Autocrine Down-Regulation of Basic Fibroblast Growth Factor Receptors Causes Mitotoxin Resistance in a Human Melanoma Cell Line

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The ability of melanoma to develop resistance to mitotoxins, growth-factor–directed anti-neoplastic agents that offer potential for the treatment of this highly refractory disease, may limit therapeutic efficacy. To address this problem, we developed a subcloned human melanoma cell line that is resistant to the mitotoxin composed of basic fibroblast growth factor conjugated to the ribosome-inactivating protein saporin. Resistance was caused by autocrine FGF ligands, which down-regulate bFGF receptors and reduce bFGF-saporin binding. Inhibiting the autocrine loop with suramin or with neutralizing antibodies to FGF up-regulated receptors and decreased resistance *in vitro*. Furthermore, suramin restored sensitivity in resistant tumor xenografts. These results suggest the potential of therapeutic modalities combining agents that neutralize growth factors with receptor-directed mitotoxins for targeting malignant melanoma either to prevent emergence of resistance or to circumvent resistance once it occurs. ***Key words: suramin/drug resistance/xenografts/toxin conjugate. J Invest Dermatol 104:916–921, 1995***

The mitotoxin basic fibroblast growth factor (bFGF)-saporin is a hybrid cytotoxic protein composed of bFGF covalently coupled to a ribosomal toxin that offers a novel approach for the treatment of neoplastic disease. This mitotoxin attaches to the cell-surface growth factor receptor *via* the ligand and internalizes by receptor-mediated endocytosis; upon its release into the cytoplasm by the lower pH of the endosome, the saporin moiety catalytically inhibits protein synthesis by rendering the 60-S subunit of ribosomes unable to bind elongation factor 2, thus mediating cell death [1]. This approach may have therapeutic utility for the treatment of malignant melanoma, in part because bFGF receptor expression in most nonproliferating normal cells appears to be very limited [2] and because many melanoma cell lines express high-affinity receptors for bFGF [3]. Given the refractory nature of melanoma to traditional therapy and the potent cytotoxicity of bFGF-saporin that we and others have demonstrated for melanoma cells both *in vitro* and *in vivo* for xenograft and metastatic models [4,5], bFGF-saporin may offer a new strategy in chemotherapy.

As with conventional chemotherapeutic agents, however, effective treatment may be limited by the emergence of resistant tumor cell populations. Tumors eventually recur in xenografted mice, and up to 40% appear resistant to further therapy with bFGF-saporin (unpublished observations). As bFGF-saporin moves closer to clinical trials, it becomes increasingly important to understand resistance and to devise strategies for combating resistance. Because production of mitotoxin-resistant cells has been described by other investigators [6–9], these strategies might be widely applicable to other mitotoxin conjugates or immunotoxin conjugates as well.

We report here that subpopulations of human melanoma cells escape bFGF-saporin targeting because of a bFGF autocrine loop, and we provide evidence that this endogenous bFGF down-regulates bFGF receptors on these cells, thus reducing their availability for mitotoxin binding and subsequent cytotoxicity. We demonstrate that this mode of resistance may be circumvented by using agents such as suramin or neutralizing antibodies to FGF, which block the autocrine loop and up-regulate receptors, before treatment with bFGF-saporin to restore sensitivity *in vivo* and *in vitro*.

MATERIALS AND METHODS

Mitotoxin bFGF-saporin was a generous gift from D.A. Lappi and A. Baird (The Whittier Institute, La Jolla, CA). Saporin was conjugated to bFGF using N-succinimidyl-3(2-pyridydithio)proprionate, and the conjugate was purified by heparin-sepharose and chromatography, as described [10,11].

Development and Cloning of bFGF-Saporin–Resistant Cells SK-MEL-5 cells (American Type Culture Collection, Rockville, MD) were subjected to increasing selective pressure *in vitro* using a procedure adapted from Teicher and Frei [12]. Briefly, semiconfluent SK-MEL-5 cells growing in minimum essential medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco-BRL) and antibiotics were treated overnight with 10 nM bFGF-saporin (sufficient to kill approximately 90% of the cells). After treatment, the cells were washed three times with phosphate-buffered saline (PBS), fresh medium was added, and incubation of cells was continued. Each time the cells reached approximately 75% confluence, bFGF-saporin was administered in a dose escalated 15% from the previous treatment. When overnight treatment with 40 nM bFGF-saporin was reached, surviving cells were recovered and grown in medium supplemented with 10% fetal bovine serum and 2 nM bFGF-saporin to

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Abbreviation: DSS, disuccinimidylsuberate.

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maintain selective pressure. After approximately 1 year of treatment, including 1 month of growth in the constant presence of bFGF-saporin, resistant cells were cloned by limiting dilution without bFGF-saporin. One colony, FSB4, was expanded in the absence of bFGF-saporin selective pressure for 2 months. Subsequent bFGF-saporin survival assays revealed that it had retained resistance despite the absence of selective pressure. Doubling time for the FSB4 clone was approximately 18 h, compared with approximately 22 h for the parental SK-MEL-5.

Colony Formation Assay Subconfluent cell monolayers, seeded at a concentration of 2×10^4 cells/cm^2, were maintained overnight in supplemented minimum essential medium as above. The following day, the cells were washed with medium and then exposed to bFGF-saporin (5–150 nM) or saporin (25–1000 nM) for 1 h in serum-free medium. After treatment, the cells were washed three times with fresh medium, suspended by treatment with trypsin/ethylenediamine tetraacetic acid, and reseeded at 3×10^3 cells/cm^2 in duplicate. After 7 d, colonies were counted. Results are reported as the concentration of bFGF-saporin or saporin that resulted in 50% reduction in colonies. Results from three independent experiments were paired by treatment and analyzed using the paired Wilcoxon test (Instat; Graph Pad, San Diego, CA).

bFGF Receptor Cross-Linking Studies Cross-linking of ^{125}I-bFGF (Amersham, Arlington Heights, IL) to receptors on cells (2×10^5) from SK-MEL-5 parent and FSB4 resistant lines with 0.15 mM disuccinimidyl-suberate (DSS; Boehringer-Mannheim, Indianapolis, IN) was performed using the method of Yayon *et al* [13]. Cell extracts (from equivalent numbers of cells) were resolved by 7% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, and bFGF receptors were visualized by autoradiography using Kodak XAR-5 presensitized film with Lightning Plus intensifying screens at −70°C for 1–8 d.

bFGF Receptor Assay ^{125}I-bFGF binding was performed using a radioreceptor assay as described by Moscatelli [14]. Briefly, cells growing in six-well plates were washed with PBS and incubated with serum-free Dulbecco's modified Eagle's medium containing 0.15% gelatin and 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.4) for 1 h at 37°C. After 1 h, the medium was replaced with fresh medium containing ^{125}I-bFGF (5–50 ng/ml). After 2 h at 4°C on a platform rocker, the cells were washed once with cold PBS and twice with 2 M NaCl in 20 mM HEPES (pH 7.4) to remove ^{125}I-bFGF bound to extracellular matrix (low-affinity receptors) [14]. ^{125}I-bFGF bound to high-affinity receptors was released by two subsequent washes with 2 M NaCl in 20 mM sodium acetate (pH 4.0). Nonspecific binding was determined in the presence of 500-fold excess of unlabeled bFGF. Released radioactivity was quantitated in a gamma scintillation counter (MULTI-PRIAS; Packard Instrument, Sterling, VA).

bFGF Competition Assay SK-MEL-5 and FSB4 cells were seeded into 96-well tissue culture plates (Costar, Cambridge, MA) at 10^3 cells/well. The next day, the medium was removed and medium containing bFGF-saporin (1–5000 pM) or bFGF-saporin with 100-fold excess bFGF (0.1–500 nM) was added to the wells. Cells in triplicate wells were cultured for 72 h with bFGF-saporin. Surviving cells were counted using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

Detection of bFGF Receptor Proteins Receptor proteins were purified from 5×10^6 cells by microbatch affinity chromatography on the C-15 polyclonal *flg* antibody (specific for FGFR1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), linked to sepharose beads, solubilized by boiling in sodium dodecylsulfate (SDS) sample buffer with reducing reagents, and resolved by 7.5% SDS-PAGE. The receptor proteins were transferred to nitrocellulose filters and incubated with C-15 antibody (2 μg/ml), the filters were washed, and bound proteins were visualized by enhanced chemiluminescence, as directed by the manufacturer (Amersham). As a control, SK-MEL-5 cells were incubated with peptide sc-121P (Santa Cruz Biotechnology, Inc.) capable of blocking anti-*flg* binding.

Suramin and Neutralizing Antibodies in Cell Survival Assays Medium on subconfluent monolayers of parental or resistant cells was replaced with fresh serum-free medium with or without suramin (1 mM) (FBA Pharmaceuticals, New York, NY) or neutralizing antibodies (10 mg/l) to bFGF (Upstate Biotechnology, Inc., Lake Placid, NY) and/or to acidic FGF (aFGF) (R&D Systems, Minneapolis, MN). Fifteen hours later, the medium was removed and the cells were washed three times with unsupplemented medium before exposing cells to bFGF-saporin (5–150 nM) for 1 h in serum-free medium. After treatment, the cells were processed as in the colony assays described above, except that individual cells were counted. Results from three independent experiments were analyzed by the paired Wilcoxon test using Instat.

Detection of bFGF Intracellular and Extracellular Protein To detect intracellular bFGF, we isolated bFGF from extracts of 80% confluent cultures of parent and resistant cells using affinity chromatography on bFGF monoclonal antibody (Santa Cruz Biotechnology, Inc.) that had been covalently coupled to Protein A Sepharose CL-4B (Pharmacia LKB Biotechnology, Inc.) using dimethylpemilimidate (Sigma). To detect extracellular bFGF, cultures of parent and resistant cells were serum starved for 8 h and then incubated overnight in serum-free medium with bFGF-neutralizing antibody (UBI) covalently coupled to Protein A Sepharose CL-4B. bFGF was eluted from the immunosorbent as above. The intracellular and extracellular samples of bFGF and, as a control, 1 ng of human recombinant bFGF (Amersham), were reduced and alkylated (with 0.2 M N-ethylmaleimide [15]) before resolving the proteins by 12% SDS-PAGE. The proteins were transferred electrophoretically onto nitrocellulose (Hybond; Amersham), which was probed using antibody to bFGF (2 μg/ml) (Santa Cruz Biotechnology, Inc.) and visualized by chemiluminescence as above.

***In Vivo* Antitumor Studies** Cells from parental and resistant lines growing in culture were implanted subcutaneously into the right rear flanks of 8-week-old male Balb/c nu/nu mice at a concentration of 2×10^6 cells in 0.1 ml of unsupplemented minimum essential medium on day 0. Nude mice were bred and maintained by the Roger Williams Cancer Center Animal Care Facility in accordance with institutional guidelines. For all studies, bFGF-saporin was dissolved in sterile PBS/0.1% bovine serum albumin, and treatment (31–125 μg/kg) was delivered by intravenous tail injection 5 d after implantation, when the tumors were approximately 50 mm^3. For studies combining bFGF-saporin with suramin, suramin was dissolved in sterile PBS, and treatment (150 mg/kg) was delivered by intravenous tail injection on days 1–3 before bFGF-saporin treatment (62 μg/kg) on day 5. The progress of each tumor was measured at least twice weekly, and tumor volumes were calculated using the formula: Volume = [(minimum measurement)^2 (maximum measurement)]/2 [16]. Median tumor volumes for the various treatment groups were compared by Instat using the Kruskal-Wallis nonparametric test, followed by Dunn post-tests to compare the effects of various treatments.

RESULTS

Development of an SK-MEL-5 Subclone Resistant to bFGF-Saporin *In vitro*, a bFGF-saporin resistant cell line was developed by systematic dose escalation and was cloned by limiting dilution. Cell survival was determined in colony-formation assays for parental SK-MEL-5 and the cloned resistant subline, FSB4, exposed to various concentrations of bFGF-saporin or saporin alone. The 50% inhibitory concentration (IC_{50}) (150 nM) for bFGF-saporin in cloned FSB4 cells was sixfold higher than the IC_{50} (25 nM) for the parental SK-MEL-5 cell line ($p < 0.01$). Treatment with saporin alone demonstrated only minimal cytotoxicity, with IC_{50}s of 900 nM or greater in both cell lines; the FSB4 resistant line demonstrated a slight increase in sensitivity compared with the parental line. By colony-formation assays, sensitivity to bFGF-saporin returned gradually over 3–5 months in the absence of selective pressure (data not shown).

The Resistant Clone Displays Fewer bFGF Receptors Preliminary studies have suggested a direct correlation between the sensitivity of cells to bFGF-saporin and the number of FGF receptors per cell [10]; therefore, we hypothesized that bFGF-saporin resistance might be attributed to reduced expression of FGF receptors. To test this hypothesis, we cross-linked ^{125}I-bFGF to FGF receptors with DSS. SDS-PAGE analysis revealed a broad, 145–170-kDa band **(Fig 1)** consistent in size with that expected for bFGF cross-linked to FGF receptors [17]. Densitometric analysis of this band in three repeated experiments showed 0% to 25% as much receptor in FSB4 cloned cells as in the parental SK-MEL-5 cell line. We observed an interesting increase in high-affinity bFGF receptors that paralleled the loss of resistance during prolonged (3–5 months) culture in the absence of bFGF-saporin selective pressure, consistent with our developing hypothesis that a decrease in FGF receptor numbers was responsible for bFGF-saporin resistance.

By ^{125}I-bFGF radioreceptor assay, parental SK-MEL-5 cells had approximately 23,000 high-affinity binding sites per cell by Scatchard analysis [18], similar to previously determined values [3]. Consistent with the reduced receptor cross-linking of FSB4 cells **(Fig 1)** and their reduced ^{125}I-bFGF binding, the resistant cells

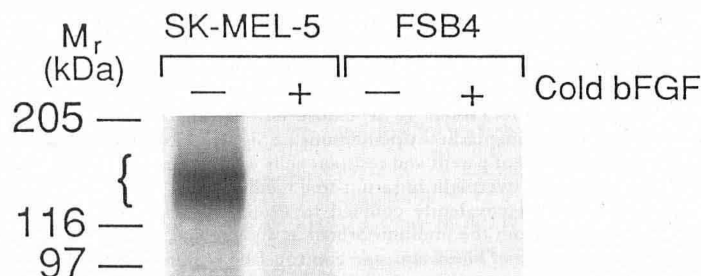


Figure 1. Resistant cells display markedly reduced amounts of FGF receptors by cross-linking of ^{125}I-bFGF. Cells were allowed to bind ^{125}I-bFGF for 2 h at 4°C with or without a 200-fold excess of unlabeled bFGF. Unbound ^{125}I-bFGF was washed away, and bound ^{125}I-bFGF was cross-linked using DSS to the FGF receptors. The cross-linked complexes were extracted from the cells, analyzed by 7% SDS-PAGE under reducing conditions, and visualized by autoradiography. *Brace* indicates position of the 145–170-kDa band.

displayed only approximately 7000 high-affinity receptors per cell, with little apparent change in dissociation constant (K_d) (155 pM) from the parental line (130 pM). Resistant FSB4 cells also expressed one third fewer low-affinity receptors (approximately 400,000) than parental cells (approximately 600,000) **(Fig 2*B*)**.

Exogenous bFGF Renders the Parental Cells Insensitive to bFGF-Saporin and Down-Regulates bFGF Receptors It is interesting that a 100-fold excess of bFGF, which competes with bFGF-saporin for binding to FGF receptors [9], shifted the parental survival curve ($p < 0.002$) to nearly coincide with that of the FSB4 cells ($p > 0.05$) **(Fig 3*A*)**. In light of these results and because autocrine bFGF has a major role in the transformation and growth of melanoma cells [19], we hypothesized that excessive bFGF produced by FSB4 cells might be down-regulating bFGF receptors. Consistent with this possibility, chronically exposing parental cells to exogenous bFGF down-regulated receptors, an effect that could be reversed within 24 h by removing the exogenous bFGF from culture **(Fig 3*B*)**.

Blocking Autocrine bFGF Increases bFGF Receptors and Abrogates bFGF-Saporin Resistance To test whether autocrine growth factors might be similarly down-regulating bFGF receptors and causing bFGF-saporin resistance in FSB4 cells, we

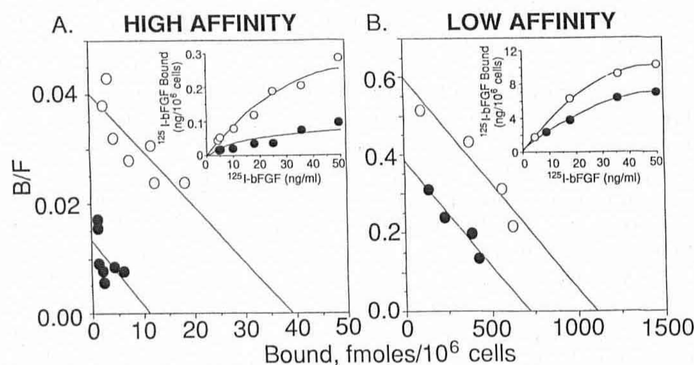
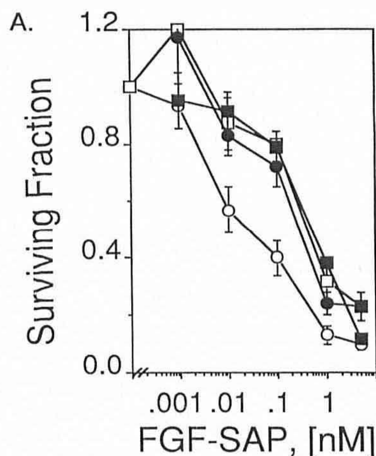


Figure 2. Resistant cells display reduced numbers of FGF receptors by Scatchard analysis. ^{125}I-bFGF binding to parental SK-MEL-5 (*open circles*) and to the resistant FSB4 (*closed circles*) cells was determined by radioreceptor assay [14]. Low-affinity binding (*B*) was designated as the amount of ^{125}I-bFGF released from the extracellular matrix by high-salt washes at pH 7.4. High-affinity binding (*A*) was designated as the amount of ^{125}I-bFGF released by subsequent high-salt washes at pH 4.0. Nonspecific binding was determined in the presence of 500-fold excess of unlabeled bFGF. The data are shown as Scatchard plots. B/F, ratio of bound ^{125}I-bFGF to unbound ^{125}I-bFGF.



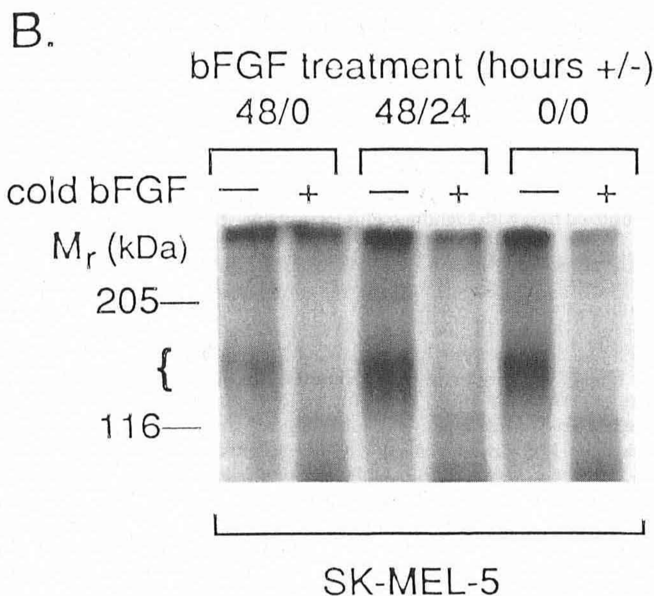


Figure 3. Exposure to exogenous bFGF reduces parental expression of bFGF receptors and renders the parental cells resistant to bFGF-saporin. *A*, SK-MEL-5 resistance to bFGF-saporin (SAP) (0.001–5 nM) in the absence (*open circles*) or presence (*closed circles*) of 100-fold excess of bFGF; FSB4-resistant cells were exposed to bFGF-saporin in the absence (*open squares*) or presence (*closed squares*) of 100-fold excess bFGF. After 3 d of treatment, the cells were suspended by trypsinization and counted by Coulter Counter. Results are expressed as the mean surviving fraction of treated cells normalized to media controls (n = 6). *Bars*, SEM. *B*, the amount of high-affinity bFGF receptor on parental cells is reduced by exposure to exogenous bFGF. SK-MEL-5 parental cells were cultured for 24 h in the presence of recombinant bFGF (18 ng/ml), then for an additional 24 h in the presence or absence (hours +/−) of bFGF. ^{125}I-bFGF binding and cross-linking to these cells was performed as in **Fig 1**. *Brace* indicates position of the 145–170-kDa band.

first examined the expression of FGF receptor type-1 (*flg*) in SK-MEL-5 and FSB4 with or without pretreatment with suramin, a polyanionic compound that blocks growth factor binding to receptors [20–23]. Receptor immunoblotting revealed receptor-specific proteins of 90 kDa, 110 kDa, 130 kDa, and 150 kDa in both SK-MEL-5 and FSB4 cells; however, the resistant FSB4 cells contained a larger proportion of bFGF receptor as the 110-kDa protein **(Fig 4)**. By pulse chase analysis, the 90-kDa protein appeared to be a precursor of the 130-kDa and 150-kDa receptor proteins, whereas the 110-kDa protein appeared to be a degradation product of the mature, 150-kDa and 130-kDa bFGF receptors (data not shown). Exposure to suramin for 15 h increased the

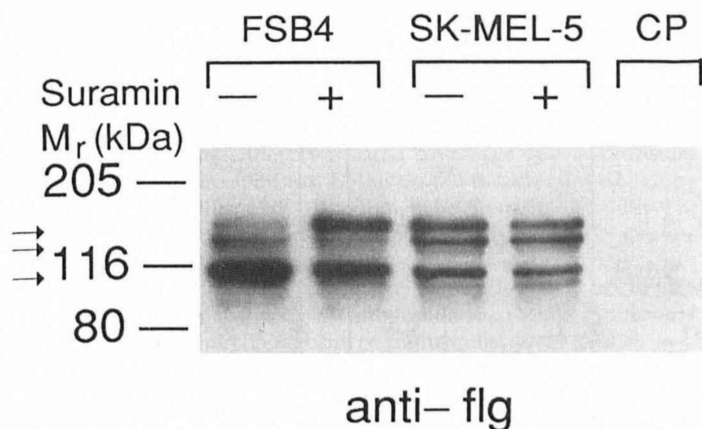


Figure 4. Suramin treatment of resistant cells increases their expression of high–molecular-weight bFGF receptor. Parental SK-MEL-5 cells or the resistant FSB4 cells were cultured for 15 h in the presence (+) or absence (−) of 1 mM suramin. bFGF receptors were immunoprecipitated with antibody to the receptors (anti-*flg*) and analyzed by chemiluminescent anti-*flg* immunoblotting. As a control, SK-MEL-5 cells were incubated with a control peptide (CP) capable of blocking anti-*flg* binding. The positions of molecular-weight standards are indicated. *Arrows* indicate positions of the 150-kDa and 130-kDa FGF receptors (*flg*) and of a 110-kDa putative degradation product.

intensity of the 150-kDa receptor while decreasing intensity of the 130-kDa receptor and the 110-kDa "degradation" product. Consistent with both these data and our developing hypothesis of autocrine-driven resistance to bFGF-saporin, pretreatment with suramin had little effect on the bFGF-saporin sensitivity of parental cells *in vitro*. However, suramin reduced by approximately 60-fold the concentration of bFGF-saporin required to yield a 50% inhibition in FSB4 survival **(Fig 5*A*)** ($p < 0.0001$). It is noteworthy that this rendered FSB4 cells nearly 10 times more sensitive than parental cells to bFGF-saporin.

To determine whether suramin might be exerting its effects by complexing with autocrine bFGF, we tested whether neutralizing antibody to bFGF would similarly restore bFGF-saporin sensitivity to FSB4 cells. SK-MEL-5 and FSB4 cells were treated overnight with neutralizing antibody specific for human bFGF, washed, and then treated with bFGF-saporin. This pretreatment increased the bFGF-saporin sensitivity of FSB4 cells so that their 7-d survival curve was indistinguishable from that of the parental cell line **(Fig 5*B*)** ($p > 0.1$). Taken together, these results suggest that suramin's ability to restore bFGF-saporin sensitivity to resistant FSB4 cells occurs, at least in part, from its ability to block the interaction between endogenous bFGF and its receptor. However, resistant cells pretreated with bFGF neutralizing antibody did not demonstrate the hypersensitivity engendered by suramin. Perhaps the resistant cells constitutively secreted another growth factor in addition to bFGF capable of competing for bFGF receptors. A likely candidate is aFGF, inasmuch as both aFGF and bFGF bind with high affinity to FGF receptor type-1 (*flg*) and to FGF receptor type-2 (*bek*) [24]. Although pretreatment of parental SK-MEL-5 cells with neutralizing antibody to bFGF did not affect their sensitivity to bFGF-saporin, pretreatment with neutralizing antibody specific for aFGF increased their sensitivity, suggesting that aFGF partially inhibits bFGF-saporin binding and cytotoxicity in parental cells **(Fig 5*B*)**. Similarly, pretreatment of FSB4 resistant cells with neutralizing antibody to aFGF increased their sensitivity to bFGF-saporin severalfold ($p < 0.0001$). Combining neutralizing antibodies to aFGF and bFGF for pretreatment of FSB4 cells further increased FSB4 sensitivity ($p < 0.0001$), comparable to FSB4 cells treated with suramin and to parental cells treated with neutralizing antibody to aFGF alone or the combination of neutralizing antibodies to aFGF and bFGF. These findings suggest that both parental and resistant cells produce autocrine FGFs; however, though autocrine aFGF plays a role in decreasing bFGF-saporin sensitivity in both cell lines, autocrine bFGF is responsible for the differential sensitivity to bFGF-saporin observed between SK-MEL-5 parental cells and FSB4 resistant cells.

Comparison of Endogenous bFGF in Resistant and Parental Cells These results also suggested that resistant FSB4 cells somehow differ in their expression of bFGF compared with parental SK-MEL-5 cells. To investigate this further, we purified bFGF from SK-MEL-5 or FSB4 cells by immunoaffinity chromatography and analyzed it by immunoblotting. An 18-kDa band co-migrating with human recombinant bFGF was detected in both the parent and resistant lines **(Fig 6)**. In addition, 22- and 24-kDa bands representing bFGF arising from an alternative translation-initiation site [25] were also detected. Densitometric analysis of the bands suggested that resistant FSB4 expressed only 35% more total bFGF than parental cells, with no relative change in isoform expression. These observations suggest an overall increase in bFGF production or in its stability as opposed to the use of a more efficient initiation codon [25] in the FSB4 cells; however, it remains to be ascertained

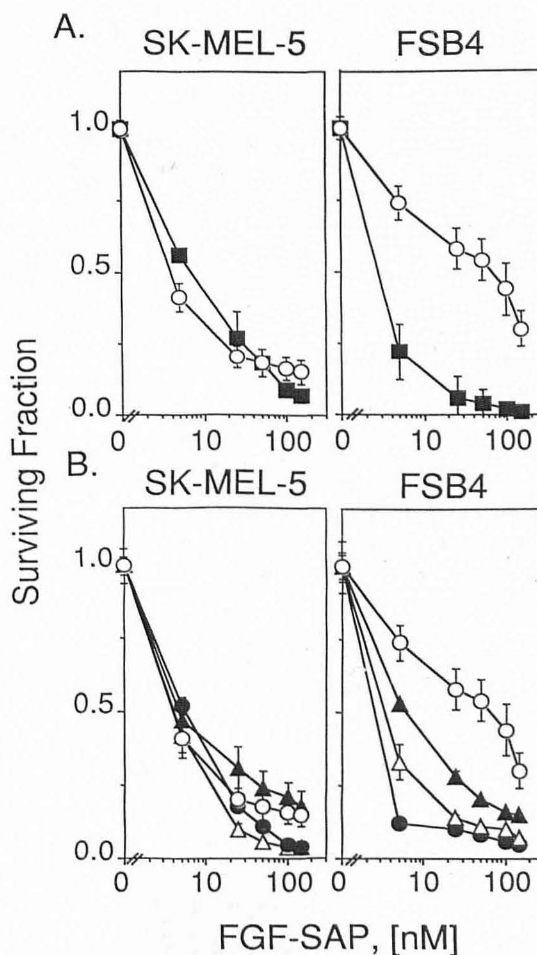


Figure 5. Resistance can be abrogated by suramin or neutralizing antibodies to FGFs. Survival of SK-MEL-5 parent cells or FSB4-resistant cells treated with bFGF-saporin (SAP) (5–150 nm) and (*A*) with (*closed squares*) or without (*open circles*) previous treatment with 1 mM suramin; and (*B*) after treatment with neutralizing antibodies (10 mg/l) to bFGF (*closed triangles*), aFGF (*open triangles*), or bFGF plus aFGF (*closed circles*), or without previous treatment (*open circles*). In both panels, parental and resistant cells were cultured for 15 h in fresh serum-free medium with or without suramin or neutralizing antibodies before exposing cells to bFGF-saporin for 1 h. After treatment, the cells were replated in duplicate for cell survival assays. After 7 d, the cells were suspended and counted by Coulter counter. Results are expressed as the mean surviving fraction of treated cells compared with untreated controls from three independent experiments ($n = 6$). *Bars*, SEM.

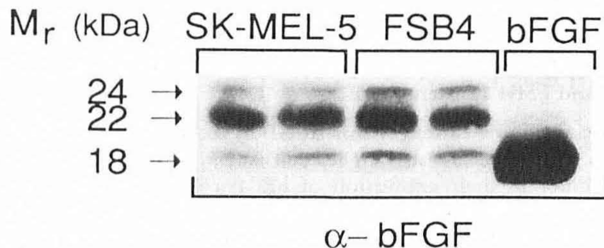


Figure 6. Resistant cells contain slightly more bFGF than the parental cells. bFGF was immunoprecipitated from parental and resistant cells using antibody specific for bFGF and then analyzed by chemiluminescent anti-bFGF immunoblotting. Human recombinant bFGF (1 ng) was used as a control. *Arrows* indicate positions of the 18-kDa bFGF species co-migrating with 1 ng of human recombinant bFGF, as well as the 22- and 24-kDa species of bFGF.

whether the observed increase in intracellular bFGF is alone responsible for the resistant phenotype. For this reason, we next examined the presence of bFGF in conditioned media. Secreted bFGF was captured by incubating monolayer cultures of SK-MEL-5 or FSB4 cells overnight with neutralizing antibody to bFGF, and the immune complexes were analyzed by anti-bFGF immunoblotting. bFGF proteins had molecular weights of 16, 18, 22, and 24 kDa, and an additional higher molecular weight form not observed in intracellular assays was detected at approximately 30 kDa in both parental SK-MEL-5 and FSB4 cells **(Fig 7)**. Densitometric analysis of bFGF proteins revealed that FSB4 secreted 20% to 30% more 18-kDa and 30-kDa bFGF, but 40% less 22-kDa bFGF than parent cells. Whether these differences represent a switch to a more biologically active isoform in the resistant FSB4 cells is not known.

We asked next whether suramin would similarly restore bFGF-saporin sensitivity in FSB4 resistant cells *in vivo*. Nude mice bearing parental SK-MEL-5 xenografts or FSB4 xenografts, which retain resistance to bFGF-saporin (31–125 μg/kg) *in vivo* **(Fig 8*A*)**, were treated with suramin intravenously for 3 d before a single intravenous injection of bFGF-saporin 5 d after tumor implantation. Suramin treatment of animals with FSB4 xenografts restored bFGF-saporin sensitivity **(Fig 8*B*)**. This was seen as a greater than 60% reduction in FSB4 tumor volumes as of day 30 after implantation, compared with tumor volumes of FSB4-bearing animals receiving bFGF-saporin treatment alone ($p < 0.05$). In contrast,

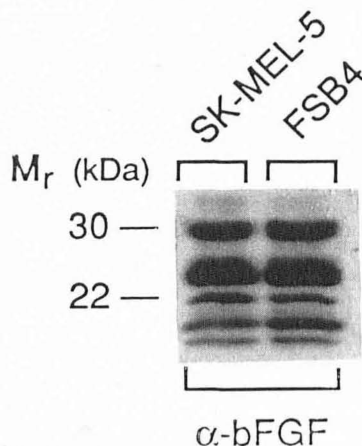


Figure 7. Resistant cells secrete bFGFs with different isoform ratios than those of parental cells. Cells were cultured overnight in serum-free medium containing bFGF neutralizing antibody covalently coupled to sepharose beads. bFGF was dissociated from the beads and analyzed by anti-bFGF immunoblotting as in **Fig 6**. The positions of the molecular-weight standards are indicated.

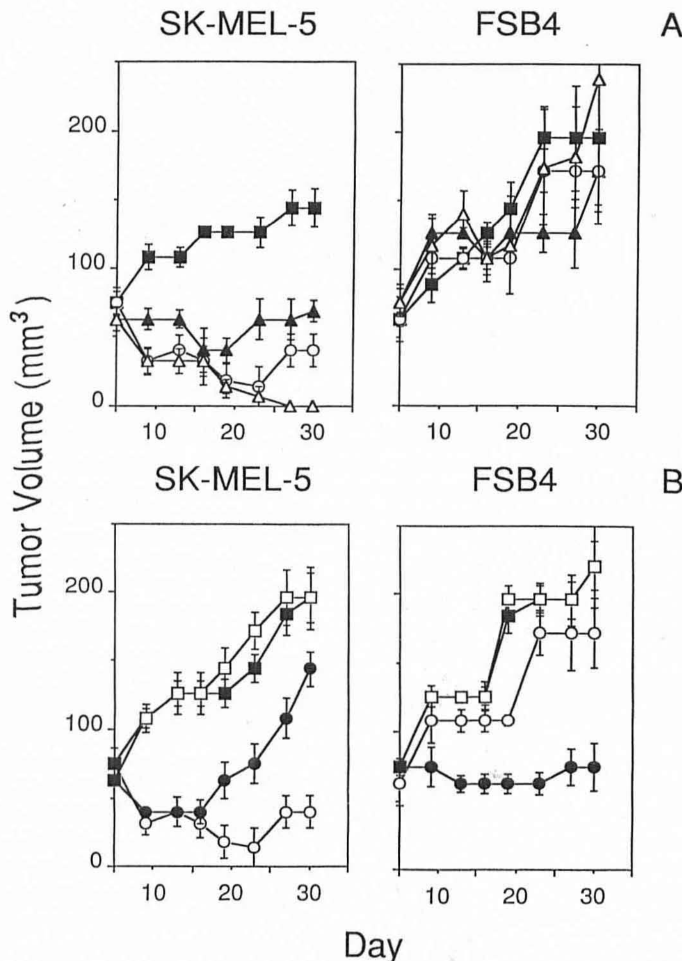


Figure 8. Suramin abrogates FSB4 resistance *in vivo*. FSB4 or SK-MEL-5 tumors were implanted subcutaneously in nude mice. On each of the next 3 d, the animals received suramin (150 mg/kg) by tail vein injection. On day 5, bFGF-saporin (62 μg/kg) was administered by tail vein injection. *A*, no suramin; bFGF-saporin concentrations were: 125 μg/kg (*open triangles*), 62 μg/kg (*open circles*), 31 μg/kg (*closed triangles*), PBS/01% bovine serum albumin (vehicle) (*closed squares*). *B*, results with suramin: suramin plus bFGF-saporin (*closed circles*), bFGF-saporin only (*open circles*), suramin only (*open squares*), PBS/01% bovine serum albumin (vehicle) (*closed squares*). Results are plotted as the medians of tumor volumes pooled from two independent experiments. *Bars*, SEM; n = 3–20 mice per treatment group.

suramin treatment alone did not significantly affect the growth of either SK-MEL-5 or FSB4 xenografts compared with vehicle-treated controls. Thirty days after tumor implantation, the median tumor volumes of bFGF-saporin–treated SK-MEL-5 xenografts measured 22% of vehicle-treated control tumors, demonstrating approximately fourfold greater sensitivity to bFGF-saporin compared with FSB4 resistant xenografts ($p < 0.01$). Suramin treatment of SK-MEL-5 xenograft-bearing animals resulted in initial bFGF-saporin–mediated tumor reduction comparable to that demonstrated in animals receiving bFGF-saporin treatment alone. However, by day 30, these tumors were more than twice as large as tumors in animals receiving bFGF-saporin alone ($p < 0.05$).

DISCUSSION

In summary, chronic exposure of SK-MEL-5 human melanoma cells to bFGF-saporin provided the selective pressure that resulted in our development of bFGF-saporin resistant cells, from which the FSB4 subline was cloned. The FSB4 line showed a sixfold decrease in bFGF-saporin sensitivity (by IC_{50}) compared with the parental

line in colony-formation assays and pronounced resistance in nude mouse xenografts. The resistance of FSB4 is due to extracellular autocrine loops between aFGF, bFGF, and cellular receptors for bFGF; these loops down-regulate bFGF receptors and thus favor survival of this variant when treated with bFGF-saporin. Of course, one might find independently isolated subclones that are resistant *via* non-autocrine mechanisms. Based on the theories developed by Coldman and Goldie [26] describing the genetic instability of neoplasms and the favored survival of phenotypic or genetic variants that are resistant to particular drugs, it is in retrospect not surprising that prolonged exposure of SK-MEL-5 cells to bFGF-saporin selected for this resistant subpopulation. Studies presented here confirm that bFGF-saporin resistant cells developed *in vitro* maintain resistance *in vivo*, and subsequent studies are currently underway to develop bFGF-saporin resistance *in vivo* to determine whether resistance arises from the same mechanism in both models. Investigations of bFGF-saporin dosing regimens *in vivo* suggest the importance of frequent administration for optimal therapeutic responses.† Because prolonged exposure to bFGF-saporin *in vivo* may similarly select resistance, investigating bFGF-saporin resistance and the mechanisms by which it occurs may be crucial to developing therapeutic regimens that will prevent or circumvent bFGF-saporin treatment failure in neoplastic disease.

Finally, the ability of suramin to restore *in vitro* sensitivity to bFGF-saporin in resistant FSB4 cells through disruption of the bFGF autocrine loop, as indicated by results obtained using neutralizing antibodies to bFGF *in vitro*, suggests that both suramin and neutralizing antibodies have potential use in combined-modality therapy with bFGF-saporin. Preliminary *in vivo* experiments presented here demonstrate that suramin is also effective in restoring sensitivity to bFGF-saporin in FSB4 xenografts; however, whether the mechanism *in vivo* is the same as we have determined for the *in vitro* model has yet to be examined. Besides the ability to block receptor-ligand interactions, suramin can inhibit DNA topoisomerase II, certain tyrosine phosphatases, and lysosomal enzymes [27–29], any one of which may influence mitotoxin sensitivity. Lappi *et al* have reported that bFGF-saporin cytotoxicity is inhibited in the presence of suramin *in vitro* [10]; the decreased sensitivity of parental xenografts treated with the combination of suramin and bFGF-saporin suggests that an antagonistic association may exist between these agents *in vivo* as well, which minimizes persistent anti-tumor activity of bFGF-saporin. Alternatively, suramin may promote malignant cell proliferation by activating an autocrine loop involving transforming growth factor-α and epidermal growth factor receptors [30], both of which are found in malignant melanoma [31,32] and tumor vasculature. We are currently optimizing therapeutic efficacy for preventing and circumventing resistance in malignant melanoma by using multiple cycles of suramin followed by bFGF-saporin.

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