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GROWTH FACTORS IN HUMAN PROSTATE CANCER CELLS: IMPLICATIONS FOR AN IMPROVED TREATMENT OF PROSTATE CANCER

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Summary—It has been previously shown that estrogens may exert their action on human breast cancer cells through coordinated control of secreted growth factors which act in an autocrine and paracrine fashion. Growth stimulation of the androgen receptor negative prostate carcinoma cell line DU-145 by dihydrotestosterone in the presence of the androgen-responsive human prostate carcinoma cell line LNCaP now indicates that androgens may regulate growth of prostate carcinoma cells through related mechanisms. A variety of androgen-regulated growth modulatory activities with autocrine and paracrine potential can be detected in conditioned media from LNCaP cells partially purified by ion exchange chromatography. Androgen-induced growth of LNCaP cells is partially inhibited by the polyanions suramin and dextran sulfates which antagonize growth factor action. These data suggest the existence of at least two different mechanisms of growth regulation by androgen which can be distinguished by their different sensitivity to growth factor inhibitory agents. We conclude that the combination of antipeptidergic substances *and* androgen withdrawal would represent a new and promising strategy for treatment of human prostate cancer.

INTRODUCTION

Since the pioneering studies by Huggins and Hodges it has been established in the scientific literature that growth of human prostate cancer is androgen dependent [1]. Furthermore, experiments in different systems have demonstrated that growth of the normal prostate is also regulated by androgens (for review see Ref. [2]). The question *how* androgens regulate growth of the normal and cancerous prostate has been only partially elucidated. The hypothesis of autocrine growth regulation of cancer cells has opened new ways to answer this question. This hypothesis includes that cancer cells (1) produce growth factors, (2) possess specific receptors for these growth factors and (3) are growth stimulated by exogenous addition of these growth factors [3]. Overexpression of autocrine growth control loops has been correlated with the expression of the malignant phenotype (for review see Ref. [4]). The detailed analysis of the steroidal regulation of autocrine growth factors and their receptors as pivotal elements in the process of steroid-induced malignant transformation has been a target of numerous investigations: for estrogen-responsive human breast cancer cells, it has been demonstrated that estrogens may regulate growth of human breast cancer through the specific regulation of a variety of growth factors with growth stimulatory and growth inhibitory potential [5–7]. The present study is designed to investigate whether androgens regulate growth of prostate carcinoma cells through a related mechanism. Although the presence of an autostimulatory loop with transforming growth factor α as an effector molecule in hormone-responsive human prostate cancer cells has been recently demonstrated [8], clear evidence that the modulation of growth factor secretion is not only a growth *associated* phenomenon but also *mediates* the growth stimulatory action of androgens in prostate cancer cells has not been provided yet. Therefore, we decided to analyze the *function* of secreted polypeptide growth factors in growth control of prostate carcinoma cells. Polyanions like suramin [9] and dextran sulfates serve as experimental tools to inactivate secreted growth factors functioning in an auto- and paracrine way [10]. The androgen-responsive human

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prostate carcinoma cell line LNCaP [11] and the androgen-independent human prostate carcinoma cell line DU-145 [12] were used as model systems. LNCaP cells have been shown to be a suitable model to study androgen action on prostate carcinoma cells: DHT and the synthetic androgen R1881 stimulate proliferation of LNCaP cells after binding to the androgen receptor; DU-145 cells do not express the androgen receptor and are not stimulated by androgens [8, 11–13]. In contrast, both cell lines express the EGF-receptor and are stimulated in their proliferation by the administration of EGF or transforming growth factor α (TGFα)[8, 13, 14].

EXPERIMENTAL

Cells and cell culture

LNCaP cells were provided by Dr Horoszewicz, Buffalo, NY, U.S.A. DU-145 cells were from American Type Culture Collection (Rockville, MD, U.S.A.). Both cell lines were passaged in Dulbecco's minimal essential medium (DMEM, Gibco, Karlsruhe, Germany), supplemented with 10% fetal bovine serum (FBS, Gibco) and routinely tested for mycoplasma contamination. For studies involving hormonal treatment, LNCaP cells were passaged at least for 1 week in DMEM supplemented with 5% sulfatase and charcoal-treated calf serum (CCS) prior to the experiment [7].

Hormones, growth factors and drugs

DHT (17β-hydroxy-5α-androstan-3-on) was supplied by Merck, Darmstadt. J-125-EGF was obtained from Amersham, U.K., unlabeled EGF (receptor grade) from Gibco/BRL. Suramin sodium, the hexasodium salt of 8,8′-[carbonyl-bis-[imino-3,1-phenylenecarbonylimino-(4-methyl-3,1-phenyl-ene)carbonylimino]]bis-1,3,5-naphthalenetrisulfonic acid, was obtained from Bayer. Dextran sulfates of different molecular weight were from Sigma (St Louis, MO, U.S.A.).

Anchorage-independent growth assays

In 35-mm tissue culture dishes (Costar, Cambridge) a 0.8 ml top layer of DMEM containing 0.4% Bacto-Agar (Difco, Detroit, MI, U.S.A.), 10% FCS, 8.000 indicator cells (LNCaP or DU-145) and the sample to be tested (hormone, growth factor, polyanion, serial dilutions of conditioned serum-free media, column fraction) was added to an already hardened bottom layer of 1.0 ml DMEM containing 0.6% agar and 10% FBS. After incubation for 14 days in a humidified 5% CO_2 atmosphere at 37°C colonies larger than 60 μm were counted microscopically.

Coculture experiments

LNCaP cells (500,000) in DMEM containing 1% CCS were plated into two compartments of a 4-compartment petri dish (100 × 15 mm, Falcon) that had been previously coated with 0.1 mg/ml poly D-lysine (Sigma) to achieve better attachment. Treatment with 10 nM DHT or ethanol vehicle only (0.1%) was started 24 h later and continued throughout the experiment. One day later the medium was removed, and the dividing walls between LNCaP and empty compartments were perforated with a hot spatula. Fresh medium was replaced; LNCaP and blank compartments were in communication. One day later 500 DU-145 cells were plated into the blank compartment. After an additional period of 5 days the medium was removed, and the number of DU-145 colonies (>50 cells) was counted after fixation with methanol and staining with amido black.

Preparation of conditioned media

Serum-free conditioned medium (CM) was prepared as described earlier [7]. In brief, near confluent monolayers of LNCaP cells that were grown in DMEM supplemented with 5% CCS in the presence of 10 nM DHT or the ethanol vehicle alone (0.1%) for at least 5 days were subsequently cultured in serum-free medium under continuous androgen treatment: 30 ml per tissue culture dish (NUNC; 150 mm diameter) of DMEM supplemented with 1 mg/l human fibronectin (Boehringer Mannheim), 2 mg/l transferrin (Collaborative Research, Lexington, MA, U.S.A.), and 20 mM Hepes buffer (pH 7.4) (Gibco). The first CM collection was removed and discarded after 12–16 h, fresh serum-free medium was conditioned by the cells for 48 h under continuous treatment. This CM was harvested, combined with 0.2% (v/v) Aprotinin (Sigma), and centrifuged at 800 *g* at 4°C for 10 min. CM was concentrated 25–200-fold by ultrafiltration in an Amicon Concentrator (cutoff 10 kDa) (Amicon, Braunschweig). After CM collections, cell monolayers were harvested with PBS, 0.02% EDTA solution (Gibco) for the determination of cell number by electronic

particle counting (Coulter ZM). Fractionation of CM-aliquots (1 mg total protein) was performed by ion exchange chromatography on a mono Q HR5/5 column (Pharmacia, Uppsala, Sweden) with a NaCl-gradient from 0 to 1 M in 20 mM Tris, pH 7.4. 1-ml fractions were collected, lyophilized, resuspended in and consequently dialyzed against PBS and tested for their growth modulatory activity in a soft agar assay with DU-145 cells as indicator cells.

EGF receptor assay

The EGF receptor assay was essentially performed as described earlier [7, 8] with some changes: LNCaP cells were plated in 24-well tissue culture dishes (NUNC) at 150,000 cells per well in DMEM containing 10% FBS. After an incubation overnight in a humidified 5% CO_2 atmosphere at 37°C, cells were carefully washed 3–4 times with binding buffer (DMEM, 1 mg/ml bovine serum albumin, BSA, Sigma, and 40 mM Hepes, pH 7.4). Binding buffer (200 µl) containing 100 pM J-125-EGF (Amersham, Braunschweig, Germany) and unlabeled EGF (Gibco/BRL, Karlsruhe, Germany) were added. After incubation at 4°C for 2 h cells were washed 4 times with ice-cold Hank's balanced salt solution (Gibco) supplemented with 1 mg/ml BSA. The cells were solubilized for 30 min at 37°C with 750 µl Triton solution (20 mM Hepes, 1% Triton X-100, 10% glycerol). Radioactivity was determined in a Berthold gammacounter.

RESULTS

Androgen stimulation of DU-145 cells in coculture with LNCaP cells

In the LNCaP/DU-145 model system we have addressed the question if secreted growth factors are involved in the process of growth regulation by androgens. The first piece of evidence for the existence of this mechanism was found in coculture experiments with LNCaP and DU-145 cells: LNCaP cells growing at high density in monolayer serve as feeder cells, the number of anchorage-dependent growing DU-145 colonies which were seeded at low density is used as an indicator for growth factor activities in the common medium. The androgen 5α-dihydrotestosterone (DHT) alone does not influence growth of DU-145 cells whereas 10 nM DHT in the presence of LNCaP cells leads to a significant growth stimulation of DU-145 cells (Fig. 1). Similar results were obtained in an

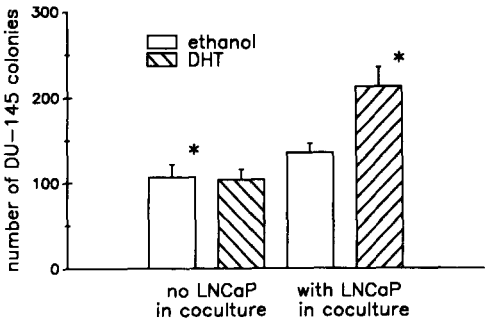

Fig. 1. Effect of secreted factors from LNCaP cells on the anchorage-dependent cloning of DU-145 cells. DU-145 cells were plated in the presence or absence of LNCaP cells treated with 10 nM DHT or ethanol vehicle alone. The number of DU-145 colonies was counted after 5 days (mean ± SD, $*P < 0.05$, $n = 5$).

experimental setting where the anchorage-independent proliferation of DU-145 cells was stimulated by the synthetic androgen R1881 in the presence of LNCaP cells (data not shown). These observations demonstrate that DHT induces the secretion of growth stimulatory factors by LNCaP cells. These factors have a growth promoting potential for human prostate carcinoma cells as measured by the stimulation of colony formation of DU-145 cells. We have chosen DU-145 cells as indicator cells to exclude any direct effects of androgens on cell growth.

Secretion of androgen-regulated growth modulatory activities by LNCaP cells

Similar observations were made by biological analysis of medium which had been conditioned by LNCaP cells. Serum-free medium was conditioned over a period of three days by LNCaP cells with and without stimulation by DHT. Conditioned medium was concentrated by ultrafiltration and tested after dialysis against fresh serum-free medium for their growth modulatory potential on DU-145 cells. Serial dilutions of conditioned media from androgen-treated LNCaP cells lead to a significant growth stimulation of DU-145 cells suggesting that LNCaP cells under androgen treatment secrete factors with higher growth stimulatory activity than untreated cells (Fig. 2). Only minimal growth stimulatory activities can be detected in media conditioned by LNCaP cells without androgen treatment. Addition of different proteases to the conditioned media preparations abolish their stimulating activity completely (data not shown). These data strongly suggest that secreted polypeptide factors are responsible for the stimulation of DU-145 cells by androgen in coculture with LNCaP cells (Fig. 1).

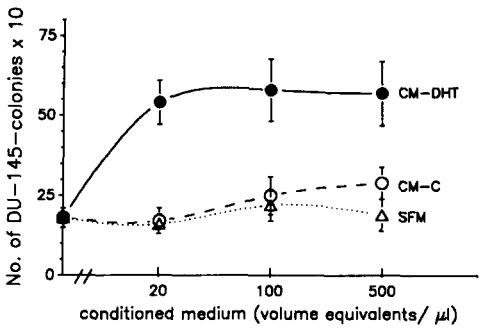

Fig. 2. Effect of serial dilutions of concentrated unconditioned serum-free medium (SFM) or concentrated serum-free medium conditioned by LNCaP cells under treatment with 10 nM DHT (CM-DHT) or ethanol (CM-C) on the anchorage independent cloning of DU-145 cells (mean ± SD of triplicate determinations in 1 representative out of 3 independent experiments). The "volume equivalent" of conditioned medium is the volume of the conditioned medium in the assay *before* concentrating by ultrafiltration.

A partial purification of the secretory products from LNCaP cells by ion exchange chromatography and analysis of the growth modulatory potential of each fraction on DU-145 cells shows that a variety of activities can be detected (Fig. 3). An almost identical pattern was obtained with LNCaP cells as indicator cells (data not shown). The secretion of some of these activities appears to be induced under androgen treatment whereas others seem to be repressed by androgen stimulation of the secreting cell. These observations clearly demonstrate that not one single factor is responsible for the stimulation of DU-145 cells (Figs 1 and 2) and eventually autocrine stimulation of LNCaP cells by androgen treatment. In contrast, the androgen regulation of a variety of growth factors seems to be important. Detailed analysis of a putative *autocrine* function of each of these factors would have to be based on their biochemical characterization.

Inhibition of anchorage-independent growth of LNCaP cells by polyanions

In order to analyze the autocrine function of androgen-regulated growth factors in general, we decided to use the polyanionic compound suramin as an experimental tool which has been shown to inhibit the action of a variety of secreted growth factors with autocrine potential including PDGF, EGF, TGFβ, FGF, IGF-I and IL-2 [9, 15, 16]. Moreover, we extended our studies on other polyanions, i.e. polysulfonated polysaccharides of the dextran sulfate type with different molecular weights. All these substances lead to a very effective growth inhibition of LNCaP cells in a dose-dependent way (Fig. 4). A similar dose–response curve is obtained with DU-145 cells (data not shown). Based on the molar concentration of each compound used, only minor differences are observed in the growth inhibitory potential of dextran sulfates of different molecular weight and suramin at concentrations between 10 and 100 μM suggesting a similar mechanism of action.

Inhibition of binding of J-125-EGF by suramin

Although the mechanism of action of suramin has not been completely understood, suramin-induced growth inhibition can be correlated with the inhibition of growth factor binding by suramin [9]. In LNCaP cells, the regulation of the system EGF/TGFα–EGF–receptor by androgens seems to be one important target and mediator of androgen action. Binding of J-125-EGF on LNCaP cells is specifically competed by

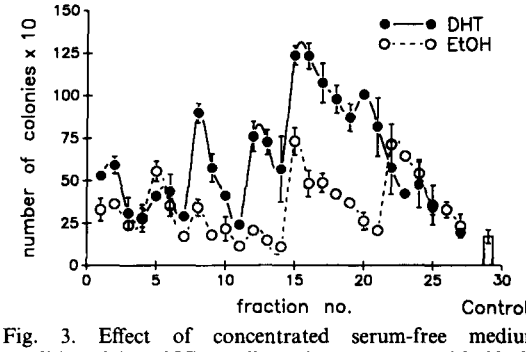

Fig. 3. Effect of concentrated serum-free medium conditioned by LNCaP cells under treatment with 10 nM DHT or ethanol (EtOH) on the anchorage-independent cloning of DU-145 cells (mean ± SD) after fractionation by ion exchange chromatography. Control: anchorage-independent cloning of DU-145 cells in the absence of CM-fractions.

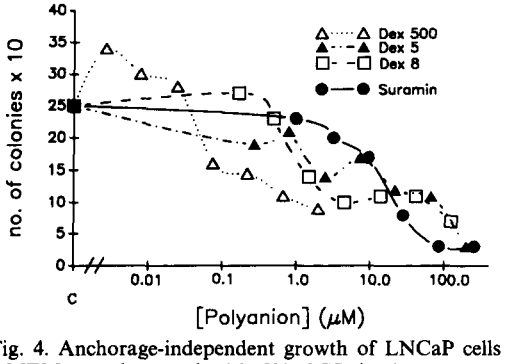

Fig. 4. Anchorage-independent growth of LNCaP cells in DMEM, supplemented with 5% CCS, in the presence of suramin or dextran sulfates with different average molecular weights (Dex 500: average mol. wt 500,000; Dex 5: average mol. wt 5000; Dex 8: average mol. wt 8000). The standard deviations of each individual triplicate determination (less than 15%) are omitted. Data are the result of 1 representative experiment out of 3 independent assays.

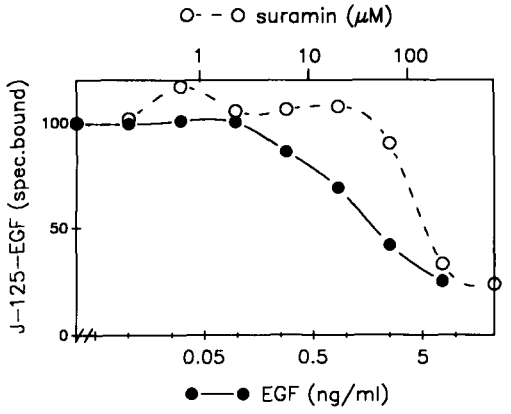

Fig. 5. Binding of J-125-EGF on LNCaP cells in the presence of different concentrations of unlabeled EGF or suramin.

unlabeled EGF (Fig. 5), confirming the presence of high affinity binding sites on their surface [8, 13]. Suramin also prevents binding of J-125-EGF to LNCaP cells in a concentration-dependent way, suggesting that suramin and other polyanions inhibit growth of LNCaP cells (Fig. 4) by the inhibition of binding of autocrine acting EGF-like material, i.e. TGFα [8].

Incomplete abolishment of androgen-induced growth stimulation of LNCaP cells by suramin

Consequently, we decided to analyze the effect of suramin alone on growth of LNCaP cells or in the simultaneous presence of 5 ng/ml EGF or 10 nM DHT (Fig. 6). Both EGF and DHT lead to a significant growth stimulation in the absence of suramin, suramin in increasing concentrations abolishes the effect of EGF completely which can be correlated with the inhibition of J-125-EGF-binding by suramin (Fig. 5). In contrast, the addition of DHT in the presence of suramin causes a growth stimulation which can be only incompletely inhibited by high concentrations of suramin. Therefore, androgens seem to exert their action through two mechanisms which can be distinguished by their different sensitivity to suramin, one which can be inhibited by suramin and most likely is mediated by the androgen-induced overexpression of autocrine growth control loops, another which is not affected by suramin.

DISCUSSION

Secreted polypeptides as regulators of prostate cancer cell growth

It has been previously postulated that estrogens regulate growth of human breast cancer cells through coordinated control of the secretion of autostimulatory and autoinhibitory polypeptides (for a review see Ref. [5]). Similar experiments for human prostate cancer have been performed only to a limited extent. Although it has been suggested that androgenic control of the secretion of a fibroblast growth factor-like activity might contribute to the mechanism of growth control of androgen-dependent Shionogi mammary carcinoma SC 115 cells [17] the hypothesis that growth control of androgen-dependent prostate carcinoma might be mediated through the regulation of specific growth factors with autocrine and paracrine potential has not been experimentally verified. Growth factor activities in prostatic tissue have been mainly characterized in terms of their fibroblast stimulating i.e. paracrine potential [18–21]. Even recent publications which focus on the identification of growth factor activities in normal and pathological prostatic tissue of the rat and man are based on the use of fibroblasts as indicators of growth modulatory activities [22, 23]. Only the regulation of the EGF receptor has been discussed with varying results in different prostatic experimental systems [13, 24].

Coculture and conditioned media experiments show (Figs 1 and 2) that growth stimulation of prostate carcinoma cells by androgens is, at least partly, mediated through secreted polypeptide factors which can act on other prostate carcinoma cells. By this means, androgen stimulation can even be transferred to androgen-independent cells in the presence of androgen-responsive cells. A TGFα-species could be one of these factors because we have previously presented evidence that LNCaP-cells

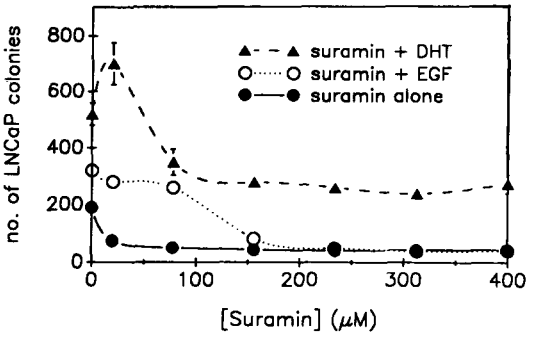

Fig. 6. Anchorage-independent growth of LNCaP cells in DMEM, supplemented with 5% CCS, in the presence of suramin alone or with the simultaneous addition of 1 nM DHT or 5 ng/ml EGF (mean ± SD of triplicate determination in 1 representative out of 5 independent experiments).

and DU-145 cells secrete a TGFα-species, express specific EGF/TGFα receptors on their cell surface and are stimulated by the addition of EGF. Moreover, androgens induce the secretion of a TGFα immunoreactive protein by LNCaP cells [8], pointing to the putative role of TGFα as one mediator of androgen action. The biological meaning of this regulatory system is also underlined by the finding that androgens also induce overexpression of the EGF receptor itself [13].

Suramin inhibits binding of J-125-EGF to its receptor (Fig. 5). Comparison of the pattern of displacement of J-125-EGF by suramin with the shift of the suramin dose–response curve by the simultaneous addition of EGF points to a causal association between these two observations: although suramin also inhibits several intracellular enzymes including DNA polymerases (for a review see Ref. [25]) it seems feasible to assume that it inhibits growth of LNCaP cells by preventing binding of autocrine acting factors, eventually EGF/TGFα, to their receptors, as it has been previously shown for PDGF [15] and IL-2 [16]. The distinct but significant growth stimulation by lower concentrations of polyanions (Figs 4 and 6) points to the differential sensitivity of different growth factors to suramin: TGFβ which exhibits autoinhibitory potential in human prostate carcinoma cells [26] can be inhibited in its action by concentrations of suramin which are much lower than the concentration necessary to prevent binding of EGF to its receptor [9]. It is therefore likely that the growth stimulation by low concentrations of dextran sulfates (Fig. 5) and suramin (Fig. 6) is mediated through the inactivation of autoinhibitory TGFβ secreted by LNCaP cells. The exact mechanism by which suramin inhibits growth factor binding is not known, by competitive binding to the receptor or direct binding of the ligand itself. Apparently a certain charge configuration of the molecule is necessary for the biological activity because both suramin and the polysulfonated dextranes are highly charged molecules which may create a local environment around the cell making them unaccessible for the peptide ligand. This hypothesis is supported by the well known fact that growth factors are dissociated from their receptors by a low pH.

Based on these findings, we propose the hypothesis that androgens exert one part of their action on androgen-dependent cells through regulation of autocrine growth control loops by at least two mechanisms, i.e. enhanced expression of the growth stimulatory ligand and induced expression of the receptor whereas androgen-independent cells constitutively overexpress these growth factors and/or receptors (Fig. 7). Binding of the ligand to its receptor leads via enhanced phosphotyrosinkinase-activity to an increased intracellular mitotic signal which subsequently is followed by the induction of DNA replication in the nucleus and cell proliferation [4]. However, androgen

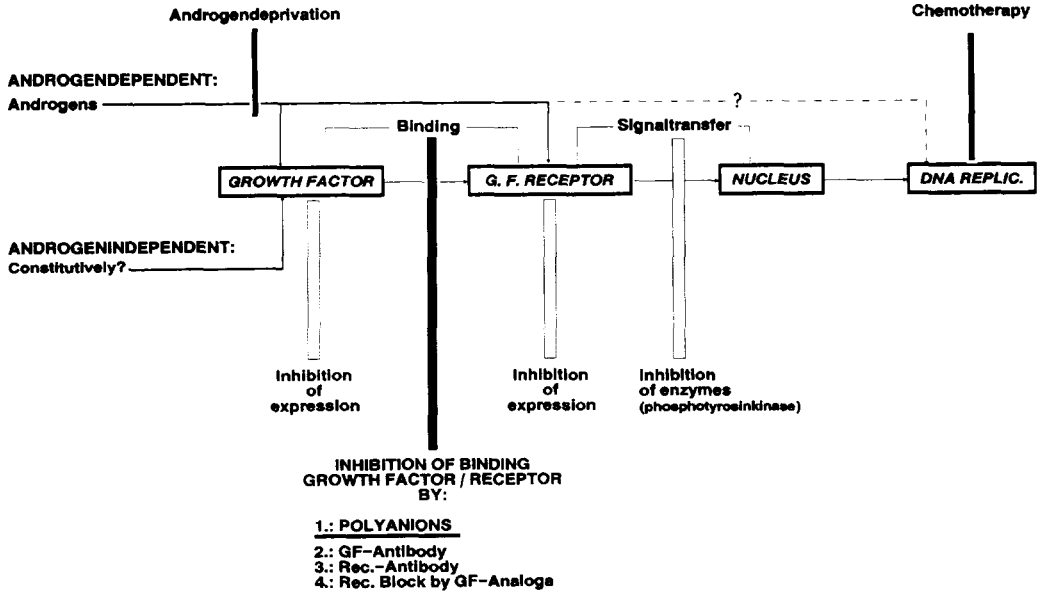

Fig. 7. Schematic diagram of the proposed mechanism of growth control of human prostate cancer cells and potential targets for therapeutical intervention.

stimulation which is accompanied by increased secretion of growth stimulating activities (Figs 1–3) is only partially inhibited by suramin. These data are in contrast to a recent report which describes a complete abolishment of the growth stimulation of LNCaP cells by the synthetic androgen R1881 through administration of suramin [27]. The reasons for these divergent findings are not clear: one possible explanation is the use of different androgens (R1181 vs DHT) and different proliferation assays (monolayer vs soft agar assays). Moreover, the concentration used in these monolayer assays (1000 μM) has a clear cytotoxic effect under anchorage-independent growth conditions in our study as judged by morphological appearance and trypan blue exclusion (data not shown). The incomplete abrogation of androgen stimulation by cytostatic concentrations of suramin in our studies points to the existence of a second mechanism which mediates androgen action but is not inhibited by suramin. One may be the enhanced expression of the receptor itself which may generate an enhanced intracellular mitotic signal, the other one may be the existence of "private" autocrine loops, i.e. a growth factor needs not to be secreted and exerts its action intracellularly [28]. A paracrine communication between neighboring cells through membrane bound growth factors which has been shown for a TGFα-species in other experimental systems [29] would probably also not be accessible to suramin. Furthermore, a mechanism distinct from growth factor action cannot be excluded, i.e. the androgen-controlled secretion of other substances like leukotrienes [30] or an immediate, intracellular influence of androgen on cell proliferation.

Antipeptidergic therapy of prostate cancer

Since the existence, androgen control and possible functional role of autocrine growth control mechanisms in human prostate cancer cells has now been demonstrated the development of a new "antipeptidergic" principle of therapy has become possible which is targeted to interfere with this mechanism of growth control (Fig. 7). The inhibition of binding of growth factors to their cell surface receptors represents a particularly hopeful approach. Polyanionic compounds like suramin which inhibit binding of most secreted polypeptide growth factors to their cell surface receptor have the advantage that not *all* relevant growth factors need to be identified which would be necessary if specific antibodies against the receptor and/or growth factor or a receptor blocking analogue were to be used. Concentrations of suramin between 100 and 300 μ M which are very effective in growth inhibition of LNCaP (Figs 4 and 6) and DU-145 cells (data not shown) can also be reached in patients [31]. However, our data clearly show (Fig. 6) that neither androgen withdrawal alone (i.e. by orchidectomy, 5α-reductase inhibitor, antiandrogen, LHRH antagonist) nor antipeptidergic blockade of growth factor action alone would lead to maximal growth inhibition but only the combination of both strategies i.e. androgen deprivation *and* antipeptidergic substances like suramin, dextran sulfates or others would represent a highly promising therapeutical approach. A further advantage of this combination therapy would be given by the ability of growth factor inactivating substances to inhibit both androgen-dependent and androgen-independent tumor cell subpopulations. Future studies will elucidate the therapeutical potential of these strategies for treatment of prostate cancer.

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