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Suramin-induced Growth Inhibition and Insulin-like Growth Factor-I Binding Blockade in Human Breast Carcinoma Cell Lines: Potentially Related Events

Federica Ravera, Loredana Miglietta, Paolo Pirani, Silvano Ferrini and Roberto E. Favoni

Suramin, a polyanionic drug used in the treatment of trypanosomiasis and onchocerciasis, inhibits growth factor-induced mitogenesis in several human tumours. We have investigated the effect of suramin on human breast cancer cell lines (HBCCL). By cell counts and thymidine incorporation we found that 50 to 400 μg/ml suramin inhibits the proliferation of HBCCL in a dose-dependent and reversible fashion ($ID_{50} \approx 200$ μg/ml for MCF-7 and MDA-MB 231). Radioreceptor and affinity cross-linking assays showed that suramin was also able to reduce the binding of insulin-like growth factor I (IGF-I) to its receptor (40–50% inhibition at 100 μg/ml). Our results indicate that the drug does not affect the IGF-I receptor (IGF-I-R), but binds directly to the IGF-I peptide. In conclusion, the strict correlation observed between suramin inhibition of proliferation and IGF-I binding on HBCCL suggests a possible therapeutic role for this molecule as an antineoplastic drug in human breast tumours.
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INTRODUCTION

SURAMIN IS a polysulphonated drug that has been used in the treatment of certain parasitic diseases, especially trypanosomiasis and onchocerciasis [1]; it affects cell metabolism in different ways and has been shown to inhibit a large number of enzymatic systems [2]. De Clercq [3] reported that suramin inhibits the reverse transcriptase activity of several avian and murine retroviruses, showing virustatic capacity. Suramin has therefore been tested in clinical trials for the acquired immunodeficiency syndrome [4]; the response noted in an HIV-associated lymphoma indicated the possible use of suramin as an antineoplastic drug [5]. Initial phase I/II studies, which were performed on patients with prostatic [6], adrenal and renal [5, 7] cancer, and also with a variety of lymphomas [5], described partial, minimal as well as no response. Moreover, a correlation between response and serum suramin levels has been shown: most occurred at blood levels above 200 μg/ml but serious toxicity was found for

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doses higher than 300 μg/ml [5, 7]. Many *in vitro* studies showed that suramin inhibits the growth of several human cell lines derived from non-small cell lung, prostate, colon and breast carcinomas and lymphoid sources [8, 9, 10, 11, 2], but the mechanism by which suramin exerts its action have not yet been clarified. The antiproliferative activity of this drug on various human tumours may occur through a single process or may be the result of a series of processes which can also occur independently. Suramin interaction with different growth factors (GF) such as platelet-derived GF (PDGF), epidermal GF, transforming GFβ, basic fibroblast GF and insulin-like GF (IGF-I and IGF-II) has recently been reported [9, 7, 12–17].

Prevention of the GF binding to specific cell membrane receptors and/or the displacement of the receptor-bound GF, as already demonstrated for PDGF [13], could indicate a biochemical mechanism of suramin action, as well as suggest its suitability as an antineoplastic drug.

Several GF act in an autocrine or paracrine fashion to control the growth of mammary epithelial cells and stimulate the malignant progression of breast tumour [18, 19]. Among them, IGF-I and IGF-II are potent mitogens for some breast cancer cells in culture [20, 21]. Moreover autonomous production of IGF-related peptides and expression of functional IGF-I and IGF-II receptors [21, 22] by human breast cancer cell lines (HBCCL) have been reported.

In the present study we demonstrate that suramin efficiently and reversibly inhibits the growth of several HBCCL. In addition we show that suramin is able to partially suppress IGF-I binding to its cell-surface receptor at the same doses required to slow down cell proliferation. These findings suggest that the inhibition of the mitogenic effect of IGF-I might be one mechanism by which suramin affects the proliferation of human breast carcinoma.

MATERIALS AND METHODS

Chemicals

Suramin (Germanin, symmetrical 3′-urea of the sodium salt of 8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5-trisulphonic acid), *Mc* = 1429.2, was donated by Bayer AG, Leverkusen-Germany. IGF-I and IGF-II were purchased from Amgen Biologicals (California, USA) and Bachem Inc., respectively.

[^{125}I] Iodotyrosil-IGF-I (somatomedin C) (ref. IMI72; s.a. = 74 TBq/mmol) and [methyl-^{3}H]thymidine (ref. TRA 120; s.a. = 185 GBq/mmol) were purchased from Amersham.

Tissue culture medium and foetal calf serum (FCS) were purchased from ICN-Biomedicals (Milan, Italy) whereas medium without phenol red was from Gibco-BRL (Milan, Italy).

Tissue culture plasticware was from Becton-Dickinson Labware (New Jersey, USA) and Greiner GmbH (Germany).

Cell cultures

MCF-7 cells (derived from pleural effusion of an adenocarcinoma) were the kind gift of Dr G. Leclercq (Institute J. Bordet, Brussels, Belgium). MDA-MB 231 cells (from a pleural effusion of an adenocarcinoma) and T47-D, ZR-75/1B, Hs578T, BT-20 cells (from pleural effusions of ductal carcinomas) were the kind gift of Dr M.E. Lippman (Georgetown University, V.T. Lombardi Cancer Research Center, Washington, DC, USA). Each cell line was routinely grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 5% heat-inactivated FCS, 2% L-glutamine (2 mmol/l), 1% penicillin–streptomycin (50 U/ml + 50 μg/ml) and 1% (100 ×) non-essential amino acids and incubated at 37°C in 5% CO_2 in air. Cells were counted in haemocytometric chamber and the viability was determined by the trypan-blue dye exclusion method. For serum-free experiments phenol red-free D-MEM supplemented with 2 mg/l transferrin, 1% (1 ×) trace elements and 20 mmol/l Hepes, was used.

Growth inhibition studies

Cell count. Cells were plated in 35-mm Petri dishes at a density of 12.5–25 × 10^3 cells/cm^2 in 2 ml of D-MEM (5% FCS) and incubated overnight. The next day, suramin (50–400 μg/ml final) was added to triplicate dishes. Cells were allowed to grow for 4 additional days prior to being harvested by trypsinisation and counting.

[methyl-^{3}H]Thymidine ([^{3}H]dThd) uptake. Cells were plated as above. 2 h before the end of each treatment [48 or 96 h in complete or serum-free medium (SFM)] the cells were pulse-labelled with 7.4 × 10^4Bq/ml of [^{3}H]dThd. [^{3}H]dThd incorporation was stopped by washing monolayers twice with phosphate-buffered saline (PBS). Cells were immediately solubilised with 0.1% sodium dodecylsulphate (SDS) at 42°C and treated with 1 N perchloric acid (PCA). After a second treatment with 0.5 N PCA, cells were incubated at 70°C for 30 min with shaking. After centrifugation the incorporated radioactivity was measured in a Packard Minaxi-4000 β-counter.

Binding studies (radioreceptor assay)

Subconfluent cultures of HBCCL were plated in 24-well dishes. After 24 h monolayers were incubated with binding buffer (D-MEM 0.1% bovine serum albumin (BSA), 40 mmol/l Hepes) for 1 h at 37°C to remove surface or receptor-bound IGF-I. Cells were then treated by one of the following: (a) simultaneously with radioligand (45 000–50 000 cpm/well; ≈ 0.04 pmoles/ml) in combination with increasing concentrations of either unlabelled competitor or suramin in a total volume of 400 μl binding buffer for 2 h at 4°C, shaking. (b) Sequentially, with the labelled growth factor for 1 h, then suramin was added for an additional hour in the presence of [^{125}I] IGF-I. (c) Sequentially, with suramin administered for 2 h and [^{125}I]IGF-I for 2 additional hours in the presence or absence of suramin.

After binding, unbound radioligand was removed; cells were washed three times with washing buffer (0.1% BSA Hanks' balanced salt solution) and lysed with Triton-X buffer (1% Triton X-100, 10% glycerol, 20 mmol/l Hepes), for 30 min at 37°C. Released radioactivity was measured in a Beckman 5500 B γ-counter. Only specific binding, after subtraction of non-specific binding occurring in the presence of a 3500-fold excess of unlabelled ligand, is shown.

Membrane preparation

Confluent MCF-7 and MDA-MB 231 cells were harvested with a cell scraper, centrifuged for 10 min at 1000 rpm, resuspended in homogenisation buffer (50 mmol/l mannitol, 5 mmol/l Hepes, 0.01% Na-azide, 0.1 mmol/l phenil-methil-sulphonil-flouride, 1 mmol/l dithiothreitol) and aspirated through a 25-gauge needle. After addition of $CaCl_2$ (10 mmol/l final concentration), cell suspension was aspirated again and centrifuged for 1 min at 13 000 rpm. The supernatant was centrifuged in a Beckman TL-100 ultracentrifuge for 10 min at 90 000 rpm. The membrane-containing pellet was resuspended in homogenisation buffer and stored at −80°C [23].

Affinity cross-linking

150 μg of MCF-7 or MDA-MB 231 membrane proteins were incubated for 1 h at room temperature with [^{125}I]IGF-I (2 × 10^5 cpm), increasing concentrations of suramin (50, 100, 200, 400 μg/ml) and excess of unlabelled IGF-I, IGF-II (1 μg/ml) or bovine insulin (10 μg/ml) in PBS containing 0.1% BSA. The samples were then centrifuged three times at 13 000 rpm at room temperature for 30 min and incubated with the cross-linker agent (0.5 mmol/l disuccinimidyl suberate) in PBS (0.1% BSA) at 4°C for 15 min. The reaction was then quenched with 10 mmol/l Tris–HCl (pH 7.4). After 30 min centrifugation at 13 000 rpm the pellets were dried and reconstituted in 100 mmol/l dithiothreitol loading buffer prior to 7.5% SDS polyacrylamide gel electrophoresis (SDS–PAGE). Parallel experiments on cell monolayers were also performed.

Statistical analysis

Results are presented as the mean percentage (± SEM) of three experiments performed in triplicate. Differences between groups (control vs. treated values) were evaluated by the Mann–Whitney test.

RESULTS

Growth effects

Growth curves were performed on the oestrogen receptor-positive (ER+) MCF-7 and the oestrogen receptor-negative (ER−) MDA-MB 231 breast cancer cell lines treated with increasing doses of suramin. Figure 1 shows that inhibition of cell growth by suramin is dose-dependent; the half-maximal effect was obtained at approximately 200 μg/ml for both MCF-7 and MDA-MB 231 cells. These results were confirmed by a [^{3}H]dThd incorporation assay where the growth inhibitory effect of suramin was even more evident on MDA-MB 231 cells (ID_{50} = 190 and 140 μg/ml for MCF-7 and MDA-MB 231, respectively, data not shown). Time-course experiments showed that the inhibition of [^{3}H]dThd uptake was near maximal after 48 h of incubation with suramin (data not shown).

The evaluation of the cell viability by a dye-exclusion assay indicated that the growth inhibitory action of suramin on both MCF-7 and MDA-MB 231 cells was not due to cytotoxicity. In addition, the removal of 100 and 200 μg/ml suramin after 48 h treatment, followed by 48 h incubation with fresh medium, partially reverted the inhibition of DNA synthesis. The recovery, compared with continuous 96-h suramin exposure, was 2–4-fold (+ 130 to 300%) on MCF-7 and 6–10-fold (+ 470 to 870%) on MDA-MB 231 (data not shown).

We further analysed the effect of suramin on IGF-I-induced cell proliferation. MCF-7 cells were cultured in SFM in the presence or absence of 10 nmol/l IGF-I for 48 h. As shown in Fig. 2, suramin at 100 μg/ml partially inhibited the IGF-I-induced cell proliferation, while at 200 μg/ml the mitogenic effect of exogenous IGF-I was almost completely abolished. In contrast to MCF-7 cultures, MDA-MB 231 cells failed to respond to exogenous IGF-I. Suramin strongly inhibited [^{3}H]dThd uptake in MDA-MB 231 both in the presence and absence of exogenous IGF-I.

Receptor studies

In order to verify the ability of suramin to inhibit IGF-I binding, we first analysed the presence of IGF-I sites on HBCCL. We found one class of specific high-affinity type I IGF receptor (dissociation constant, K_d = 0.5–0.7 nmol/l) in all cell lines examined as already reported by others [22]. Subsequently, monolayers of various ER+ (MCF-7, T47D, ZR75/1B) and ER− (MDA-MB 231, Hs578T, BT20) HBCCL were incubated with [^{125}I] IGF-I and increasing concentrations of suramin (50, 100, 200, 400 μg/ml). As shown in Fig. 3a, b, a dose-related binding inhibition of [^{125}I] IGF-I to IGF-I receptor was observed in the presence of suramin (40–50% of inhibition at 100 μg/ml). Furthermore, Scatchard analysis revealed that K_d were not modified whereas the number of occupied receptors (B max) were significantly decreased. We then analysed whether suramin was also able to displace IGF-I bound to its receptor. MCF-7 and MDA-MB 231 cell lines were treated with [^{125}I]IGF-I for 1 h at 4°C (in preliminary time-course experiments we already found that maximal binding of IGF-I was reached after 1 h of incubation at 4°C); several doses of suramin (50, 100, 200, 400, 800, 1600 μg/ml) were subsequently added to the plates for a further hour. As shown in Fig. 4, suramin was able to dissociate, in a dose-dependent manner, the cell-bound GF. Concentrations of 200 μg/ml were needed to dissociate 50% of the prebound IGF-I in both MCF-7 and MDA-MB 231 cell lines. In order to verify whether or not suramin inhibits the IGF-I/receptor interaction by competition for the receptor or by direct obstruction of the GF itself, we performed IGF-I binding experiments on suramin-pretreated cells. Preincubation of MCF-7 and MDA-MB 231 monolayers with 200 μg/ml suramin for 2 h did not

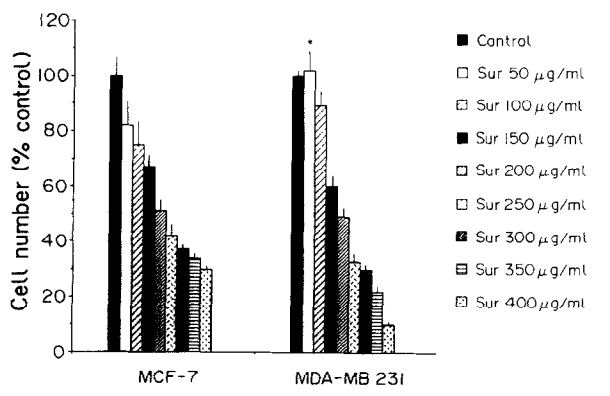

Fig. 1. Effect of suramin (96 h of exposure) on the growth of oestrogen receptor-positive (MCF-7) and -negative (MDA-MB 231) human breast cancer cell lines. Columns: Mean of three independent experiments performed in triplicate. Bars: SEM; P = 0.05; * = not significantly different from untreated cells.

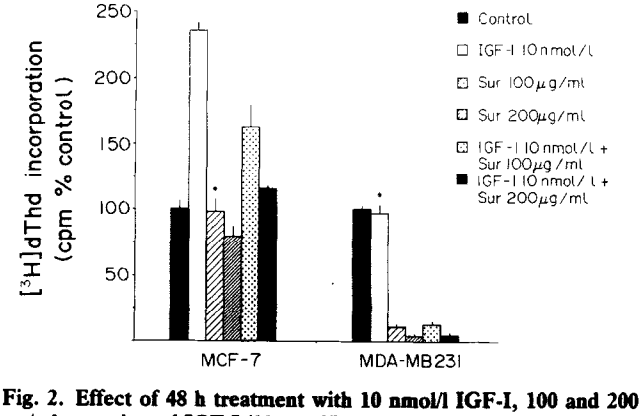

Fig. 2. Effect of 48 h treatment with 10 nmol/l IGF-I, 100 and 200 μg/ml suramin and IGF-I (10 nmol/l) + suramin (100 and 200 μg/ml) (simultaneous administration) in serum-free medium on the growth of MCF-7 and MDA-MB 231 cell lines. Columns: Mean of three independent experiments performed in triplicate. Bars: SEM; P = 0.05; * = not significantly different from untreated cells.

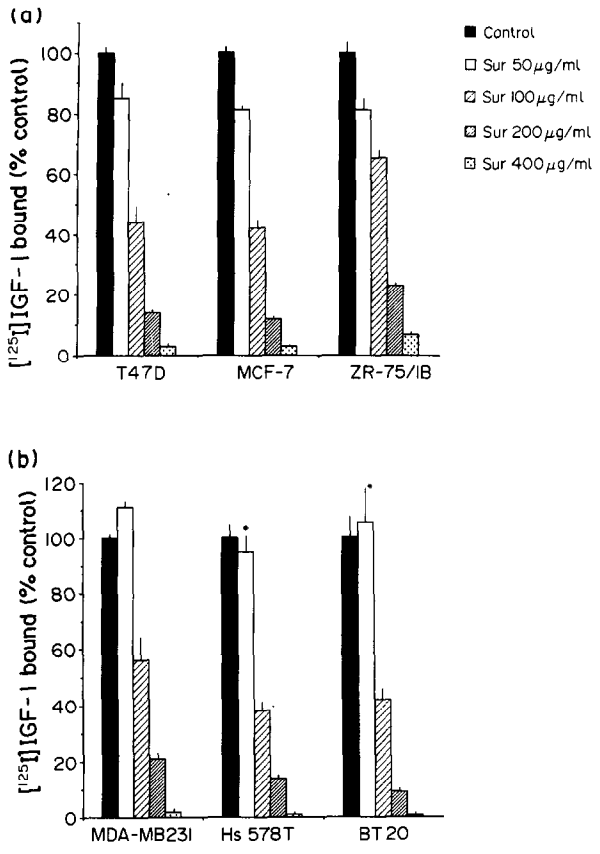

Fig. 3. Dose-dependent reduction of [^{125}I] IGF-I binding to ER+ (a) and ER− (b) human breast cancer cell surface receptors in the presence of suramin (simultaneous administration). Columns: Mean of three independent experiments performed in triplicate. Bars: SEM; $P = 0.05$; * = not significantly different from untreated cells.

significantly inhibit the binding of [^{125}I]IGF-I added to the cells for a further 2 h. In contrast, the binding was strongly inhibited when suramin was still present during the incubation with [^{125}I]IGF-I (data not shown). This result and the finding that suramin does not affect the GF/receptor mutual affinity suggests that this drug does not bind directly to the IGF-I receptor but to the soluble or receptor-bound IGF-I thus preventing its interaction with the receptor.

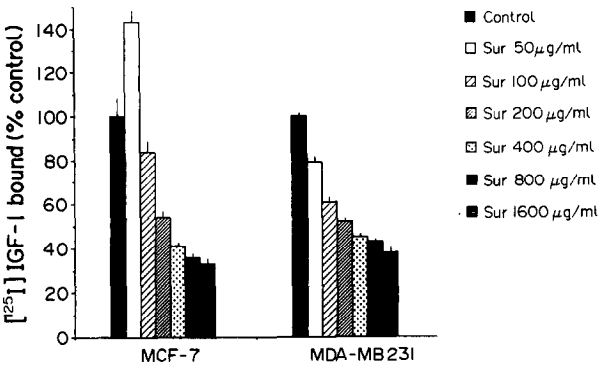

Fig. 4. Dose-dependent reduction of [^{125}I]IGF-I binding to ER+/ER− human breast cancer cell membrane receptors in the presence of suramin (sequential administration). Columns: Mean of three independent experiments performed in triplicate. Bars: SEM; $P = 0.05$; * = not significantly different from untreated cells.

Cross-linking experiments

Further evidence for the inhibitory effect of suramin on IGF-I binding was provided by affinity cross-linking experiments. Membranes and monolayers of MCF-7 and MDA-MB 231 cell lines were incubated with [^{125}I]IGF-I and increasing concentrations of suramin; the GF was covalently linked to its binding site. Autoradiograms of proteins resolved by 7.5% SDS–PAGE (Fig. 5a, b), revealed a major band of approximately 300 kDa corresponding to the whole IGF-I receptor molecule. An additional band, detected at 130 kDa, represents the receptor α-subunit bound to the iodinated peptide. Densitometric analysis revealed a negative correlation between the amount of suramin added and the IGF-I bound to its receptor (data not shown).

It is therefore evident that suramin was able to inhibit IGF-I binding to its specific receptor molecule. In addition, bovine heparin (200 μg/ml), a molecule that is structurally related to suramin, revealed a non-significant inhibition of IGF-I binding to its receptor on MDA-MB 231 cells.

Excess of unlabelled IGF-I, IGF-II (1 μg/ml) and insulin (10 μg/ml) also competed with the tracer for the receptor resulting in 100, 80 and 20% inhibition of binding respectively, in accordance with the decreasing affinity of those molecules for the IGF-I receptor.

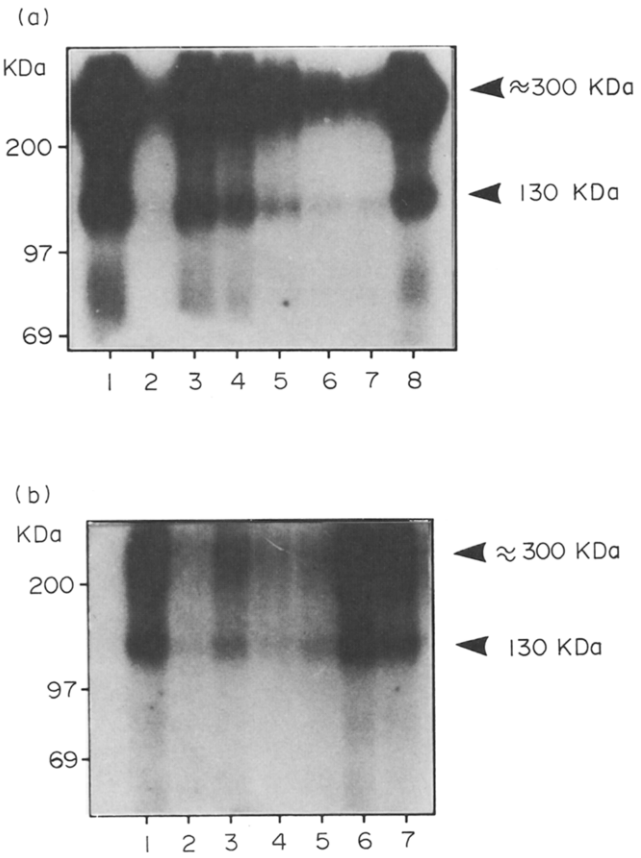

Fig. 5. Affinity cross-linking of [^{125}I] IGF-I to MCF-7 and MDA-MB 231 breast cancer cell line membrane preparations. The arrows indicate the position of [^{125}I] IGF-I covalently bound to the whole IGF-I receptor molecule (≈ 300 kDa) and to the IGF-I receptor α-subunit (130 kDa). (a) Lane 1 = [^{125}I] IGF-I; 2 = IGF-I; 3–6 = suramin 50, 100, 200, 400 μg/ml; 7 = IGF-II; 8 = bovine insulin; (b) Lane 1 = [^{125}I] IGF-I; 2 = IGF-I; 3, 4 = suramin 100, 200 μg/ml; 5 = IGF-II; 6 = bovine insulin; 7 = bovine heparin.

DISCUSSION

In the present study we provide evidence for a reversible cytostatic effect of the antiparasitic drug suramin on HBCCL *in vitro*. This effect may be related to the capability of this drug to inhibit the binding of IGF-I to its specific cell surface receptor, resulting in the suppression of its mitogenic effect.

Recent studies indicated that suramin inhibits the IGF-I-induced proliferation of human osteosarcoma cells [17] and the IGF-I and oestradiol-induced mitogenic action on HBCCL [30].

A number of polypeptide GF, acting by an autocrine or paracrine mechanism, have been shown to have mitogenic activity on human breast cancer cells [18, 19]. The IGF system is a complex group of cross-reacting peptidic hormones, receptors and serum binding proteins (BP) [21, 22, 24–27]. IGF-I is highly mitogenic for breast cancer cells in culture [20, 22]; this process is mediated by interaction of the GF with its specific transmembrane receptor which displays tyrosine kinase activity and a K_d of 0.5–4 nmol/l [22]. Recent studies have shown that an antibody which blocks the IGF-I receptor is able to inhibit human breast cancer cell proliferation in a clonogenic assay [28] as well as tumour growth *in vivo* [29], suggesting an autocrine role for this factor. Basing on these previous observations we have evaluated the antiproliferative activity of suramin on HBCCL and its action on the IGF-I/IGF-I receptor complex in this tumour model.

We demonstrated that therapeutic concentrations of suramin inhibited the proliferation of both ER+ and ER− HBCCL. This effect was cytostatic since the cell viability after suramin treatment was not affected. Moreover, the removal of the drug from the cultures resulted in the recovery of cell proliferation.

The previously described antagonistic effect of suramin on GF activity prompted us to examine whether this drug could inhibit the mitogenic action of IGF-I on HBCCL. The enhancement of ER+ MCF-7 cell proliferation induced by exogenous IGF-I in SFM was inhibited in a dose-dependent manner by suramin. The proliferation of the ER− MDA-MB 231 cells, under the same experimental conditions, was not enhanced by exogenous IGF-I, probably because of the constitutive production of IGF-I by this cell line [21]. Thus, it is conceivable that the inhibitory activity of suramin on the proliferation of MDA-MB 231 may be related to an interference with autocrine GF/GF receptor loop. The suramin interaction with the IGF-I/IGF-I receptor binding was also confirmed by [^{125}I]IGF-I binding experiments.

The drug induced a dose-dependent inhibition of [^{125}I]IGF-I binding in all the HBCCL examined. In addition, the concentrations of suramin required for IGF-I binding inhibition were in the same order of magnitude as those inhibiting cell proliferation, indicating a possible relationship between these two effects of the drug.

Suramin was able to displace [^{125}I]IGF-I already bound to its receptor only at concentrations 4-fold higher than those required to inhibit the binding of [^{125}I]IGF-I simultaneously administered with suramin. The finding that pretreatment of HBCCL with suramin did not affect IGF-I binding to its receptor, suggests that suramin is not acting directly by competing at the receptor level but rather by capturing IGF-I. It has also been hypothesised that IGF binding proteins (IGF-BP), which are produced by many HBCCL [25–27] could play a critical role in modulating the interaction between IGF-I and its receptor [24]. Therefore, it is supposed that IGF-BP could be also implicated in the events leading to the suramin-induced IGF-I/IGF-I receptor binding inhibition. Although a possible involvement of IGF-BP cannot be excluded, in the current studies it would seem unlikely since conditioned medium (containing the cell-secreted BP) was removed and monolayers were extensively washed before radioreceptor assays were performed.

Suramin inhibition of the IGF-I binding to its specific surface receptor molecule on HBCCL was also confirmed by affinity cross-linking assays. These experiments revealed the presence of bands corresponding to the typical size of the whole IGF-I receptor molecule and to its α-subunit, that were sharply reduced by addition of suramin.

The action of suramin appears to be specific since heparin (a structurally related polysulphonated compound), showed only a non-significant decrease in IGF-I binding on both MCF-7 and MDA-MB 231 cell lines.

In conclusion, our data indicate that suramin exerts antiproliferative activity and inhibitory influence on IGF-I binding in human breast cancer cells. The cytostatic effect, resulting from these potentially related events, implies that the eventual clinical usefulness of suramin in the adjuvant treatment of human breast cancer should be explored further.

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Fine Specificity of Antibody Recognition of Carcinoma-associated Epithelial Mucins: Antibody Binding to Synthetic Peptide Epitopes

Shaun Briggs, Michael R. Price and Saul J.B. Tendler

The protein core of polymorphic epithelial mucins consists predominantly of a repeating 20 amino acid peptide motif. Many monoclonal antibodies reactive with breast carcinomas recognise determinants located within the mucin protein core, and epitope mapping techniques have demonstrated that these antibodies bind to epitopes of three, four or five amino acids within the hydrophilic sequence, P D T R P A P. Each of these mucin core-reactive antibodies map to epitopes containing the central arginine residue. The fine specificity of a panel of anti-mucin antibodies binding to the tetrameric peptides P D T R or R P A P (synthesised on the heads of polyethylene pins) was examined by systematically replacing each amino acid in turn with all other 19 natural amino acids, and then testing these analogues for antibody binding. We have (i) identified those amino acids in epitopes which are essential for antibody binding, (ii) shown that for each epitope there is a hierarchy of residues required for immune recognition—certain amino acids may be replaced with little or no loss of antibody binding, while the presence of others is essential, and (iii) concluded that antibody specificity is further regulated by the residue(s) flanking an epitope motif which may impose conformational constraints upon the presentation of the epitope to an antibody.
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INTRODUCTION

HUMAN EPITHELIAL mucins identified using monoclonal antibodies are widely expressed molecules which are associated with secretory epithelia and with carcinomas of the breast and ovary [1–3]. These high molecular weight glycoproteins are extensively glycosylated with oligosaccharides attached in O-linkage to serine and threonine residues of the single chain polypeptide core [4,5]. Furthermore, the complexity of their banding pattern in electrophoretic gels is attributable to a genetic polymorphism which has been defined at both the protein and DNA level [6, 7].

Many murine monoclonal antibodies produced against human