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Research Article

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Suramin, an Experimental Chemotherapeutic Drug, Activates the Receptor for Epidermal Growth Factor and Promotes Growth of Certain Malignant Cells

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Abstract

Previous studies have shown that suramin is capable of disrupting autocrine growth involving coexpression of platelet-derived growth factor and its receptors in a fibroblast model for mesenchymal oncogenesis. Suramin is currently in use as an experimental drug for the treatment of patients with epithelial cell tumors. In the present study, we have investigated the efficacy of suramin in a carcinoma model system. Our findings demonstrate that suramin enhances cell surface signaling in A431 cells by activating an autocrine loop involving the receptor for epidermal growth factor (EGFR). The mechanism of suramin action was shown to be indirect, not affecting the ability of ligand to bind and activate the EGFR. Instead, suramin induced the release of membrane-bound transforming growth factor α, thereby increasing its potential to activate cell surface EGFRs. Since suramin potently blocks tyrosine phosphorylation induced by platelet-derived growth factor but can activate the growth pathway regulated by the EGFR, biological responses of tumor cells to suramin treatment may differ dramatically. (*J. Clin. Invest.* 1992. 89:1242–1247.) Key words: epithelial malignancy • suramin • autocrine growth • tyrosine phosphorylation • transforming growth factor-α

Introduction

Suramin is a polyanionic molecule that has been used for over 60 yr in the treatment of both human and bovine trypanosomiasis (1). More recently the drug has been used experimentally in the treatment of cancer patients (2, 3). Although suramin has significant antitumor activity (2–4), its mechanism of action is not known. Recent in vitro studies have shown that suramin interacts with the class of growth factors that bind to heparin (5) and that suramin inhibits growth induced by this class of factors (5–8). Furthermore, exposure of v-*sis*/platelet-derived growth factor (PDGF)[1]-transformed cells to suramin causes their reversion to a contact-inhibited state by a mechanism that involves direct inhibition of PDGF binding to its receptor (5–8). Such findings have suggested that the antitumor activity of suramin may involve a block of growth factor–mediated cell proliferation.

Epithelial cells express receptors for epidermal growth factor (EGFR). Known ligands for EGFR include epidermal growth factor and transforming growth factor α (TGFα) (9), neither of which are members of the heparin binding class of factors. Furthermore, many epithelial cell tumors coexpress the EGFR and its TGFα ligand (10, 11). These findings raise the possibility that autocrine stimulation may be an important component of epithelial cell carcinogenesis. In the present study, we have examined the biochemical and biological effects of suramin in two cell models for squamous cell carcinoma.

Methods

Cells. The epidermoid squamous cell carcinoma lines A431 (12) and KB (13) were grown in DME supplemented with 10% bovine serum or 10% fetal bovine serum, respectively. A serum-free medium consisting of DME, 10 nM sodium selenite, and 10 μg/ml transferrin was also used (8).

Antibodies and immunodetection assays. Antiphosphotyrosine mouse monoclonal antibody PY20 was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA), anti-human EGFR rabbit polyclonal antibody Ab-4 was purchased from Oncogene Science, Inc. (Manhasset, NY), and affinity purified rabbit anti-mouse IgG was purchased from Organon Teknika (West Chester, PA). For immunoblotting assays, cells were washed with cold phosphate buffered saline and disrupted at 4°C in a buffer containing 50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1 mM vanadate, 1 mM phenylmethylsulfonylfluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Lysates were clarified by centrifugation at 15,000 *g* and their protein concentrations were determined by the method of Bradford (14) using commercially prepared reagents (Bio-Rad Laboratories, Richmond, CA). Protein extracts were fractionated by SDS–PAGE on a slab of 8% separating and 5% stacking gels. Proteins were transferred to nitrocellulose filters and incubated overnight in a buffer containing 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween 20, and 4% bovine serum albumin (protease free). Treated filters were incubated with antibody and washed as described (15). Rabbit polyclonal anti-human EGFR antibody was visualized using ^{125}I-labeled protein A. When antiphosphotyrosine mouse monoclonal antibody was employed, rabbit anti-mouse IgG was used to enhance binding of ^{125}I-labeled protein A.

Detection and quantitation of TGFα. A431 cells were grown to confluence in 10-cm dishes and the medium was replaced with DME supplemented with 10 nM sodium selenite and 10 μg/ml transferrin, but not serum (8). After 16 h, suramin (kindly provided by the Cancer Treatment Evaluation Program, National Cancer Institute) was added for varying periods of time. Medium thus conditioned was collected, clarified by centrifugation, dialyzed against 100 vol of 0.1 M acetic acid, and lyophilyzed. Proteins were reconstituted in 0.15 M NaCl/0.1 M acetic acid and the pH was adjusted to 7.2 with 1 M Tris. All samples were assayed using a human TGFα radioimmunoassay kit (Biomed. Technol., Inc., Stoughton, MA). Epidermal growth factor was purchased from Gibco Laboratories (Grand Island, NY).

Mitogenic assay. Cells were grown to confluence in 24-well plates, washed twice with DME, and cultured for 24 h in serum-free DME as described (8). Cells were labeled with [^{3}H]thymidine (1 μCi/ml) for the last 6 h of incubation, washed twice with ice-cold phosphate-buffered

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1. *Abbreviations used in this paper:* EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PDGF, platelet-derived growth factor; TGFα, transforming growth factor-α.

saline, and washed twice with ice-cold 6% (wt/vol) trichloroacetic acid. DNA was solubilized in 0.25 M NaOH, and its radioactivity was measured by liquid-scintillation counting.

Results

Effect of suramin on tyrosine phosphorylation. In fibroblast models, suramin has been shown to inhibit tyrosine phosphorylation of certain growth factor receptors by interfering with growth factor receptor binding. Thus, we chose to examine the effect of suramin on tyrosine phosphorylation in carcinoma cells before and after treatment. A431 cells, maintained in serum-free medium, were treated for up to 3 d with concentrations of the drug approximating those used in cancer patients. Protein extracts were examined by immunoblotting for changes in cellular tyrosine phosphorylation. As shown in Fig. 1 *A*, a prominent band of 170 kD was detected in lysates of A431 cells whether treated or not. This band has been shown to represent a tyrosine phosphorylated form of the EGFR. A number of lower molecular weight bands ranging in size from 75 to 170 kD were readily detected in suramin-treated but not untreated cells. Maximum intensity of the lower molecular weight proteins was observed at 72 h of treatment, and by 96 h their intensities were diminished. These findings suggested an enhancement of substrate tyrosine phosphorylation by suramin treatment.

A431 cells are known to express abundant EGFRs. Thus, we examined possible activation of the EGFR in suramin-treated cells by immunoblotting with antibody against EGFR. As shown in Fig. 1 *B*, at 72 and 96 h the abundance of EGFR detected in suramin-treated cells was dramatically reduced compared with that detected in untreated cells. Moreover, we observed a shift in the mobility of EGFR in lysates of cells treated for 72 or 96 h. Such shifts are characterized of activated EGFRs. These findings demonstrated a suramin-induced down regulation of the EGFR and directly implicated the EGFR in the increased tyrosine phosphorylation of lower molecular weight proteins observed in suramin-treated cells.

The effect of suramin on the EGFR is indirect. To investigate the possibilities that suramin was acting as a ligand for EGFR or might be promoting its activation by an indirect mechanism, A431 cells were treated with suramin, epidermal growth factor (EGF), or a combination of both for periods of time ranging from 5 to 60 min. Cell lysates were examined by immunoblotting using antiphosphotyrosine antibody. As shown in Fig. 2, the antibody recognized the EGFR as a 170-kD protein in untreated cells. The effect of EGF was characterized by increased tyrosine phosphorylation of the EGFR itself as well as lower molecular weight species. Suramin alone had little effect on the EGFR or its response to EGF over the 60-min time course of the experiment. Thus, suramin does not have EGF-like activity, and the drug does not appreciably interfere with the ability of EGF to bind and activate its receptor. We conclude that the relationship between suramin and the EGFR must be indirect.

Suramin increases the availability of ligand for the EGFR. TGFα, like EGF, is an activating ligand for the EGFR (9, 16–18) and expression of its mRNA has been detected in A431 cells (10, 11). The TGFα translational product is a membrane-spanning precursor molecule with a cytoplasmic component and an extracellular domain containing mature TGFα as well as flanking regions at both termini (9). Thus, proteolytic activity is required to liberate the mature form of TGFα from the cell surface. Previous studies of PDGF B chain have shown that this molecule is tightly associated with the extracellular surface of fibroblasts (19) and that suramin interrupts this association, inducing the accumulation of PDGF molecules in the extracellular fluid (20). The mechanism of suramin action involves proteolytic removal of a PDGF B domain that anchors the molecule to the cell surface (20).

We reasoned that the delayed effect of suramin on the EGFR was consistent with a mechanism whereby the drug facil-

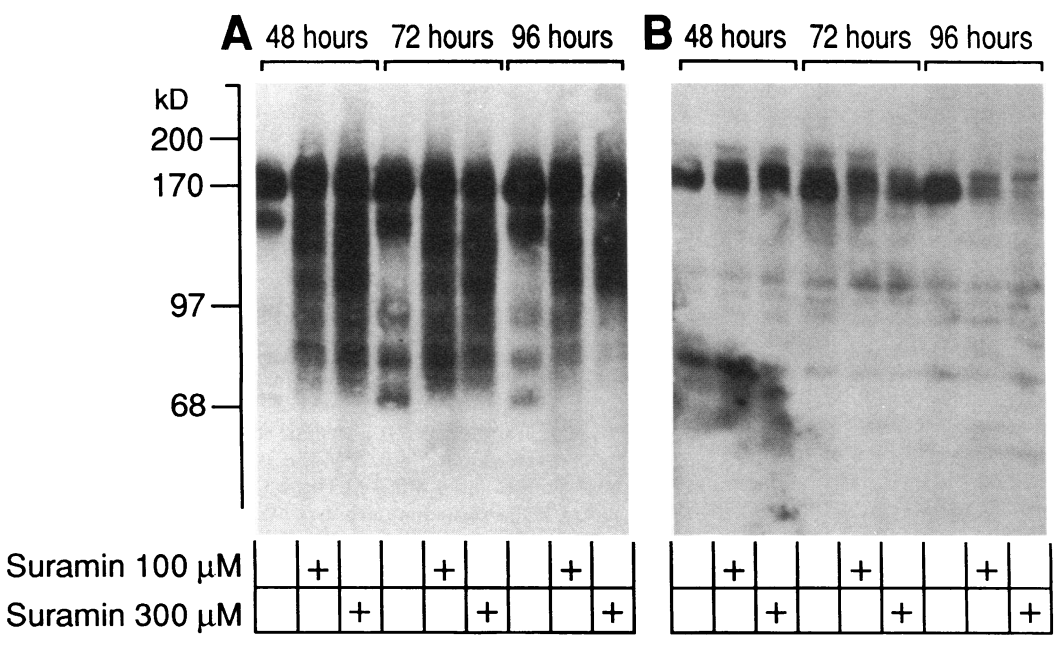

Figure 1. Tyrosine phosphorylation of cellular proteins after treatment with suramin. A431 cells were grown to confluence, and culture fluids were replaced with serum-free medium. After 16 h, cells were incubated, for the time increments shown, in the absence of suramin or in the presence of 100 or 300 μM suramin. Cells were lysed, and extracts, normalized by protein concentration, were fractionated by SDS–PAGE. Proteins were transferred to nitrocellulose filters, incubated with antiphosphotyrosine PY20 (*A*) or anti-EGFR antibodies (*B*), and subsequently treated with ^{125}I-labeled protein A. Proteins were visualized by autoradiography.

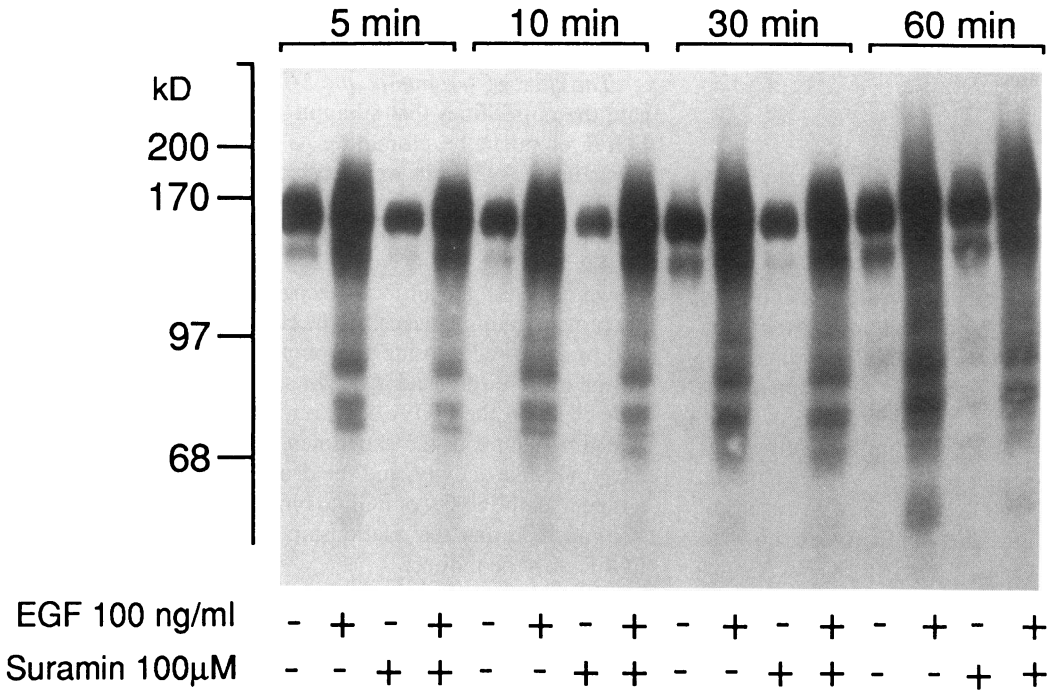

Figure 2. Acute treatment of A431 cells with EGF and suramin, alone or in combination. A431 cells were grown to confluence, and culture fluids were replaced with serum-free medium. After 16 h, culture medium was supplemented with EGF or suramin as indicated. After treatment, cells were lysed, and protein extracts were examined by immunoblotting using anti-phosphotyrosine antibody, PY20. Bands were visualized by autoradiography.

itated excision of TGFα from its precursor and thereby increased its availability for activating EGFRs. To test this hypothesis, we assayed for TGFα in medium conditioned by A431 cells treated with suramin for varying lengths of time. As shown in Fig. 3, no TGFα was detectably released from untreated A431 cells cultured for up to 3 d. In contrast, by 72 h, 3 ng TGFα was detected in medium conditioned by A431 cells treated with 100 μM suramin. Moreover, by 3 d, a time at which suramin-mediated down regulation of the EGF receptor was observed (Fig. 1 *B*), > 12 ng TGFα was detected in medium of cells treated with suramin at the higher dose. These concentrations (1–2 ng/ml) approximate the half-maximal effective dose for TGFα-induced mitogenesis in typical epithelial cells (21). We conclude that suramin mediates the accumulation of TGFα in culture fluids of A431 cells.

Inhibition of A431 growth by suramin treatment. Previous work has documented the growth inhibitory effect of EGF on A431 cells (22). To determine whether suramin-mediated accumulation of TGFα had a similar biological effect on A431 cells, suramin-treated cultures were examined for their ability to incorporate [^{3}H]thymidine into high molecular weight DNA. As shown in Fig. 4, DNA synthesis decreased as a function of suramin concentration at 24, 48, and 72 h of exposure to the drug. However, treated cells continued to incorporate [^{3}H]thymidine even after 72 h of exposure. Parallel cultures were examined microscopically. As shown in Fig. 5, suramin treatment had a dramatic effect on the morphological appearance of A431 cells. This morphologic effect was reversed when cultures treated with the drug for 72 h were subsequently incubated in the absence of suramin (data not shown). Thus, suramin reduced the growth rate of A431 cells and altered their morphological appearance as well.

Suramin enhances growth of carcinoma cells expressing TGFα and normal levels of EGFR. In contrast to its effect on A431 cells, EGF acts as a proliferative stimulus for normal epithelial cells. Thus, we sought to test the effect of suramin on carcinoma cells coexpressing TGFα and normal levels of EGFRs. KB was identified as a carcinoma cell line that expressed TGFα (10) but did not overexpress EGFR as determined by immunoblotting (data not shown). When KB cells were treated with suramin, enhanced tyrosine phosphorylation of a 170-kD protein, as well as several lower molecular weight species, were observed (Fig. 6). The tyrosine phosphorylation response was both time and concentration dependent. Thus,

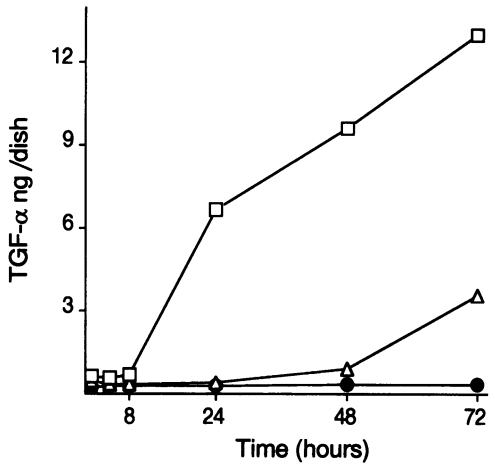

Figure 3. Detection of TGFα in medium conditioned by A431 cells treated with suramin. A431 cells (●——●) were treated with 100 (△——△) or 300 (□——□) μM suramin for the time increments shown. Medium conditioned by treated cells was clarified by centrifugation, dialyzed against 100 vol of 0.1 M acetic acid, and lyophilyzed. Proteins were reconstituted in 0.15 M NaCl/0.1 M acetic acid and the pH was adjusted to 7.2 with 1 M Tris. All samples were assayed using a human TGFα radioimmunoassay kit (Biomed. Technol., Inc., Stoughton, MA). Recoveries of TGFα were between 50 and 60%. The experiment shown is representative of three independent trials. Values obtained for duplicate samples varied by < 10%.

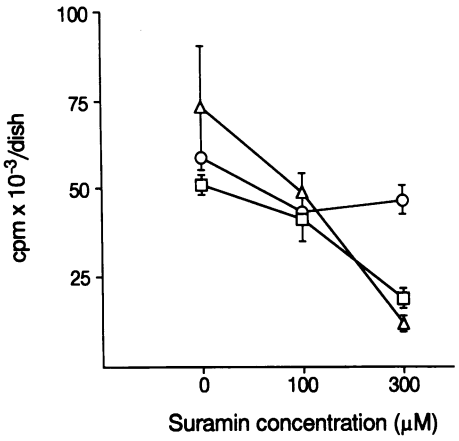

Figure 4. DNA synthesis in A431 cells treated with suramin. A431 cells were grown in 24-well plates, washed twice with DME, and cultured in the presence of suramin at the concentrations indicated for 48 (○——○), 72 (□——□), or 96 (△——△) h. Uptake of [^{3}H]thymidine was measured as described in the methods.

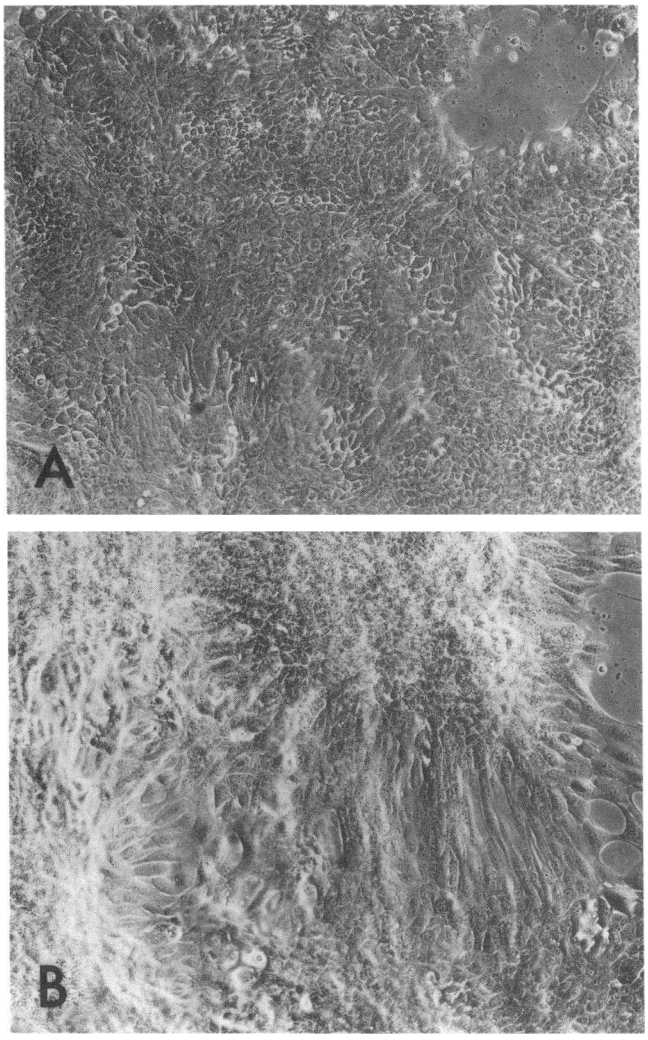

Figure 5. Morphologic appearance of A431 cells. (*A*) Untreated A431 cells or (*B*) cultures treated for 24 h with 300 μM suramin were photographed at a magnification of ×100.

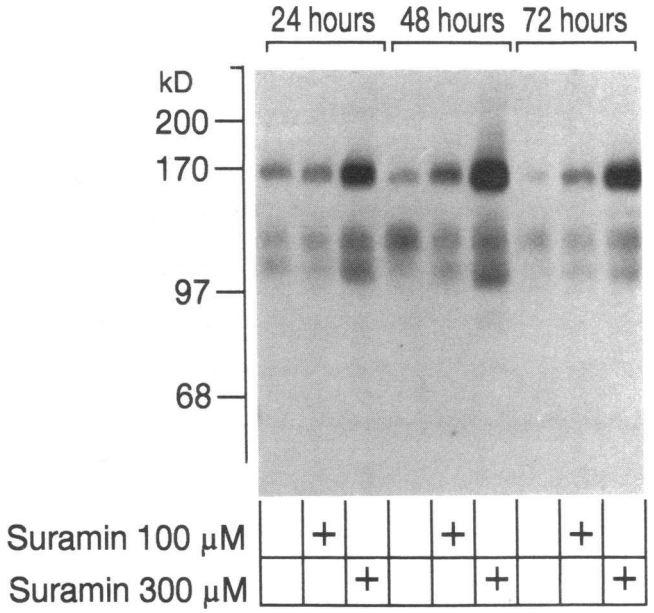

Figure 6. Tyrosine phosphorylation of KB cell proteins after treatment with suramin. KB cells were grown to confluence, and culture fluids were replaced with serum-free medium. After 16 h, cells were incubated for the time increments shown in the presence or absence of suramin as indicated. Cells were lysed, and extracts, normalized by protein concentration, were fractionated by SDS–PAGE. Proteins were transferred to nitrocellulose filters, incubated with anti phosphotyrosine antibody, PY20, and subsequently treated with ^{125}I-labeled protein A. Proteins were visualized by autoradiography.

suramin induces enhanced tyrosine phosphorylation in settings where TGFα is coexpressed with normal levels of EGFR.

To investigate the biological response of KB cells to suramin treatment, subconfluent cultures were maintained for up to 48 h in serum-free conditions with or without suramin. As shown in Fig. 7, KB cells did not grow well in the absence of serum. By 48 h, their appearance had become rounded and some cells had detached from the culture dish (Fig. 7 *C*). In contrast, when supplemented with suramin alone, cells were confluent at 24 h (Fig. 7 *B*) and by 48 h had continued to grow, piling up in several areas (Fig. 7 *D*). From these findings, we conclude that suramin is able to sustain the growth of KB carcinoma cells cultured under serum-free conditions.

Discussion

The idea of developing new anticancer agents that might interfere with autocrine growth stimulation was given impetus when growth factors such as PDGF, EGF, and colony-stimulating factor-1 (CSF-1), in cooperation with their receptors, were recognized as having oncogenic potential. Because the critical interaction of ligand and receptor appears to occur on the cell surface, reagents such as antibodies against ligand binding domains or ligands themselves have been evaluated in model systems with some success in slowing the growth rates of cells having autocrine loops (7, 8). Another approach has focused on the idea of engineering mutated ligands that might act as competitive antagonists. A third approach has involved the use of drugs, such as suramin, to interfere with ligand-induced recep-

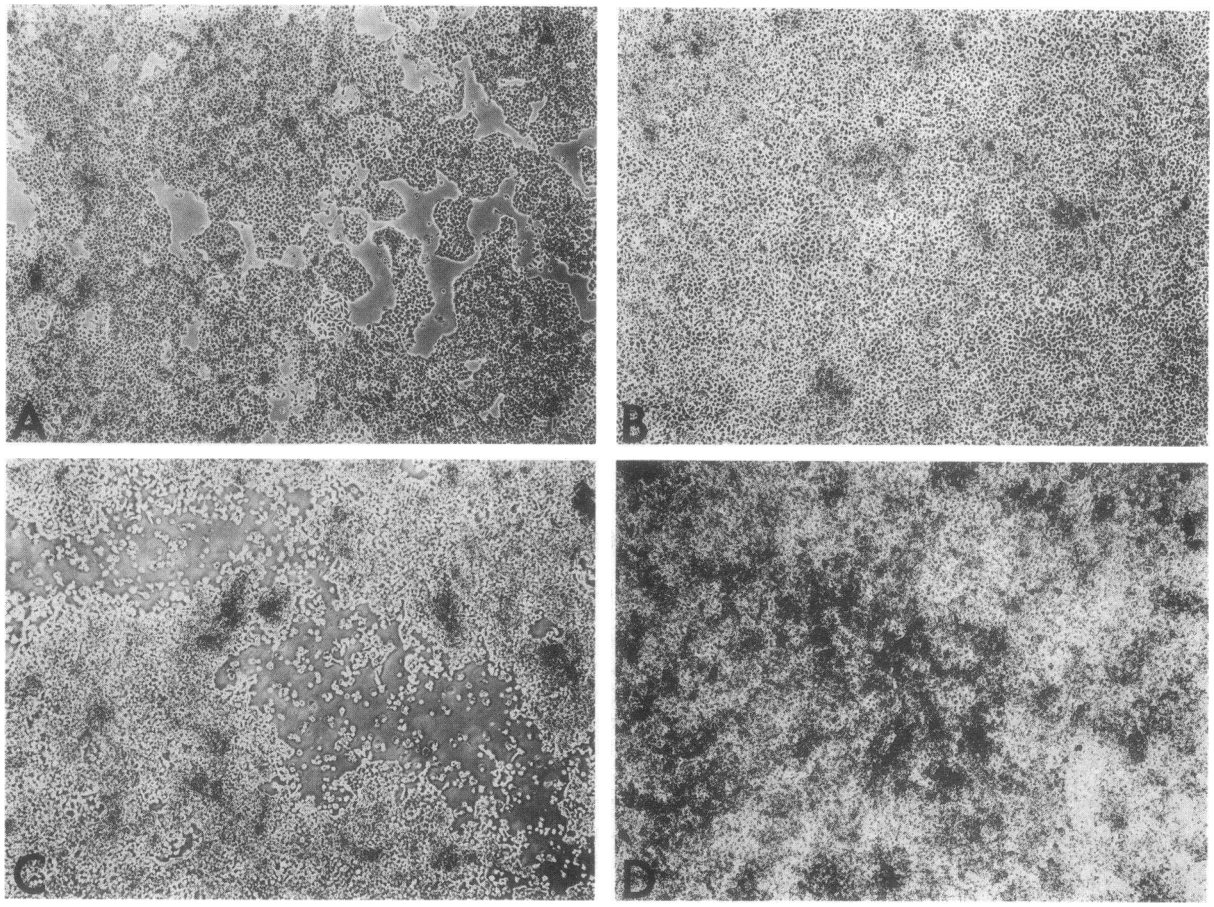

Figure 7. Suramin enhances growth of KB cells in serum-free conditions. KB cells were grown in complete medium to 80% confluence, and culture fluids were replaced with serum-free medium for 24 (*A* and *B*) or 48 (*C* and *D*) h. Suramin was added at time 0 (*B* and *D*) to a final concentration of 300 μM. ×50.

tor activation. The efficacy of suramin in the disruption of autocrine loops involving PDGF and its receptors has provided an additional rationale for clinical testing of suramin as an antitumor agent (2, 3).

In fibroblasts expressing PDGFs, suramin inhibits PDGF binding to its receptor and thereby inhibits PDGF-induced tyrosine phosphorylation of the receptor and its substrates (5, 6, 8). In the present study, we have shown that suramin can elicit a dramatically different biochemical response, namely increased tyrosine phosphorylation, in carcinoma cells expressing EGFR and surface bound TGFα ligand. We propose that the mechanisms underlying increased signaling through the EGFR involve release of TGFα from the surface and the inability of the drug to appreciably interfere with the binding of TGFα to the EGFR.

The A431 cell line is well characterized and unique among carcinoma cells. EGFR expression is roughly 30-fold higher in A431 cells relative to other epithelial cells whether nontransformed or tumor derived. Unlike typical epithelial cells, which are stimulated to grow by EGF or TGFα, A431 cells are growth inhibited (22). Thus, our observation that suramin inhibits growth of A431 cells is consistent with the known biological response of this cell to EGFR activation. Similarly, the effect of suramin on KB cells also reflects the known biological response of these cells to EGF. EGFR expression is typically unaltered during the development of epithelial cell malignancies and coexpression of EGFR and its TGFα ligand is not uncommon in carcinomas (10, 11). Thus, with respect to EGFR expression, the KB line is more representative of carcinoma cells than A431. The tyrosine phosphorylation response of both A431 and KB cell lines was similar, although its extent was more muted in KB cells, an observation that likely reflects the number of EGFRs expressed on the surface of each. Levels of EGFR expression may also account for the dramatically different biological responses of A431 and KB cells to suramin treatment. In any case, our current findings demonstrate that under certain conditions, suramin can act as a growth-promoting agent for malignant epithelial cells.

Other studies have shown that the TGFα precursor is capable of stimulating EGFRs located on the surface of adjacent cells (23, 24). Physically limited by the area of cell-to-cell contact, only a portion of a cell's EGFRs could be activated by a form of TGFα bound to the membrane of another cell. In contrast, a soluble TGFα would have greater freedom to act as an autocrine or paracrine factor. The present study shows that suramin induces an increase in soluble TGFα, dramatically enhances EGFR autophosphorylation and tyrosine phosphorylation of lower molecular weight proteins, and at the same time profoundly affects the biology of exposed cells. Although it is tempting to speculate that TGFα alone might account for these effects, further study will be required to assess the role of TGFα in the cellular response to suramin. Factors such as amphiregulin, which is also anchored to the cell surface by a transmembrane domain (25), or other EGF-related factors may also

play a role in activating the EGFR and mediating the biological effects observed. We are currently testing the hypothesis that TGFα alone can account for the biochemical and biological effects of suramin.

Our findings also have implications with regard to the clinical use of suramin as an antitumor agent. For the unusual tumor that expresses both TGFα and abundant EGFR, suramin may be growth inhibitory. However, suramin is likely to stimulate growth of the more common epithelial cell malignancies that express both TGFα and a normal complement of EGFR. TGFα also possesses angiogenic activity (21), which may enhance vascularization of tumors expressing the factor or of tumors that might be affected by liberation of TGFα from natural sources. Thus, information regarding expression of TGFα and perhaps the EGFR might be useful in choosing chemotherapeutic approaches for the treatment of epithelial malignancies.

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