Suramin Inhibits Growth of Human Osteosarcoma Xenografts in Nude Mice

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ABSTRACT

The effect of suramin on tumor growth and morphology in two different human osteosarcoma xenografts (L-I OSM and L-II OSM) grown in BALB/c-\(\text{ca}-\text{nu/nu}\) mice was studied. Suramin (total dose, 720 mg/kg) given by i.p. injection (60 mg/kg/dose) for up to 9 weeks significantly inhibited osteosarcoma cell growth in both tumors, suramin-treated tumors showing only one-third or less of the volume of nontreated controls. Cell cycle distribution of tumor cells measured by DNA flow cytometry demonstrated that suramin treatment caused accumulation of cells in the S and G2 phases of the cell cycle, in both L-I OSM and L-II OSM. In the aneuploid L-II OSM tumor suramin preferentially inhibited the growth of aneuploid cells, leading to a decrease in the ratio of aneuploid to diploid cells. Both osteosarcomas retained their histological appearance and the liver, spleen, heart, and kidneys of the treated animals were unaffected by suramin. These results are compatible with the view that suramin inhibits the growth of human osteosarcomas by cytostatic effects.

INTRODUCTION

Suramin is a polyamionic drug commonly used in the treatment of Rhodesian trypanosomiasis and oncocerciasis (1). It is an inhibitor of DNA polymerases (2) and reverse transcriptase (3, 4) as well as many other enzymatic systems (1, 5-7). Suramin reversibly reverts the phenotype of simian sarcoma virus-transformed fibroblasts in culture and it is also able to induce cell differentiation in several experimental systems (8-11). The drug has been reported to inhibit in vitro cell growth of normal as well as of neoplastic cell lines, possibly by interaction with the binding of a number of growth factors including platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, insulin-like growth factor, and transforming growth factor \(\beta\) to their specific cell surface receptors (10, 12-18). Moreover, suramin is able to inhibit receptor binding of other ligands like low density lipoprotein (19) and transferrin (20). In vitro, suramin inhibits the cytopathic effect of human immunodeficiency virus and has hence been used in clinical trials in the treatment of acquired immunodeficiency syndrome, albeit with limited success (21-24). Recently, encouraging results were published from two independent clinical trials indicating that suramin may be useful as an antineoplastic agent in the treatment of certain advanced human malignancies (25, 26). In the present study, we undertook the present study to determine its antiproliferative effect in two different human osteosarcoma xenografts in nude mice.

MATERIALS AND METHODS

Sarcoma Tissue. Two different human, genuine osteosarcomas were obtained from fresh surgical specimens upon open biopsy. One chemo-, radio-, or immunotherapy was given prior to surgery. One osteosarcoma originated from the distal femur of a 13-year-old boy. Histopathological examination revealed a grade III osteogenic sarcoma characterized by very pleomorphic cells and small foci of pathological new bone formation. The other specimen was obtained from the proximal tibia of a 12-year-old girl. This tumor was classified as a grade III osteosarcoma of chondroblastic type with high cellularity and areas of relatively large amounts of osteoid. Immediately after removal, pieces of sarcoma tissue were placed in nutrient medium at 4°C and inoculated into nude mice within 2 h. The established osteosarcoma xenografts in nude mice will be referred to in the text as L-I OSM and L-II OSM, respectively.

Animals. Four- to 6-week-old male, congenitally dysthymic BALB/c-\(\text{ca}-\text{nu/nu}\) mice (Bomholtgård Breeding and Research Ltd., Ry, Denmark) were housed in sterilized cages in laminar air flow clean benches at 26-28°C in humidified air (45-55%) with 12-h light and dark cycles. One to 4 animals were kept per cage. Sterile food (F12 pellets; Astraw, Södertälje, Sweden) and water was given ad libitum. The mice were given a minimum of 10 days to adapt to the laboratory environment before manipulation.

Transplantation. Viable-appearing parts of the sarcoma specimens were cut into blocks measuring 2 x 2 x 2 mm and inoculated s.c. into the dorsum of 6-9 mice. Four pieces of sarcoma tissue were inoculated per animal under general xylazine (5 mg/kg body weight) and ketamine (50 mg/kg body weight) anesthesia. When the transferred sarcomas had reached a volume of about 1.5 cm³ the animals were killed and the tumor tissue was removed and set aside for histological examination, DNA flow cytometry, and transplantation into new mice. Serial transplantations were made under sterile conditions using plugs from about 5-7 mm³ that were transferred s.c. (4-6 pieces/animal) into the recipient animals (4-12 mice/ passage). The same technique was used for successive serial transplantations to maintain a stock of osteosarcoma-bearing mice. For histological examination, pieces of tumor tissue were fixed in 4% buffered formaldehyde for at least 48 h, dehydrated in graded ethanol series and embedded in paraffin; histological sections were prepared and stained with hematoxylin and eosin and according to van Gieson. The cell morphology of original sarcomas and serially transplanted sarcoma xenografts was examined. Fresh tumor tissue was frozen in liquid nitrogen in passage 7 (L-I OSM) and in passage 5 (L-II OSM) and processed for the demonstration of alkaline phosphatase using a diazonium technique (27).

Suramin. Suramin (Germanin-Bayer, Bayer 205); hexaammonium salt of 3,3-urenylene bis-[3-(3-benzamido-4-methylbenzamido)-1,3,5-naphthale- trisulfonic acid; C₁₇H₁₄O₂₃N₆Na₂M, M₁₄29, was a generous gift from Bayer AG, Leverkusen, Germany. Suramin was dissolved in sterile 0.15 M NaCl and injected within 30 min after preparation.

Experimental Design. Each mouse received one tumor block of approximately 10 mm³ s.c. through a 3-mm skin incision in the dorsum. The subsequent sarcoma growth was evaluated measuring two perpendicular tumor diameters \((a = \text{length}; b = \text{width})\) once a week with a slide caliper. Tumor volume was calculated according to the method of the National Cancer Institute (28):

\[
\frac{a \times b^2}{2} = V
\]

Suramin treatment started when the transplants showed reliable tumor growth, i.e., when tumor volume was more than 40 mm³. Within 5-7 weeks of transplantation, tumor growth was visible in 30 animals of the L-I OSM series and in 24 animals of the L-II OSM series. The
tumors used in this study were in their 8th (L-I OSM) and 7th (L-II OSM) passages, respectively. The animals were randomized into control and treatment groups. In the treated group, each mouse received i.p. injections of 1.8 mg suramin (60 mg/kg/dose, equivalent to 300 mg/m², in 0.1 ml 0.15 M NaCl) on day 1, 3, 6, 9, 14 and then weekly for up to 9 weeks. Day 1 is defined as the first day of suramin treatment. Control mice received i.p. injections of 0.1 ml of 0.15 M NaCl according to the same regimen. At the end of the treatment period all mice were killed and autopsied with recording of organ weights (liver, spleen, heart, and kidneys). All tissues were fixed in 4% buffered formaldehyde for at least 48 h and processed for histological examination as described above. Organs were prepared for histological sections and stained with hematoxylin and eosin and periodic acid-Schiff stain. Sections (5 μm) of the tumors were stained with hematoxylin and eosin and according to van Gieson. The mitotic index of the sarcoma xenografts was calculated as the number of mitotic figures in 10 consecutive high power fields (corresponding to 2500–6000 cells) of vision from random areas in each transplant.

Preparation of Cells from Fresh Osteosarcoma Tissue. Immediately after removal of the sarcoma xenografts, tumor material was obtained by scraping the entire cross-section area of the tumors with a sharp curet. Tumor tissue samples were placed in 0.4 ml citrate buffer (250 mM succrose, 40 mM trisodium citrate, 5% (v/v) dimethyl sulfoxide), pH 7.6, and kept frozen at ~80°C. Suspensions of single cell nuclei were obtained using the detergent-trypsin procedure described by Vin-deløv et al. (29). After addition of RNase the nuclei were stabilized by spermine tetrahydrochloride, filtered through a 41 μm nylon mesh, and stained with propidium iodide. Trout and chicken erythrocytes were added as internal marker cells.

Preparation of Paraffin-embedded Material. Sections (50 μm) from the routine, paraffin-embedded original osteosarcomas fixed in neutral buffered formaldehyde were deparaffinized in xylene, rehydrated in graded ethanol series, and washed twice in distilled water. The single-cell suspension was obtained according to the method of Schutte et al. (30) by treatment of the tissue with 0.25% trypsin in citrate buffer overnight at 37°C in a shaking water bath. After filtration through nylon mesh, the suspension was stained with propidium iodide as described above.

DNA Flow Cytofluorometry. The DNA content of the cell nuclei was determined with a Leitz MPV Flow (Leitz Gmbh, Wetzlar, Germany) (31) flow cytometer equipped with a mercury lamp and connected with a Monroe OC 8888 microcomputer (Litton Business). Histograms including at least 20,000 cells, with a coefficient of variation of less or equal to 6.6%, were recorded. The cellular ploidy was calculated assuming that chicken and trout RBC have 35% and 80%, respectively, of the DNA content of human diploid cells. After manual selection by the operator the number of tumor cells, modal channel number, coefficient of variation of each peak, and the fraction of S-phase cells were determined by the computer program. The DNA content of brain cells from nude mice was identical to that of normal diploid human cells.

Statistics. The significance of differences between tumor volumes in treated and control groups were analyzed at the end of the experiments using the two-tailed rank sum tests combining results from different series with Elteren’s method (32). In one of the experimental series, there was a dropout in the control group. In this case the rank number was set pessimistically so as to minimize the significance of the test. Mean mitotic index, organ weights and flow cytometric data were analyzed by the two-tailed Student t test. P < 0.05 was considered significant.

RESULTS AND DISCUSSION

The growth of the human osteosarcoma xenografts L-I OSM and L-II OSM in nude mice was significantly inhibited by i.p injections of suramin at 60 mg/kg/dose (Fig. 1). The effect was evident within 10 days of treatment and increased throughout the experiment. At the time of termination of the experiment treated animals had tumors with a volume of one-third or less of that of nontreated ones. The growth-inhibitory effect of suramin was not complete since the tumors displayed a low but steady volume increase despite ongoing treatment. Further, suramin did not influence the histopathological appearance of the sarcomas. Thus, both sarcomas grew as lobulated tumors within a pseudocapsule and demonstrated a moderately pleomorphic appearance built up of mainly spindle-shaped cells arranged in bundles and whorls and tumors showed occasional areas with osteoid. Vascularization was pronounced.

If suramin has a direct toxic effect on tissues (33), this was not reflected by the present findings. Analogously the normal tissues of the experimental animals were unaffected by suramin. A number of organs (liver, spleen, heart, and kidneys) retained their normal weights and histological appearances throughout the experiment. Although some animals developed a transient maculopapular skin rash during the first week of suramin administration, the general condition of the mice was not affected and all animals seemed to thrive despite treatment. The xenografts did not grow invasively into adjacent tissues nor did metastatic spread occur. The serially passaged osteo-
sarcoma xenografts retained the original tumor morphology well as compared to the original tumors (Fig. 2). Flow cytometry demonstrated that L-I OSM was diploid with a mean DNA index of 1.03 (range, 1.00–1.07) and L-II OSM was aneuploid with a mean DNA index of 1.32 (range, 1.27–1.40); both retained their ploidy levels upon repeated transplantations (Fig. 3). Both sarcomas stained alkaline phosphatase positive, as expected.

L-I OSM tumors showed a mitotic index, expressed as the mean number of mitotic figures per 100 cells, of 7 ± 1.3 (SE) and L-II OSM 7 ± 0.8; the values were not significantly changed after suramin treatment (6 ± 0.9 and 6 ± 0.6, respectively). Cell cycle analysis by flow cytometry revealed that suramin administration results in a changed distribution of tumor cells in the respective cell cycle compartments. The percentage of cells in S and G2-M was significantly increased in the two different suramin-treated sarcomas (Table 1). The higher percentage of cells in these cell cycle phases found in the treated tumors together with the unchanged mitotic indexes suggest a selective effect of the drug in the S and G2 period. Similarly, Forsbeck et al. (20) have shown that certain established human cell lines are arrested in S phase by suramin. The results of cell cycle analysis in conjunction with the histological findings in suramin-treated sarcomas may support the view that the antitumor effect is cytostatic rather than cytotoxic. Analysis of cell numbers deduced from the flow cytometric data shows that suramin also clearly reduced the proportion of aneuploid cells (from 88 to 80%) with a concurrent increase in the relative count of diploid cells (from 12 to 20%) in the L-II OSM tumors. This may indicate a selective suppression of aneuploid cells in a heterogeneous population of tumor cells. However, growth inhibition was evident also in the inherently diploid L-I OSM tumor, indicating that also diploid tumor cells were responsive to suramin treatment. There was no observed increase in infiltrating inflammatory cells in the tumor or any increased amount of tumor stroma formation which could explain the relative increase in diploid cells in the suramin-treated L-II OSM tumors. Calculations of total cell numbers from data derived from...
from the mean tumor volumes and flow histograms showed that suramin inhibited the aneuploid cell population by approximately 66% and the diploid by 30%.

The antiproliferative mode of action of suramin in experimental systems including the present one is unclear. It has been proposed that suramin acts via interference with ligand-receptor interaction including growth factors and transferrin or with GAG\(^3\) turnover. Growth factors have been ascribed a role in the abnormal growth of certain human tumors, including sarcomas (17). The fact that suramin is able to displace growth factors from their receptors suggests that suramin may affect the growth of sarcoma transplants by interfering with a ligand-receptor-mediated mitogenic pathway.

It has been reported that suramin causes lysosomal accumulation of heparan sulfate and dermatan sulfate by inhibition of one of the lysosomal enzymes - iduronate sulfatase - involved in the turnover of these substances (34). Heparin-like GAGs such as heparan sulfate and dermatan sulfate are considered to be angiostatic and it has subsequently been proposed that suramin might exert its antitumoral activity by inducing an accumulation of these GAGs (25). Histopathological examination excluded any difference in the degree of vascularization of the two suramin-treated osteosarcoma lines as compared to controls. Since no signs of vascular impairment could be disclosed the antiproliferative activity of suramin might be mediated through some other major pathway.

The present study demonstrates for the first time an inhibitory effect of suramin on the growth of human transplantable osteosarcoma tissue in nude mice. It extends earlier observations on the antiproliferative effects of suramin in vivo; previous studies have exclusively related to a heterogeneous material of spontaneously occurring human malignancies. Our experimental model offers a highly reproducible system which may prove useful for further attempts to clarify the tumoristatic mechanism of action of the drug.

**REFERENCES**


**Table 1**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>(G_0)</th>
<th>S</th>
<th>(G_2-M)</th>
</tr>
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<tbody>
<tr>
<td>L-1 OSM Control (8)*</td>
<td>77.89 ± 1.47*</td>
<td>8.26 ± 0.35</td>
<td>12.77 ± 0.69</td>
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<tr>
<td>Suramin (6)</td>
<td>76.72 ± 1.19</td>
<td>9.45 ± 0.41</td>
<td>15.13 ± 0.87*</td>
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<td>L-11 OSM Control (12)</td>
<td>82.28 ± 1.52</td>
<td>11.83 ± 0.65</td>
<td>8.52 ± 0.44</td>
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<tr>
<td>Suramin (13)</td>
<td>66.72 ± 1.48</td>
<td>20.44 ± 1.29</td>
<td>13.48 ± 0.75*</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, total number of sarcomas examined.

*Mean ± SE.

*Values refer to the aneuploid tumor cell population.

\(^*\) \(P < 0.001\).

**Fig. 3.** Representative DNA histograms obtained from L-I OSM (A) or from L-II OSM (B) sarcomas. Essentially similar patterns were obtained with the original tumors (not shown).
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