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Suramin Inhibits Growth and Transforming Growth Factor-β1 (TGF-β1) Binding in Osteosarcoma Cell Lines

P. Kloen, C.L. Jennings, M.C. Gebhardt, D.S. Springfield and H.J. Mankin

Autocrine production of growth factors has been shown to be involved in the multistep process of tumorigenesis. The ability of suramin, a polyanionic anti-parasitic drug, to block growth factor-induced cell proliferation makes it a potential antineoplastic drug. We studied the effects of suramin on seven osteosarcoma cell lines. Using clinically achievable concentrations of suramin (50–400 μg/ml), we found a time- and dose-dependent inhibition of [^{3}H]thymidine incorporation. We also showed that suramin is able, dose-dependently, to prevent binding of transforming growth factor (TGF)-β1 to its receptors. DNA synthesis inhibition by suramin was attenuated by TGF-β1 in some cell lines. Two cell lines that were inhibited by TGF-β1 were affected similarly by suramin as cell lines that were stimulated by TGF-β1. In conclusion, in five out of seven osteosarcoma cell lines, we showed a correlation between inhibition of growth factor-stimulated mitogenesis and binding of TGF-β1 to its receptor. Similar effects in TGF-β1-inhibited osteosarcoma cell lines suggest involvement of other mechanisms and/or growth factors. However, suramin proves to be a potent inhibitor of osteosarcoma cell proliferation *in vitro*.

Key words: suramin, transforming growth factor-β1, osteosarcoma, receptor, autocrine growth
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INTRODUCTION

THE GROWTH of normal cells and tissues is a delicately balanced system in which a key role is played by multiple polypeptide growth factors. One of these factors is transforming growth factor-β (TGF-β) a 25-kDa homodimer, originally described as being able to induce phenotypic transformation of normal rat fibroblasts [1–3]. Subsequently, TGF-β has been shown to be multifunctional, and to be expressed ubiquituously, being important for cell growth and differentiation as well as wound healing. In contrast to its well characterised inhibitory effects on epithelial derived cells, TGF-β stimulates proliferation of mesenchymal cells [1–4]. Although the pathways of TGF-β are

still unclear, they are mediated through three different cell surface receptors: type I (53 kDa) and type II (70–100 kDa), both proteoglycans, and type III (280–330 kDa), a betaglycan [5].

In 1978, Todaro and DeLarco suggested that endogenous production of growth factors may be involved in tumorigenesis [6]. Sporn and Todaro modified this concept in the so-called "autocrine hypothesis", which postulates that production of growth factors by cells themselves might contribute to tumour formation [7]. Overexpression of growth factors and/or their receptors has since been reported in many neoplastic cell types [8–10].

Although TGF-β is produced by almost all cells, on a tissue/mass basis, bone is both the largest producer and storage site [11], and it has become clear that TGF-β plays an important role in the metabolism of bone [12]. We became interested in the possibility that an autocrine loop involving TGF-β may be involved in the aetiology and proliferation of osteosarcomas. We have previously demonstrated *in vitro* that the prerequisites needed for an autocrine loop involving TGF-β exist for human osteosarcoma cells, i.e. TGF-β receptors, production of TGF-β by the cells and effects on DNA synthesis by exogenously added TGF-β [13]. If an autocrine loop involving TGF-β is crucial for the growth of osteosarcoma cells, then having the ability to interfere with such an autocrine loop may represent a potential avenue for therapeutic intervention. This could theoretically be done by inhibiting expression of growth factors and/or growth factor receptors, or by preventing binding of the growth factor to its receptor. One of the prototypes of drugs currently being investigated in this respect is suramin, a polysulphonated compound originally developed nearly 75 years ago for use as an antitrypanocidal agent [14–16]. Suramin was found not only to inhibit a variety of key enzymes, such as DNA polymerase [17] and reverse transcriptase [18], but also to prevent mitogenic effects of growth factors [14–16]. Either of these effects elicited by suramin could explain the inhibition of *in vitro* growth of a number of cell lines by suramin. We set out to study the effect of suramin on DNA synthesis in seven different osteosarcoma cell lines, and its interference with TGF-β effects on these cells.

MATERIALS AND METHODS

Cell lines

The human osteosarcoma cell lines KHOS-312H, KHOS-240S, HOS, U-20S, SAOS-2 and MG-63 were a gift from S.H. Friend (MGH Cancer Center, Charlestown, Massachusetts, U.S.A.). A mouse osteosarcoma cell line MGH-OSA was established earlier in our laboratory. Cells were routinely cultured in DMEM containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin. Suramin was obtained from FBA Pharmaceutical (New Haven, Connecticut, U.S.A.), TGF-β1 was obtained from R&D Systems (Minneapolis, Minnesota, U.S.A.) and ^{125}I-labelled TGF-β1 was a gift from M.B. Sporn (NIH, Bethesda, Maryland, U.S.A.)

Effects of suramin and/or TGF-β1 on DNA synthesis

The effect of different doses of suramin was tested on seven osteosarcoma cell lines. We used suramin concentrations of 0, 50, 200 and 400 μg/ml, which have been shown to be clinically achievable [14–16]. Cells were plated at 1×10^4 cells/well in 96-well plates in DMEM + 10% FCS. After 24 h, wells were washed extensively and serum-free DMEM was added. Cells remained in serum-free medium for 48 h, after which different concentrations of suramin in serum-free medium were added. [^{3}H]Thymidine was added (1.6 μCi/well) after 20 h. After a 24-h incubation, DNA synthesis was measured by washing the cell layers with phosphate buffered saline (PBS), precipitation of DNA with 10% trichloroacetic acid (TCA), and dissolving in sodium hydroxide. Aliquots were counted using a liquid scintillation counter. Experiments were performed in triplicate. Since our main interest was in a possible autocrine loop in osteosarcoma cells involving TGF-β, we investigated the effects of suramin on DNA synthesis in these cells in the presence and absence of TGF-β1. Two representative human osteosarcoma cell lines were studied: KHOS-240S, for which DNA synthesis was shown to be stimulated 1.8-fold by TGF-β1, and MG-63, for which DNA synthesis was shown to be inhibited down to 60% by TGF-β1 [13]. The kinetics of [^{3}H]thymidine incorporation into DNA in MG-63 and KHOS-240S cells that were treated with TGF-β1 and/or suramin were measured. Cells were plated at 1×10^4 cells/well in 96-well plates in DMEM + 10% FCS for 24 h. Cells were then washed extensively and left in serum-free medium for 48 h. Suramin (200 μm/ml) and/or TGF-β1 (1 ng/ml) was added in serum-free medium. Then 4-h pulses of [^{3}H]thymidine were added after 6-h intervals for a period of 44 h. DNA synthesis was measured as described above. Experiments were performed in triplicate.

Effects of suramin on TGF-β1 binding

TGF-β receptors were affinity-labelled as described by Massague [19]. Briefly, confluent cells in six wells were washed with bicarbonate-free MEM, 25 mM Hepes pH 7.4, and 1 mg/ml bovine serum albumin (BSA). ^{125}I-labelled TGF-β1 (100 pM) was added in the presence or absence of different amounts of suramin (25, 600 μg/ml). The plate was put on a rotating platform for 2.5 h at 4°C. To show specificity of the binding, we incubated a control with ^{125}I-labelled TGF-β1 and 100-fold excess of unlabelled TGF-β1. Cells were washed and disuccinimydil suberate (cross-linking agent) was added. After 1 h, the cells were solubilised using a mixture of Triton-X, Tris and EDTA. The supernatant was centrifuged and stored at −70°C until use. The solubilised proteins were separated using SDS–polyacrylamide gel electrophoresis under reduced conditions on a precast 4–15% linear gradient gel (BioRad, New York, U.S.A.). Gels were dried and X-ray exposed for 14 days at −70°C using an intensifying screen.

RESULTS

Effects of suramin on DNA synthesis

Suramin markedly inhibited DNA synthesis in a dose-dependent manner in all seven osteosarcoma cell lines tested, as shown in Figure 1. Even at the low concentration of 50 μg/ml, there was a significant inhibitory effect. Effects were shown to be cytostatic rather than cytotoxic, as evaluated by direct microscopic evaluation of cell viability using methylene blue staining (data not shown).

Figure 2 shows a time course of the effects of suramin (200 μg/ml) and/or TGF-β1 (1 ng/ml) in two osteosarcoma cell lines. These cell lines were representatives of cell lines with different responses to TGF-β1. They were chosen after previous investigations had shown that MG-63 was inhibited by TGF-β1,

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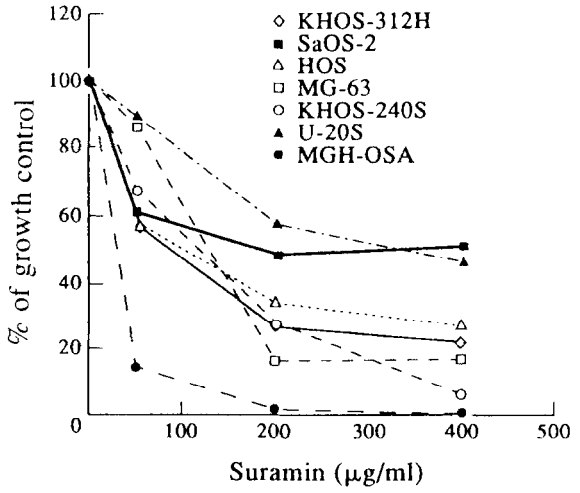

Figure 1. Dose-dependent effect of suramin on seven osteosarcoma cell lines as determined by [^{3}H]thymidine incorporation. Control (untreated cells) is 100%. % of control=cpm (treated cells)/cpm (untreated cells). Results represent mean of triplicate experiments with a S.E.M. less than 5%.

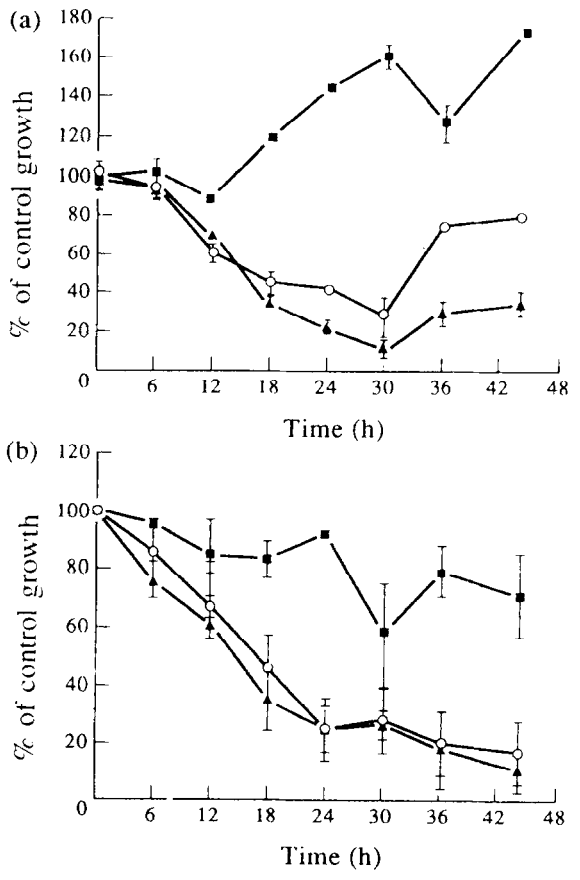

Figure 2. (a) Time-course on effect of suramin (200 μg/ml) and/or TGF-β1 (1 ng/ml) on DNA synthesis in KHOS-240S osteosarcoma cells as measured by [^{3}H]thymidine incorporation. Control (untreated cells) is 100%. % of control=cpm (treated cells)/cpm (untreated cells). Results represent mean of triplicate experiments with a S.E.M. less than 5%. ■ TGF-β1; ▲ suramin; ○ TGF-β1 and suramin. (b) Same for MG-63 osteosarcoma cells.

whereas KHOS-240S was stimulated by TGF-β1 [13]. The maximum DNA inhibitory effect of suramin on KHOS-240S cells occurred after 30 h incubation, at which time the DNA synthesis was only 10% of the untreated cells. After 30 h incubation, we saw a gradual increase in DNA synthesis to a value of 30% of its control value after 44 h. The inhibitory effects of suramin were clearly attenuated by the presence of TGF-β1 (1 ng/ml). TGF-β1 by itself showed a delayed kinetics response with a peak after about 30 h, at which time the DNA synthesis was 1.6 times that of its control value. This was followed by a decline in mitogenic activity to 1.25 times that of its control at 36 h, followed by an increase to 1.75 times of its control.

Addition of suramin to MG-63 showed a gradual inhibition of DNA synthesis over time. After 44 h of incubation with suramin (200 μg/ml), DNA synthesis was only 10% of its value in the untreated cells. The inhibitory effect of 1 ng/ml TGF-β1 alone on MG-63 cells was maximum (40% inhibition) at 30 h after addition, after which a gradual increase was seen. There was virtually no difference between DNA synthesis inhibition in MG-63 by treatment with suramin (200 μg/ml) alone or in combination with TGF-β1 (1 ng/ml).

Effect of suramin on binding of TGF-β1 to its receptor

In earlier experiments, we showed the presence of TGF-β1 binding receptors on the osteosarcoma cells tested [13]. We sought to provide evidence that the inhibitory effects of suramin on DNA synthesis were caused by inhibition of TGF-β binding to its receptor. We cross-linked ^{125}I-labelled TGF-β1 to its receptors, in the presence and absence of different concentrations of suramin. Figure 3 shows that suramin is able, dose-dependently to prevent binding of TGF-β1 to its receptor on MGH-OSA cells. Of interest is the fact that DNA synthesis in MGH-OSA is inhibited by TGF-β1. Similar results were seen in cell lines that were stimulated by TGF-β1 such as SAOS-2 (data

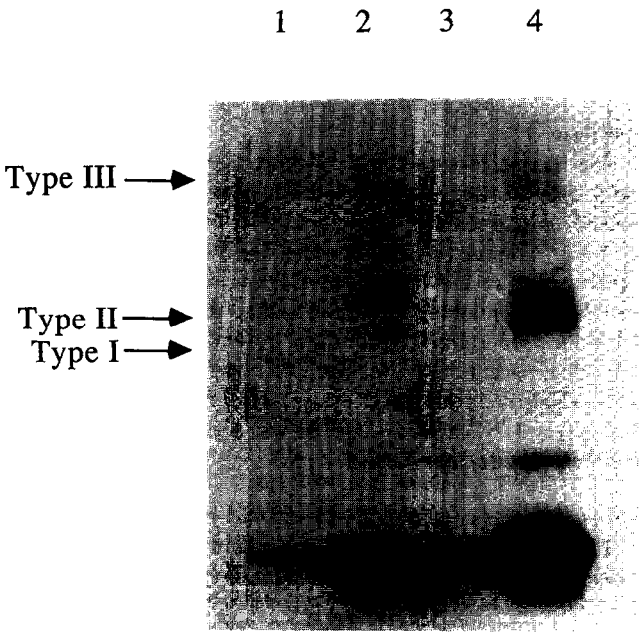

Figure 3. Affinity cross-linking of [^{125}I]TGF-β1 to MGH-OSA cells. Lane 1=[^{125}I]TGF-β1 + 600 μg/ml suramin; lane 2=[^{125}I]TGF-β1 + 25 μg/ml suramin; lane 3=[^{125}I]TGF-β1 +excess unlabelled TGF-β1; lane 4=[^{125}I]TGF-β1. The different TGF-β receptors (types I, II and III) are marked.

not shown). As is shown in Figure 3, excess unlabelled TGF-β1 prevented binding of ^{125}I-labelled TGF-β1 to its receptor showing specificity of the binding.

DISCUSSION

The importance of autocrine growth control of malignant cells, as popularised by Sporn and Todaro [7], is clearly recognised as one of the promoters or initiators in certain tumours [8–10]. Interference at the level of growth factor production or growth factor stimulation could be a very attractive approach in these tumours. Currently, however, treatments based on interference of specific growth factor-mediated pathways are practically unavailable. A prototype of a potentially useful drug is suramin, an old anti-parasitic drug, which has been shown not only to block the activity of enzymes that are critical for cell growth and proliferation, such as DNA polymerase [17] and reverse transcriptase [18], but more importantly, to inhibit growth factor binding [14–16]. Among the growth factors it has been shown to inhibit are epidermal growth factor (EGF) [20], fibroblast growth factor (FGF) [20], platelet-derived growth factor (PDGF) [21], TGF-β [20,22] and insulin-like growth factor (IGF-I) [23–26]. Multiple studies have been published on the inhibitory effects of suramin *in vitro* on DNA synthesis in many different cell lines, all of which revealed similar results, in that suramin had reversible inhibitory effects, most probably due to interference of growth factor binding [20–27]. A few studies have investigated the effects of suramin *in vivo*, results of which were generally modest, although warranting further investigation [14,15,28–30].

With bone being a large producer and storage site for TGF-β [12], and the known ability for auto-induction [2], we felt that this growth factor was a logical candidate for autocrine growth control in bone. Derangement of normal growth control by abberant expression of TGF-β and/or receptors could lead to tumorigenesis of bone cells, e.g. osteosarcoma. Previously we have shown all the prerequisites for a TGF-β involving autocrine loop to be present in osteosarcoma cells [13]. The involvement of autocrine processes is furthermore suggested by the ability of the osteosarcoma cells to grow in a defined serum-free medium containing no added growth factors.

If autocrine control has a role in the growth of osteosarcoma, interference with binding of the key growth factor could be a hypothetical therapeutic approach. Therefore, we investigated the effects of suramin on seven different osteosarcoma cell lines. Our results showed that suramin has a dose-dependent inhibitory effect on all osteosarcoma cell lines tested. Our experiments clearly showed that the cell lines tested express specific cell surface binding sites for TGF-β, to which binding of radioactively labelled TGF-β1 was inhibited in the presence of suramin. Whether this is caused by interference at the receptor level or by binding of the growth factor in solution was not addressed. The concentrations needed to prevent binding of TGF-β1 to its receptor were in the same range as those inhibiting DNA synthesis. These findings suggest that the DNA synthesis inhibitory activity of suramin could be related to interference of binding of TGF-β in osteosarcoma cell lines that are stimulated by TGF-β (four of seven cell lines tested [13]). However, in osteosarcoma cell lines that are inhibited by TGF-β1, such as MG-63 and MGH-OSA, the inhibitory effect of suramin and its relationship to TGF-β is not as easy to explain. If TGF-β1 is the only autocrine factor controlling MG-63 and MGH-OSA cell growth, inhibition of TGF-β1 binding by suramin should theoretically lead to growth stimulation. In our experiments, this was not the case, since suramin resulted in growth inhibition of MG-63 and MGH-OSA. This may be explained by suramin's "overruling" effect on cellular enzymes, such as DNA polymerase, thereby diminishing the effect of TGF-β blockage. There might be other autocrine growth factors (in addition to the inhibitory TGF-β) important for growth of these cell lines, which is also suggested by the ability of MG-63 and MGH-OSA to reach confluence under serum-free conditions (data not shown). Recently authors have reported on suramin's ability to interrupt autocrine loops; Pollak reported that suramin blocks the IGF-I-stimulated proliferation of MG-63 cells by interference with the interaction of IGF-I and its receptor [23]; Morocz speculated on suramin's growth factor interference in lung cell lines [26]; Minniti attributes suramin's inhibitory effects on rhabdomyosarcoma cells to interruption of IGF-II autocrine growth loop [25]; and Ravera suggested a possible therapeutic role for suramin in human breast tumours by interruption of IGF-I binding [24]. Our current findings and theirs are not contradictory, but emphasise that, *in vitro* and even more so *in vivo*, a very complex system of growth stimulating and growth inhibiting factors is present, and binding of most of these growth factors will be influenced by suramin. Moreover, suramin also has multiple other effects on important enzyme systems, which makes it hard to attribute effects of this drug to a specific cellular location. To our knowledge, this is the first report on the interaction of suramin and TGF-β1 on osteosarcoma cell lines. We would like to emphasise that, although suramin can have a potential role in management of autocrine controlled tumorigenesis, its precise effects need further elucidation. However, at this time, suramin is a useful tool in cytokine and growth factor research.

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Growth Inhibition of Oestrogen Receptor-positive Human Ovarian Carcinoma by Anti-oestrogens *In Vitro* and in a Xenograft Model

S.P. Langdon, A.J. Crew, A.A. Ritchie, M. Muir, A. Wakeling, J.F. Smyth and W.R. Miller

This paper presents results of the *in vitro* and *in vivo* effects of anti-oestrogens on the growth of human ovarian cancer cells. Tamoxifen and the "pure" anti-oestrogens, ICI 164,384 and ICI 182,780, inhibited the oestrogen-stimulated growth of the oestrogen receptor (ER)-positive PE04 and PE01 cell lines grown in culture, the latter two compounds being more potent than tamoxifen. In the absence of 17β-oestradiol (E_2), tamoxifen, but not the pure anti-oestrogens, produced a small degree of growth stimulation in the PE01 and PE04 lines at concentrations between 10^{-7} and 10^{-9} M. In contrast, growth of the ER-negative PE014 line was unaffected by E_2 and all three anti-oestrogens. The effects of tamoxifen and ICI 182,780 on PE04 cells grown as xenografts in nude mice were also studied. Both anti-oestrogens produce significant growth inhibitory effects. These results indicate that ovarian carcinoma cells may be sensitive to anti-oestrogens *in vitro* and *in vivo*, and support the view that anti-oestrogens merit further clinical studies in patients with ER-positive tumours.
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INTRODUCTION

THE ROLE of anti-oestrogens in the treatment of ovarian cancer remains controversial. The presence of oestrogen receptors (ER) in 67% of ovarian adenocarcinomas (reviewed in [1]) suggests that this disease might be responsive to anti-oestrogen therapy, and several small clinical trials with tamoxifen have reported response rates of between 6 and 36% [2–7], although others have not observed any benefit [8–11]. Many of these studies concluded that, although the response rate to tamoxifen is low, there is a group of patients who could benefit from anti-oestrogen therapy. In this respect, it is interesting that an association between the presence of ER and tumour response has been