential for regulating apoptosis, it is of interest to know if it can affect the response of cells to DNA-damaging agents and chemotherapy drugs. Most studies in this area have described the ability of FGF-2 to enhance cell survival after drug treatment and/or DNA damage. Overexpression of a full length FGF-2 cDNA in NIH 3T3 mouse fibroblasts produces resistance to *N*-(phosphonacetyl)-L-aspartate [13], as well as to etoposide and 5-fluorouracil [14]. Furthermore, expression in NIH 3T3 cells of a secreted form of FGF-2 causes continuous activation of FGF receptors through an autocrine loop, which is thought to block cisplatin-induced apoptosis [15]. Similarly, FGF-2 exported by rat prostate tumor cells is capable of mediating resistance to paclitaxel, doxorubicin, and 5-fluorouracil [16]. Overexpression of FGF-2 in a human bladder cancer cell line also causes resistance to cisplatin, both *in vitro* and in tumor cells implanted into nude mice [17].

Exogenous FGF-2 can also affect the survival of cells exposed to cytotoxic agents. For example, addition of exogenous FGF-2 to endothelial cells inhibits apoptosis induced by ionizing radiation, both *in vitro* and in mice [18]. A direct link between FGF-2 and clinical resistance to chemotherapy has yet to be proven. However, high intracellular levels of FGF-2 in chronic lymphocytic leukemia correlate with resistance to apoptosis and higher survival when these leukemia cells are treated with fludarabine *in vitro* [19].

More recently, it has been demonstrated that FGF-2 can also have the opposite effect on cell survival, meaning it can sensitize cells to apoptotic stimuli and chemotherapy agents. For example, in the absence of drug treatment, FGF-2 induces apoptosis in Ewing's sarcoma cell lines [20]. Another study showed that FGF-2 and FGF-1, both neurotrophic survival factors, enhance apoptosis from oxidative stress in neuronal PC12 cells, whereas nerve growth factor and insulin protect these cells from this stress [21]. In MCF7 breast cancer cells, FGF-2 suppresses cell growth and alters the ratio of the mitochondrial proteins Bax and Bcl-2 such that cells are rendered more susceptible to apoptosis [22]. Another study with MCF7 cells has shown that exogenous addition of FGF-2 or ectopic expression of an FGF-2 cDNA increases the sensitivity of the cells to cisplatin and enhances cisplatin-induced apoptosis [23]. Finally, we have shown that prolonged exposure to exogenous FGF-2 sensitizes NIH 3T3 cells to cisplatin-induced cytotoxicity and apoptosis [24].

We now report that exogenous FGF-2 can also increase the sensitivity of human tumor cell lines to a variety of chemotherapy drugs. We used MCF7 cells and the ovarian tumor cell lines A2780 and SKOV3, in addition to mouse NIH 3T3 cells, and tested sensitivity to cisplatin, carboplatin, doxorubicin, etoposide, and docetaxel. We also tested a panel of seven pancreatic cancer cell lines with cisplatin. We found that FGF-2 pretreatment produced a strong and statistically significant sensitization to cisplatin and carboplatin in NIH 3T3 and MCF7 cells, while smaller effects on doxorubicin and docetaxel sensitivity were observed. Of the two ovarian tumor cell lines, only A2780 cells showed significant sensitization with FGF-2 pretreatment, and only to cisplatin. FGF-2 had no significant effect on etoposide toxicity in any of the cell lines tested, nor did it have an effect on the cisplatin sensitivity of any of the pancreatic cell lines. Importantly, the effect of FGF-2 on cisplatin sensitivity did not correlate with its effect on cell growth. This effect was also independent of S-phase accumulation and p53 status in mouse fibroblasts. Thus, the sensitizing activity of FGF-2 does not appear to be a secondary effect of its proliferative activity.

2. Materials and methods

2.1. Reagents and cell culture

Cell lines were obtained from the following sources: NIH 3T3 cells from Dr. M. Gottesman (National Cancer Institute); MCF7 and 3T3 10-1 cells from Dr. J. Momand (California State University); A2780 cells from Dr. T. Hamilton (Fox Chase Cancer Center); ASPC, BxPC, CaPan2, HS766T, HTB147, MIA, and Panc1 cells from P. Paty (Memorial Sloan-Kettering Cancer Center); and SKOV3 cells from the American Type Culture Collection. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (PS). The 3T3 10-1 cells were grown in identical medium except that 10% fetal bovine serum (FBS) was used instead of CS. Tumor cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS, 5 μg/mL of insulin (the pancreatic cell lines were grown without insulin), and PS.

Recombinant human FGF-2 (18-kDa isoform) was purchased from Promega, and stock solutions were prepared in PBS with 0.5% BSA. Drug stock solutions were prepared as follows: cisplatin (Sigma) at 1 mg/mL in saline was stored at room temperature and protected from light; carboplatin (Sigma) at 1 mg/mL in PBS was prepared immediately before each experiment; doxorubicin (Sigma) at 1 mg/mL in DMSO was stored at −20°; etoposide (Sigma) at 10 mg/mL in DMSO was stored at −80°, and was diluted in PBS before use; and docetaxel (Rhone-Poulenc) at 10 mg/mL in ethanol was stored at −80°.

2.2. Colony-forming assays

A total of $(4-10) \times 10^4$ cells were seeded in each well of a 6-well cluster plate in their standard growth medium,

and were allowed to attach overnight. The cells were incubated with 10 ng/mL of FGF-2 or PBS carrier for only 24 hr and then were exposed to different concentrations of chemotherapy drugs for 1 hr (cisplatin, doxorubicin, etoposide) or 24 hr (carboplatin, docetaxel), in the continued presence or absence of FGF-2. Pancreatic cell lines were exposed to cisplatin for 3 hr. The drug was then washed away, and the cells from each treatment were trypsinized and replated into triplicate 6-cm plates at 400–1200 cells per plate, depending on the cell line. The cells were incubated for 6–15 days in the maintained presence or absence of FGF-2, except for MCF7 cells and the pancreatic cell lines which were plated in the absence of FGF-2. Colonies were stained with 0.5% methylene blue in 50% ethanol. Plating efficiencies were calculated as the number of colonies formed in the absence of drug divided by the number of cells plated. Percent survival was calculated as the number of colonies formed at each drug concentration divided by the number of colonies formed in the absence of drug (±FGF-2), ×100.

2.3. TUNEL assays

For each cell line, 4×10^5 cells were seeded in 10-cm plates and allowed to attach overnight. The next day the cells were refed with standard growth medium containing 10 ng/mL of FGF-2 or PBS carrier only and incubated for 24 hr. Next, the cells were exposed to cisplatin in their standard growth medium (±FGF-2) for 8 hr (MCF7) or 1 hr (A2780), after which the cisplatin medium was removed and the cells were rinsed with PBS. After incubation for an additional 24 hr (MCF7) or 72 hr (A2780) in medium without cisplatin (±FGF-2), the cells were harvested. Floating and trypsinized adherent cells were collected and pelleted together. The cell pellet was resuspended in 0.5 mL of growth medium, and the suspension was added drop-wise to 5 mL of cold 1% paraformaldehyde and incubated for 15 min on ice. The cells were then washed once with PBS, resuspended in 5 mL of ice-cold 70% ethanol, and stored at −20° for 4 days before analysis. The fixed cells were stained for DNA cleavage by TUNEL and analyzed by flow cytometry, as described previously [24].

2.4. Proliferation assays

Cell growth was measured by seeding 2.5×10^4 cells (NIH 3T3) or 5×10^4 cells (MCF7) into 6-cm plates; or 1×10^4 cells per well in 24-well cluster plates (A2780 and SKOV3). The next day (day 0) each cell line was refed with its standard growth medium containing either 10 ng/mL of FGF-2 or the PBS carrier. The number of cells in triplicate plates (or wells) was determined at different time points after plating, including day 0, by trypsinization, staining with trypan blue and counting viable cells with a hemocytometer.

2.5. Cell cycle analysis

NIH 3T3 cells were synchronized by arrest in G_1/G_0 using contact inhibition and serum starvation. Actively growing plates of cells that had reached 90% confluence were rinsed with PBS and placed in DMEM containing 0.1% CS, and incubated for 48 hr. At this time, one plate was harvested and analyzed to ensure that the cells had arrested, while the remaining plates were trypsinized and plated at low density in regular growth medium containing 10 ng/mL of FGF-2 or PBS carrier only (12 plates each). At 15, 18, and 21 hr after release of growth arrest, one plate with FGF-2 and one plate without FGF-2 were trypsinized for cell cycle analysis. Another three plates each (±FGF-2) were analyzed for cisplatin sensitivity by exposing cells to 0, 6.7, or 10 μM cisplatin for 1 hr (±FGF-2), and then replating in the presence or absence of FGF-2 to allow colony formation, as described above. To measure cell cycle distribution, trypsinized cells were added drop-wise to ice-cold 70% ethanol, for fixation, and stored at −20°. For analysis, cells were pelleted from the ethanol by centrifugation (320 *g*, 5 min, 4°), washed once with Hanks' Balanced Salt Solution (HBSS) with 0.1% BSA, and resuspended in 0.5 to 1 mL HBSS with 5 μg/mL of propidium iodide (PI) and 50 units of ribonuclease A (DNAse free, Sigma). The samples were run and analyzed on a MoFlo (Cytomation) flow cytometer. Data were acquired and analyzed with the Summit program (Cytomation). Raw signal data were gated from a PI fluorescence vs. integrated-PI fluorescence scatter plot to exclude doublets and small debris.

3. Results

3.1. Cisplatin sensitization of MCF7 and A2780 cell lines

We have shown previously that 24-hr pretreatment with FGF-2 enhances cisplatin cytotoxicity in NIH 3T3 cells [24]. We expanded our investigation to examine this effect of FGF-2 on human tumor cell lines: MCF7 breast cancer cells, A2780 and SKOV3 ovarian cancer cells, and a panel of seven pancreatic cancer cell lines. NIH 3T3 cells were included in these studies as a control. The cell lines were cultured for 24 hr in the presence or absence of 10 ng/mL of FGF-2 and then exposed to various concentrations of cisplatin for 1 hr. Cytotoxicity was measured by a colony-forming assay as described in Section 2. Results are shown in Fig. 1 and Table 1.

FGF-2 enhanced cisplatin cytotoxicity in MCF7 and A2780 cells, as in NIH 3T3 cells (Fig. 1; Table 1). For MCF7 cells, the average IC_{50} from three independent experiments was reduced 2.5-fold in cells pretreated with FGF-2 compared with cells treated with cisplatin alone (Fig. 1B; Table 1). Over the course of four experiments with A2780 cells, the average IC_{50} for cisplatin was reduced 1.8-fold with FGF-2 (Fig. 1C; Table 1). These differences

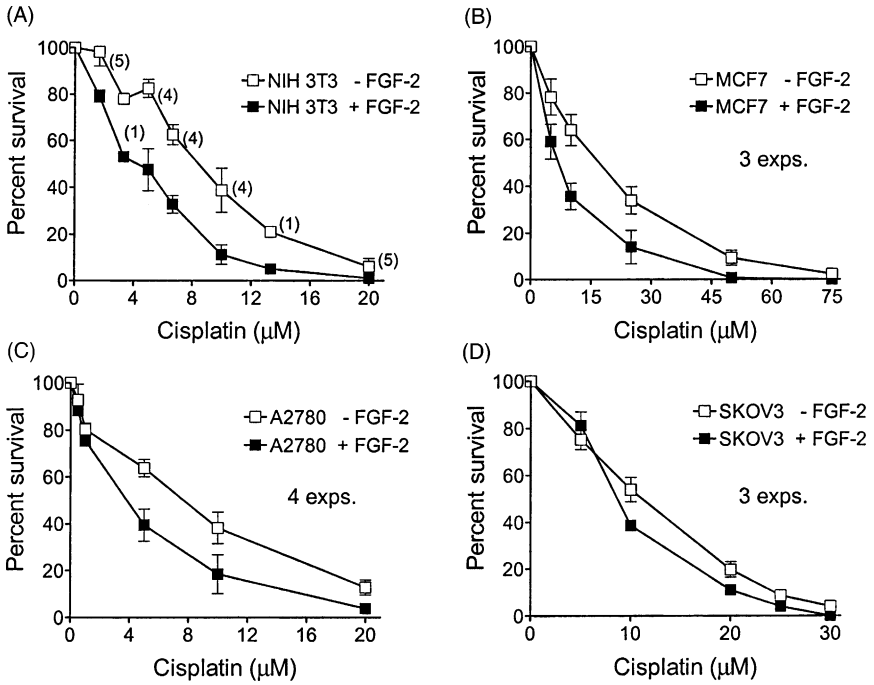

Fig. 1. Colony-forming assays with cisplatin in multiple cell lines. NIH 3T3 (A), MCF7 (B), A2780 (C), and SKOV3 (D) cells were incubated in the presence (closed symbols) or absence (open symbols) of 10 ng/mL of FGF-2 for 24 hr before exposure to various concentrations of cisplatin for 1 hr, and cell survival was measured by the colony-forming assay. Each point represents the average percent survival from one to four experiments, each plated in triplicate. The number of experiments performed is indicated in each panel (exps.) or at each data point (in parentheses). Percent survival within an experiment is the average number (from triplicates) of colonies formed at each cisplatin concentration divided by the average number of colonies formed in the absence of drug (±FGF-2), ×100. Error bars represent the SEM from the combined experiments.

Table 1
Cisplatin IC_{50} values derived from colony-forming assays

	−FGF-2		+FGF-2[b]		
Cell line[a]	IC_{50}[d] (μM)	P.E.[e]	IC_{50} (μM)	P.E.	N[c]
NIH 3T3	8.8 ± 0.5	29 ± 7	4.6 ± 0.4**	18 ± 8	5
MCF7	16.9 ± 1.4	44 ± 9	6.7 ± 1.0**	26 ± 11	3
A2780	8.0 ± 0.9	41 ± 6	4.4 ± 1.1*	27 ± 6*	4
SKOV3	11.3 ± 1.6	25 ± 5	8.6 ± 0.1***	15 ± 4	3
MIA	6.2 ± 0.9	31 ± 1	5.3 ± 0.0	28 ± 0	2
BxPC	1.5 ± 0.3	21 ± 1	1.5 ± 0.0	20 ± 3	2
HTB147	2.1 ± 0.4	15 ± 4	1.2 ± 0.3***	12 ± 3	3
HS766T	0.1	9	0.1	9	1
Panc 1	1.7	47	1.7	42	1
ASPC	1.7	49	1.7	51	1
CaPan2	2.2	39	2.2	41	1

[a] The cell lines listed below the gap are all pancreatic cancer cell lines.
[b] Data sets with three or more experiments were compared (+FGF-2 vs. −FGF-2) using paired, two-tailed *t*-tests: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; and *** not significant.
[c] Indicates the number of independent experiments performed with each cell line, each done in triplicate.
[d] Average cisplatin IC_{50} (μM) for each cell line. Values were derived from the results of colony-forming assays performed in the absence (−) or presence (+) of 10 ng/mL of FGF-2. Errors signify SEM (N > 2) or range (N = 2).
[e] Average plating efficiences (±SD) in the presence and absence of FGF-2 for each set of experiments. For MCF7 cells, all cells were incubated in the absence of FGF-2 during the colony-forming phase of the experiment.

were statistically significant for both cell lines. SKOV3 cells showed only a small increase in cisplatin sensitivity with FGF-2 treatment (Fig. 1D; Table 1), and this difference was not statistically significant. For the pancreatic cell lines, six showed absolutely no sensitization by FGF-2 and one, HTB147, showed a small, statistically insignificant effect of FGF-2 on cisplatin sensitivity (Table 1).

It should be noted that FGF-2 suppressed the plating efficiency of some of the cell lines tested. In the cisplatin experiments, the average plating efficiencies were lower for NIH 3T3, A2780, and SKOV3 cells pretreated and plated in FGF-2 (Table 1). A negative effect on plating efficiency and growth of MCF7 cells has been reported [22], and for these cells we found it necessary to plate all cells out of FGF-2 for the colony-forming phase of the experiment. Under these conditions, average plating efficiencies were still lower for cells pretreated with FGF-2 (Table 1). Essentially there were no differences in the plating efficiencies for the seven pancreatic cell lines, which were all plated in the absence of FGF-2 for the colony-forming phase of the assay (Table 1).

3.2. TUNEL assays in MCF7 and A2780 cells

To determine if the effect of FGF-2 on cisplatin colony formation corresponded to a similar effect on the cisplatin-induced death response, as we found previously with NIH

3T3 cells, we performed TUNEL assays with MCF7 and A2780 cells. Conditions of the assay are described in Section 2, and representative results are shown in Fig. 2. In MCF7 cells, the percentage of cells undergoing a death response in this experiment increased from 0.9% with cisplatin alone and 2.9% with FGF-2 alone (over a background level of 0.4%) to 11.2% with cisplatin plus FGF-2 (Fig. 2A). (Note that we refer to cell death because the TUNEL assay does not rigorously distinguish between apoptosis and necrosis.) A similar enhancement of cisplatin-induced death was observed over the course of three independent experiments, with an average 11-fold (±2.9-fold) enhancement of the

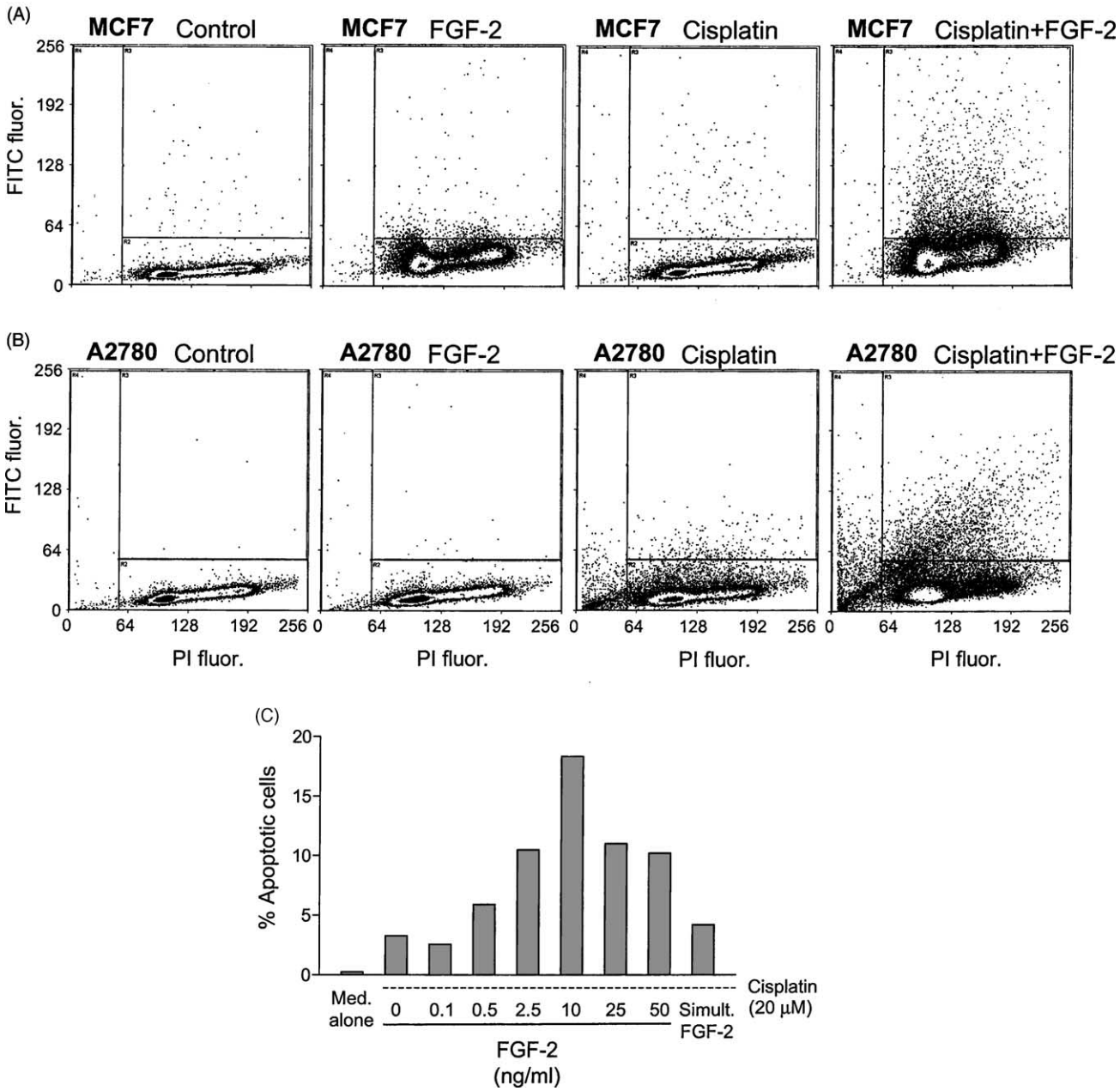

Fig. 2. TUNEL assays with cisplatin. The amount of cisplatin-induced cell death was measured by TUNEL staining, as described in Section 2 and analysis on a flow cytometer. TUNEL staining per cell is indicated by fluorescein isothiocyanate (FITC) fluorescence on the *y*-axis of the scatter plots, and the amount of DNA per cell is indicated by PI fluorescence 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. Total apoptosis was taken as the percentage of cells in the R3 + R4 regions. (A) MCF7 cells were pretreated with 10 ng/mL of FGF-2 or PBS carrier for 24 hr and then exposed to 33 μM cisplatin (±FGF-2) for 8 hr. The cells were incubated for an additional 24 hr (±FGF-2) and then harvested for TUNEL staining. (B) A2780 cells were analyzed as the MCF7 cells were, except that cisplatin was at 20 μM for 1 hr, and the cells were harvested 72 hr later. (C) A2780 cells were pretreated with different concentrations of FGF-2 for 24 hr and then exposed to cisplatin, as above. The presence or absence of FGF-2 was maintained for 72 hr after cisplatin exposure until the cells were harvested. Other cells received medium alone (−FGF-2, −cisplatin) for 48 hr before harvesting, or received FGF-2 (10 ng/mL) and cisplatin simultaneously for 1 hr, followed by FGF-2 for 72 hr and harvesting. Bars indicate the percentage of apoptotic cells from a single representative experiment, taken from the sum of cells staining TUNEL positive and cells with a sub-G_1 DNA content.

death response in FGF-2-treated cells over cells exposed to cisplatin alone.

FGF-2 also enhanced cisplatin-induced death in A2780 cells. In the experiment shown in Fig. 2B, cell death increased from 6.5% with cisplatin alone and 0.5% with FGF-2 alone (over a background of 0.4%) to 19.5% with cisplatin plus FGF-2. Over the course of five experiments, FGF-2 produced an average 3.7-fold (±0.4-fold) increase in cisplatin-induced death response in A2780 cells.

FGF-2 showed a concentration-dependent effect on cisplatin-induced death in A2780 cells, with a maximal enhancement at 10 ng/mL at FGF-2 (Fig. 2C). This effect also required preincubation with FGF-2, as simultaneous exposure to FGF-2 and cisplatin did not produce a meaningful increase in the death response over that produced by cisplatin alone (compare the "simult. FGF-2" bar (4.2% death) with the "0 FGF-2" bar (3.3% death) in Fig. 2C). The concentration- and time-dependent effects in A2780 cells were consistent with our previous results in NIH 3T3 cells [24].

3.3. Sensitization to other chemotherapeutic agents

We next determined the spectrum of chemotherapeutic agents for which FGF-2 could modulate cytotoxicity. Using colony-forming assays, we measured the effect of FGF-2 on the sensitivity of NIH 3T3, MCF7, A2780, and SKOV3 cells to carboplatin, doxorubicin, etoposide, and docetaxel, all common chemotherapy agents. FGF-2 incubations and drug treatments were identical to the conditions used with cisplatin, except that exposures to carboplatin and docetaxel were for 24 hr. Some results are shown in Fig. 3, and all IC_{50} data are summarized in Table 2. Like cisplatin, carboplatin reacts directly with

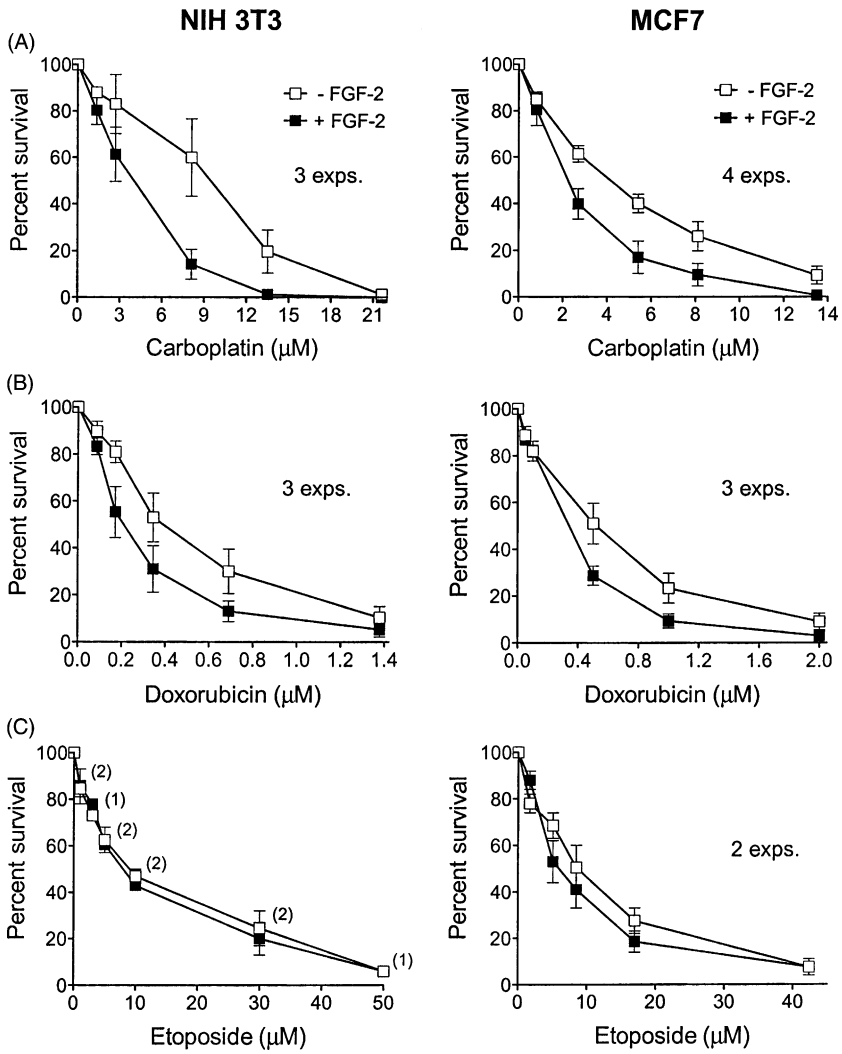

Fig. 3. Colony-forming assays with multiple chemotherapeutic agents. NIH 3T3 and MCF7 cells were incubated in the presence or absence of FGF-2 for 24 hr before exposure to carboplatin (A), doxorubicin (B), or etoposide (C). Cells were exposed to drugs for 1 hr (doxorubicin and etoposide) or 24 hr (carboplatin). Cytotoxicity was measured by the colony-forming assay, as in Fig. 1. The number of experiments performed with each drug is indicated. Each point represents the average percent survival ± SEM. Error bars represent ranges in the cases where only two experiments were performed. All results with these and other drugs are summarized in Table 2.

Table 2
IC_{50} values for other chemotherapy drugs

	Plating[a]		Carboplatin (μM)[b]			Doxorubicin (μM)			Etoposide (μM)			Docetaxel (nM)		
Cell line	−	+	−	+[c]	N[d]	−	+	N	−	+	N	−	+	N
NIH 3T3	33 ± 5	20 ± 5	3.3 ± 0.6	1.5 ± 0.3*	3	0.4 ± 0.1	0.2 ± 0.0*	3	9.4 ± 0.4	7.9 ± 0.4	2	7.1 ± 1.3	4.0 ± 0.2	4
MCF7	34 ± 9	17 ± 7	1.5 ± 0.1	0.8 ± 0.2*	4	0.6 ± 0.1	0.3 ± 0.0	3	9.5 ± 2.3	6.3 ± 1.8	2	0.8 ± 0.1	0.5 ± 0.1*	3
A2780	37 ± 4	28 ± 8	1.6 ± 0.0	1.1 ± 0.1	2	0.1 ± 0.0	0.1 ± 0.0	3	0.7 ± 0.1	0.5 ± 0.1	4	2.3 ± 0.1	2.0 ± 0.2	4
SKOV3	23 ± 2	9 ± 2	2.8 ± 0.9	2.0 ± 0.3	2	0.6 ± 0.1	0.5 ± 0.1	2	6.7 ± 1.1	6.2 ± 1.0	3	0.8 ± 0.3	0.9 ± 0.3	3

[a] Average plating efficiencies (±SD) in the presence and absence of FGF-2 for the entire set of experiments. For MCF7 cells, all cells were incubated in the absence of FGF-2 during the colony-forming phase of the experiment.
[b] Average IC_{50} values fore each cell line and each drug. Values were derived from the results of colony-forming assays performed in the presence (+) or absence (−) of FGF-2. Errors signify SEM (N > 2) or range (N = 2).
[c] Data sets with three or more experiments were compared (+FGF-2 vs. −FGF-2) using paired, two-tailed *t*-tests and were not significantly different unless indicated: $^*P \leq 0.05$.
[d] Indicates the number of independent experiments performed with each cell line and each drug, each done in triplicate.

DNA to form platinum adducts, so we expected to see a similar modulation of carboplatin cytotoxicity by FGF-2. FGF-2 did produce a statistically significant sensitization to carboplatin in NIH 3T3 cells ($P = 0.05$, 3 experiments) and MCF7 cells ($P = 0.03$, 4 experiments) (Fig. 3A). A2780 and SKOV3 cells (2 experiments each) showed only modest trends towards sensitization to carboplatin (Table 2).

With doxorubicin, FGF-2 sensitized NIH 3T3 cells ($P < 0.05$), and produced a trend (not statistically significant) towards greater sensitivity in MCF7 cells (Fig. 3B and Table 2). Essentially no sensitization was observed for A2780 or SKOV3 cells with doxorubicin (Table 2). With etoposide, FGF-2 did not sensitize any of the four cell lines tested (Fig. 3C and Table 2). With docetaxel, FGF-2 significantly sensitized MCF7 cells ($P = 0.013$), it had a small effect on NIH 3T3 cells (not statistically significant), and it had no effect on A2780 or SKOV3 cells (Table 2).

3.4. Drug sensitization vs. growth stimulation

FGF-2 is a mitogen for many cell types [1]. NIH 3T3 cells are growth-stimulated by FGF-2, although at a lower concentration than that needed for cisplatin sensitization [24]. To determine if the drug-sensitizing effect of FGF-2 correlated with its proliferative effect, we measured the effect of FGF-2 (10 ng/mL) on population growth in NIH 3T3, MCF7, A2780, and SKOV3 cells. Culturing in 10 ng/mL

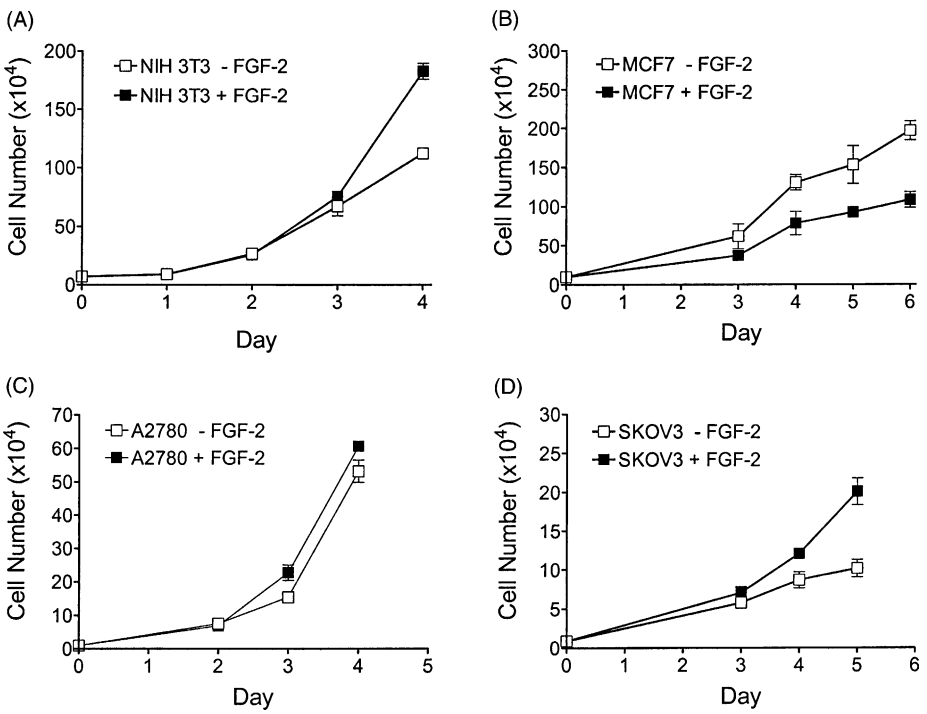

Fig. 4. Proliferation assays with multiple cell lines. Growth in the presence and absence of FGF-2 (10 ng/mL) was monitored in NIH 3T3 (A), MCF7 (B), A2780 (C), and SKOV3 (D) cells. Cells were plated in multiple wells and allowed to attach overnight. Cells were collected, stained with trypan blue, and counted on the following day (day 0), and on progressive days following. Data points are the averages from triplicate wells at each time point ± SEM.

of FGF-2 supported the growth of NIH 3T3 cells (Fig. 4A), consistent with our previous results in a [^{3}H]-thymidine incorporation assay [24]. FGF-2 produced a modest suppression of growth in MCF7 cells (Fig. 4B), consistent with previous reports [25]. A2780 cells showed a slight growth stimulation with FGF-2 (Fig. 4C), whereas SKOV3 cells experienced a more pronounced stimulation of growth (Fig. 4D). Therefore, there was no correlation between the effects of FGF-2 on cell growth and its effects on drug sensitivity: FGF-2 caused growth inhibition and sensitization to multiple drugs in MCF7 cells, whereas it caused growth stimulation but no sensitization in SKOV3 cells. Taken together, these data indicate that growth stimulation by FGF-2 is neither necessary nor sufficient for its effect on drug sensitivity.

We also wanted to know if other growth factors might have the same effect as FGF-2 on drug sensitivity. Since FGF-1 stimulates growth through the same family of receptors as FGF-2, we chose FGF-1 for further analysis in NIH 3T3 cells. FGF-1 did stimulate DNA synthesis in NIH 3T3 cells at concentrations above 10 ng/mL, as measured by [^{3}H]-thymidine incorporation assays (data not shown). However, FGF-1 (up to 100 ng/mL) had no effect, or a slight protective effect, on cisplatin-induced cytotoxicity and TUNEL staining in these cells (Fig. 5A and data not shown). For A2780 cells, we tested the effect of EGF, a classic mitogen for epithelial cells. EGF stimulated the growth of A2780 cells in a cell proliferation assay (data not shown), but had no effect on the level of cisplatin-induced death in A2780 cells (Fig. 5B). Therefore, growth stimulation by other growth factors was not sufficient to reproduce a sensitizing effect.

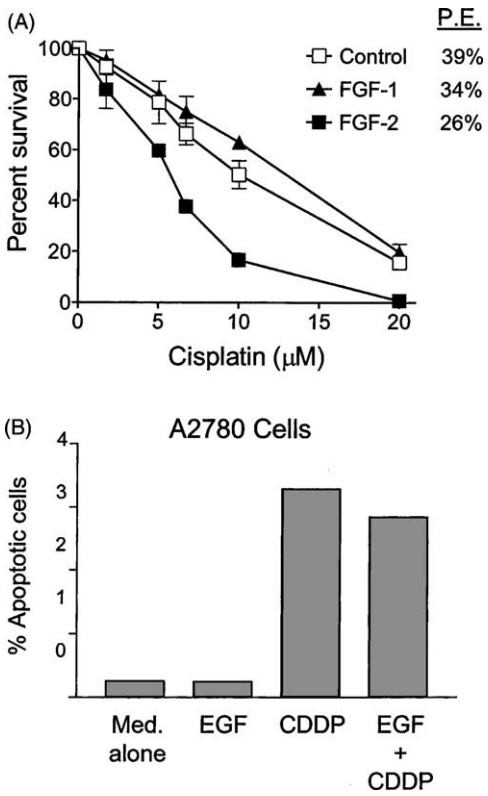

Fig. 5. Cisplatin assays with FGF-1 and EGF. (A) NIH 3T3 cells were incubated with 10 ng/mL of FGF-2, 100 ng/mL of FGF-1, or PBS carrier for 24 hr before exposure to increasing concentrations of cisplatin for 1 hr. Cisplatin cytotoxicity was measured by the colony-forming assay. A single representative experiment is shown, with error bars indicating SEM for triplicate plates at each drug concentration. The presence or absence of growth factors was maintained during colony growth. Plating efficiencies (P.E.) for each condition are indicated on the figure. (B) A2780 cells were pretreated with or without 25 ng/mL of EGF for 24 hr, and then incubated with cisplatin (±EGF) for 1 hr. The number of apoptotic cells was determined by the TUNEL assay, 72 hr after exposure to cisplatin (±EGF), as described in Fig. 2. Controls received medium alone (Med. alone) for 48 hr or EGF alone (EGF) for 48 hr before harvesting. CDDP = cisplatin alone; CDDP + EGF = cisplatin + EGF.

3.5. Drug sensitization throughout the cell cycle

Although stimulation of cell growth was not a necessary prerequisite for sensitization to cisplatin, it was still possible that FGF-2 might be exerting its effect by altering the cell cycle distribution of a cell population in a way that impacts drug sensitivity. We have demonstrated that FGF-2 actually delays entry into S-phase after G_1-synchronization [24] and therefore could influence the overall cell cycle distribution of a cell population. We sought to determine if cisplatin sensitization by FGF-2 was a function of a population's accumulation in S-phase or some other phase of the cell cycle. NIH 3T3 cells were synchronized in G_1 by contact inhibition and serum starvation, and then released into normal growth medium ± FGF-2. Cells were collected at various time points, and aliquots were removed to determine the cell cycle profile and sensitivity to cisplatin. Results are shown in Fig. 6. As previously demonstrated [24], cells released into FGF-2-containing medium took 3 hr longer to enter S-phase than untreated cells. Nevertheless, at each time point, cells released into medium containing FGF-2 showed a greater sensitivity to cisplatin than cells released into medium without FGF-2. Importantly, both synchronized populations remained predominantly in G_1 at 15 hr and cells at this time point were still sensitized by FGF-2. Moreover, they showed about the same magnitude of cisplatin sensitization as unsynchronized cells. Comparing the time points that showed comparable S-phase accumulation for the two synchronized populations (18 hr for cells lacking FGF-2 and 21 hr for cells exposed to FGF-2), there was generally less overall sensitivity to cisplatin in S-phase and only a slight sensitization to cisplatin in the presence of FGF-2. Overall, these data suggest that the effect of FGF-2 on cisplatin sensitivity is not a function of driving cells into S-phase and may be more pronounced during the G_1 phase of the cell cycle.

3.6. Drug sensitization as a function of p53 status

In some cell types, p53 appears to protect cells from cisplatin-mediated DNA damage [26–28]. Wild-type NIH

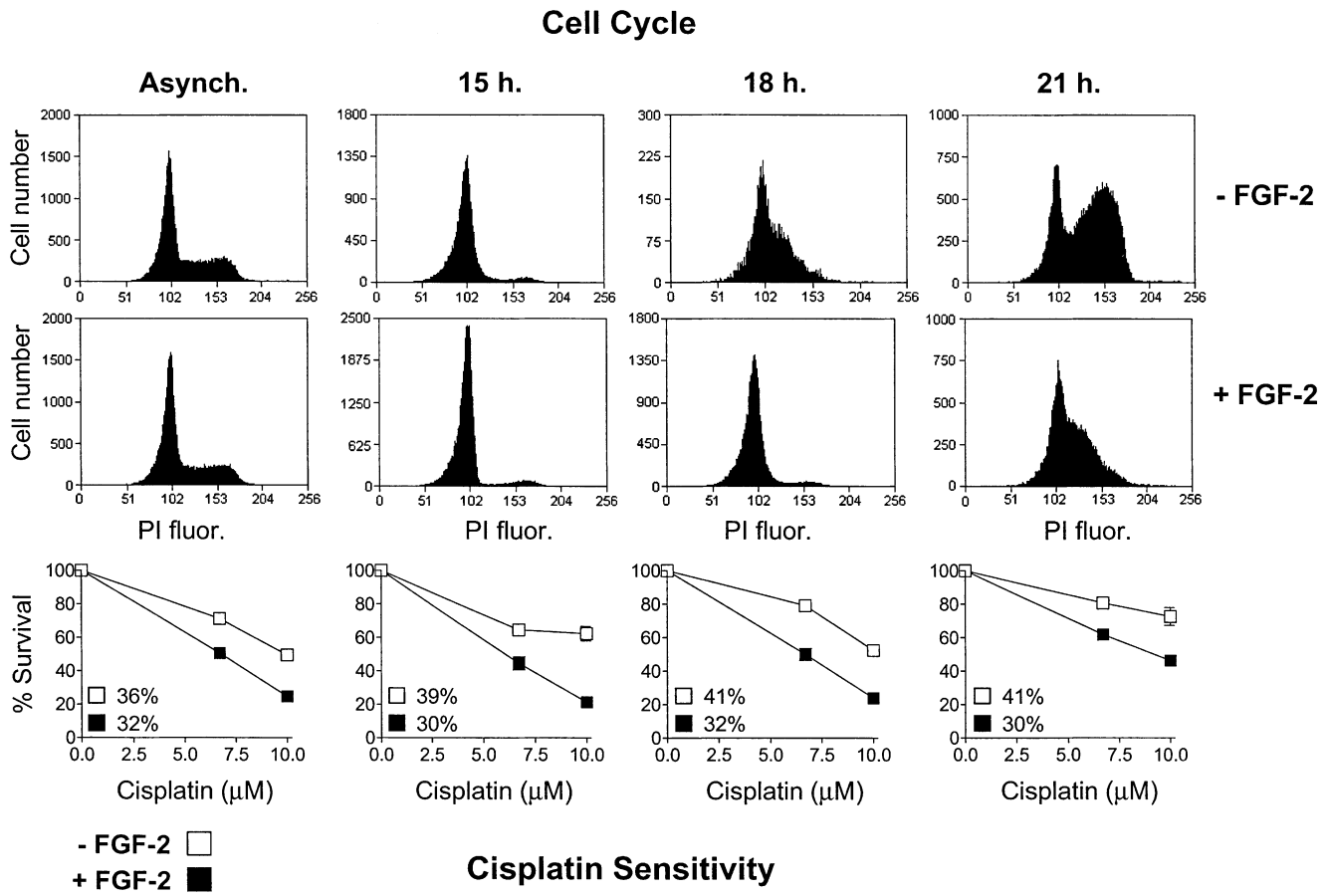

Fig. 6. Cisplatin colony-forming assays as a function of cell cycle. NIH 3T3 cells were growth arrested by contact inhibition and serum starvation for 48 hr, followed by trypsinization and replating at a lower density in full growth medium with or without FGF-2 (10 ng/mL). Cell cycle profiles were determined 15, 18, and 21 hr after release from synchronization, using PI staining and flow cytometry analysis. At each time point, parallel plates (±FGF-2) were exposed to 0, 6.7, or 10 μM cisplatin for 1 hr and replated at 400 cells per dish in triplicate to allow colony formation. Each point represents the average number of colonies formed at each cisplatin concentration divided by the average number of colonies in 0 drug (±FGF-2), ×100. Error bars represent SEM. Plating efficiencies in the presence and absence of FGF-2 are shown as insets of each concentration–response graph.

3T3 cells, MCF7 cells, and A2780 cells all have functional p53 and were sensitized to varying degrees by FGF-2; SKOV3 cells are p53-null, and the pancreatic cell lines shown in Table 1 are p53-mutant, and none of these were sensitized by FGF-2. Therefore, to determine if wild-type

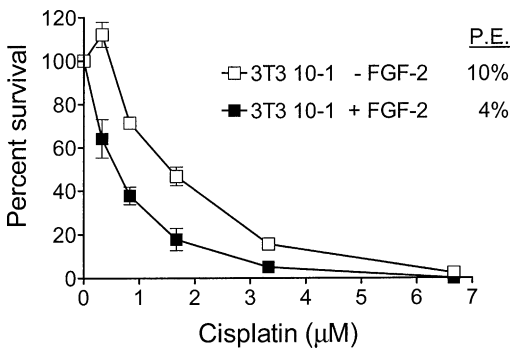

Fig. 7. Cisplatin colony-forming assays with p53-negative 3T3 10-1 fibroblasts. 3T3 10-1 cells were incubated with or without FGF-2 (10 ng/mL) for 24 hr before exposure to cisplatin for 1 hr. A single representative colony-forming assay is shown, with error bars indicating SEM for triplicate plates at each drug concentration. Plating efficiencies (P.E.) are indicated on the figure.

p53 function is required for the sensitizing effect of FGF-2, we measured cisplatin cytotoxicity in 3T3 10-1 cells, mouse fibroblasts that have a homozygous deletion of p53 [29]. Representative results are shown in Fig. 7. Preincubation with FGF-2 increased the sensitivity of 3T3 10-1 cells to cisplatin by 2.7-fold in this experiment (Fig. 7) and by 2.6-fold in a repeat experiment (data not shown). This suggests that functional p53 is not required for FGF-2 to sensitize 3T3 fibroblasts to the cytotoxic effects of cisplatin.

4. Discussion

We have followed up on our original report with NIH 3T3 mouse fibroblasts [24] to demonstrate that FGF-2 can modulate the cisplatin sensitivity of MCF7 breast cancer and A2780 ovarian cancer cell lines (Figs. 1 and 2; Table 1). No such sensitization was seen with SKOV3 ovarian cancer cells or with a panel of pancreatic cell lines (Fig. 1; Table 1). FGF-2 also sensitized NIH 3T3 cells to carboplatin and doxorubicin and showed a trend towards sensitizing to docetaxel. MCF7 cells were sensitized

significantly to carboplatin and docetaxel, with a trend towards sensitization to doxorubicin (Fig. 3; Table 2). There was no correlation between the sensitizing effects of FGF-2 and its proliferative effects on the cell lines tested, since FGF-2 caused proliferation but not sensitization of SKOV3 cells and sensitization but a slight growth inhibition of MCF7 cells (Fig. 4). Cisplatin sensitization was specific to FGF-2, as pretreatment with FGF-1 did not sensitize NIH 3T3 cells and pretreatment with EGF did not sensitize A2780 cells (Fig. 5). Moreover, the effect of FGF-2 on cisplatin sensitivity of NIH 3T3 cells did not depend on the cells being in S-phase of the cell cycle and might have been more biased towards cells in G_1 (Fig. 6). Taken together, these results suggest that cell proliferation is neither necessary nor sufficient to explain the effects of FGF-2 on cisplatin sensitivity. Finally, the ability of FGF-2 to sensitize 3T3 fibroblasts to cisplatin appeared to be p53-independent (Fig. 7).

FGF-2 appeared to have a small but reproducible effect on plating efficiencies in the colony-forming assays, for all but the pancreatic cell lines. The drug-sensitizing activity of FGF-2 cannot just be an artifact of its effect on plating, however, for several reasons. First, this effect did not correlate with the effect of FGF-2 on drug sensitivity: SKOV3 cells were not sensitized to cisplatin even though they showed the largest reduction in plating efficiency with FGF-2; A2780 cells showed only slightly reduced plating efficiency but they were sensitized significantly by FGF-2. Second, sensitization in the colony-forming assay always coincided with an enhanced death response in the TUNEL assay (see below), which does not involve re-plating of the cells at any point in the assay. Third, FGF-2 had no inherent cytotoxic activity towards most of the cell lines tested, as determined by the colony-forming assay, the TUNEL assay, and the proliferation assay and thus would not be expected to synergize with the cytotoxic drugs used in this study.

The case with MCF7 cells may be somewhat different. FGF-2 had an inhibitory effect on the growth of these cells (Fig. 4 and [25]), and their plating efficiency was reduced dramatically (greater than 10-fold) in medium containing FGF-2 (data not shown). An accurate assessment of the effect of FGF-2 on cisplatin sensitivity in MCF7 cells required plating in the absence of FGF-2 during the colony-forming stage of the toxicity assay. It is possible that more rigorous combination effect analyses will reveal interesting synergies between FGF-2 and chemotherapeutic agents in these cells.

4.1. FGF-2 sensitization and drug-induced death response

FGF-2 increased the sensitivity of MCF7, A2780, and NIH 3T3 cells to cisplatin by 2-fold or greater (Fig. 1 and Table 1), and this corresponded to an increased death response to cisplatin in each of these cell lines (Fig. 2 and [24]). Fenig *et al.* [23] also demonstrated that exogenous FGF-2 increases cisplatin cytotoxicity and apoptosis in MCF7 cells. FGF-2 alters the ratio of Bax and Bcl-2 in MCF7 cells [22], rendering them more susceptible to apoptosis, and perhaps accounting for the increased number of TUNEL-positive cells with FGF-2 (in the absence of cisplatin) in this cell line. A prolonged incubation with FGF-2 was necessary to enhance cisplatin-induced death in A2780 cells, as simultaneous addition of FGF-2 and cisplatin had no effect in the TUNEL assay (Fig. 2C). This appears to be consistent between cell lines, as we have reported a similar time-dependence in NIH 3T3 cells [24]. We do not know the minimum pretreatment time with FGF-2 that will produce the sensitizing effect, but exposure to FGF-2 for as few as 4 hr appears to render NIH 3T3 cells more sensitive to cisplatin (Coleman A and Kane S, unpublished observations).

SKOV3 cells and the panel of pancreatic cancer cell lines did not show any significant sensitization to cisplatin with FGF-2 treatment. The reason for the cell line variability is not known, but it further suggests a specific molecular cause for sensitization that may be better understood by comparing the effects of FGF-2 in susceptible and non-susceptible cell lines.

4.2. FGF-2 sensitization and cell proliferation

To begin to explore the mechanism by which FGF-2 sensitizes cells to cisplatin, we first wanted to rule out the trivial explanation that the phenomenon was simply a secondary effect of the proliferative activity of FGF-2. Three pieces of data argue against such an explanation. First, there was no correlation between the proliferative response of cell lines to FGF-2 and the ability of FGF-2 to cause chemosensitization. FGF-2 significantly enhanced cisplatin cytotoxicity in MCF7 cells but inhibited the growth of these cells (Fig. 4B). Conversely, FGF-2 stimulated the growth of SKOV3 cells but did not sensitize them to cisplatin. Second, the effect on sensitivity appeared to be specific to FGF-2. FGF-1 did not sensitize NIH 3T3 cells and EGF did not sensitize A2780 cells to cisplatin cytotoxicity or the death response (Fig. 5). Both growth factors were as mitogenic as FGF-2 for the cell line on which they were tested. Third, cisplatin sensitization by FGF-2 was not dependent upon NIH 3T3 cells being in S-phase of the cell cycle and was perhaps more pronounced for cells in G_1 (Fig. 6). Therefore, increased cisplatin cytotoxicity in FGF-2-treated cells must not simply be a result of FGF-2 driving more cells into S-phase during cisplatin treatment. On the other hand, we cannot rule out the possibility that slowed cell cycle progression or G_1 accumulation in the presence of FGF-2 might enhance sensitivity to some drugs.

4.3. Possible mechanisms of drug sensitization by FGF-2

The activation of p53 is a cornerstone of the cellular response to DNA-damaging agents. The p53 mediates the

response to DNA damage by activating the transcription of genes that regulate cell cycle arrest, apoptosis, and DNA repair [30]. At least one of these genes, GADD45, is a component of nucleotide excision repair, a pathway that functions in the repair of cisplatin-induced DNA damage [31]. Loss of functional p53 in some cells, including human foreskin fibroblasts, MCF7 cells, and A2780 cells, makes them more sensitive to cisplatin [26–28]. Thus, it is possible that FGF-2 treatment somehow interferes with the ability of p53 to mediate a DNA damage survival response, thus rendering the cells more sensitive to cisplatin. However, p53 does not seem to be involved in the sensitizing effect of FGF-2, at least in mouse fibroblasts, since FGF-2 sensitized both p53-wild type (NIH 3T3) and p53-null (3T3 10-1) fibroblasts to cisplatin to similar extents (2.7-fold for 3T3 10-1 cells (Fig. 7) vs. an average 1.9-fold for NIH 3T3 cells (Table 1)). As expected for p53-null cells, 3T3 10-1 cells were about 6-fold more sensitive to cisplatin, both with and without FGF-2, than were NIH 3T3 cells.

To explore the mechanism of drug sensitization further, we wanted to know if there was a pattern in the types of drugs that were susceptible to sensitization by FGF-2. Such a pattern could indicate a common type of DNA damage that was susceptible to the effects of FGF-2. We first tested carboplatin, which, like cisplatin, reacts with DNA to form platinum adducts and base cross-linking. FGF-2 sensitized NIH 3T3 and MCF7 cells to carboplatin to an extent similar to that of cisplatin (2-fold or greater) (Fig. 3A). A2780 cells were sensitized to cisplatin but showed essentially no increase in carboplatin cytotoxicity with FGF-2 treatment. We next tested doxorubicin and etoposide. Both are topoisomerase II inhibitors that facilitate double-strand DNA breaks [32], although doxorubicin can also cause oxidative DNA damage through the production of free radicals [33]. FGF-2 sensitized NIH 3T3 and MCF7 cells to doxorubicin (statistically significant ($P < 0.5$) only for NIH 3T3), although the effect was smaller than for cisplatin and carboplatin (Fig. 3B). There was no effect of FGF-2 on the sensitivity of NIH 3T3 and MCF7 cells to etoposide (Fig. 3C). FGF-2 also produced a modest sensitization to docetaxel, a microtubule-stabilizing agent, in NIH 3T3 and MCF7 cells (statistically significant ($P < 0.5$) only in MCF7 cells, Table 2). Again, the effect was smaller with docetaxel than with cisplatin or carboplatin, but this suggests that the sensitization effect may not be limited to DNA-damaging drugs.

The mechanism by which FGF-2 produced sensitization to these agents is not known, but there is precedence for growth factors and cytokines acting as intermediaries in response to DNA damage. Both FGF-2 and interleukin-1α (IL-1α) are up-regulated and exported when normal human fibroblasts and HeLa cells are UV irradiated, and these factors in turn induce downstream components of the UV radiation response, including secretion of proteases [34]. UV radiation and cisplatin both cause DNA damage that is repaired by nucleotide excision repair, and in this light it is interesting that exogenous IL-1α can also sensitize cells to cisplatin and carboplatin [35]. In these studies, IL-1α reportedly decreases the repair of DNA–platinum adducts. Furthermore, MAPK signaling pathways have been implicated in the induction of GADD45 gene transcription by a p53-independent mechanism [36]. Survival signals from MAPKs such as ERK1/2 also modulate cisplatin cytotoxicity [37,38]. Thus, although there is not yet any direct link between FGF-2 and DNA repair, it is possible to speculate that prolonged incubation with FGF-2 leads to down-regulation of a MAPK-associated survival response to cisplatin/DNA damage and that this leads to greater drug sensitivity. Clearly, other mechanisms that involve the intersection of FGF-2 signaling and specific drug responses are also possible. For example, the observed effects of FGF-2 on plating efficiencies in some cell lines point to a possible involvement of cell adhesion molecules. Indeed, it is known that FGF-2 is capable of interacting with and/or affecting the expression of various cell adhesion molecules and extracellular matrix components in ways that affect cell adhesion (reviewed in [1]). Separate from an effect on plating, however, it is possible that interactions with cell adhesion proteins could somehow translate into an enhanced potential for cell death in some cell lines and that this contributes to the mechanism of sensitization by FGF-2 in some cell lines. Importantly, the results with SKOV3 cells, which showed reduced plating efficiency with FGF-2 but were not drug-sensitized by it, suggest that an effect on cell adhesion proteins might not be sufficient for achieving enhanced toxicity.

We have demonstrated that FGF-2 can affect the response of human tumor cells to cisplatin *in vitro*. Whether FGF-2 plays a role in the response of a tumor to chemotherapy remains to be determined. However, understanding the mechanism by which FGF-2 facilitates chemosensitization *in vitro* should provide insight into whether and how FGF-2 might affect chemosensitivity *in vivo*, as well. In the long term, it may be possible to exploit this function of FGF-2 to improve the efficacy of conventional chemotherapy for the treatment of some types of cancer.

Acknowledgments

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