Retinoic Acid Modulates Extracellular Urokinase-Type Plasminogen Activator Activity in DU-145 Human Prostatic Carcinoma Cells

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ABSTRACT
Effects of all-trans retinoic acid (RA) on the net enzymatic activity of secreted, extracellular urokinase-type plasminogen activator (u-PA) in DU-145 human prostatic carcinoma cells were examined to assess the potential use of retinoids in human prostate cancer prevention and treatment. u-PA is associated with tumor progression involving invasion and metastasis. Based on a chromogenic substrate assay, results show that DU-145 cells secrete five times more u-PA than normal human prostatic epithelium. DU-145 cells were treated with 0.1 to 10 μM RA for 48 h. This short treatment of cells with RA did not inhibit growth. After a 48-h treatment of cultures with RA, serum-free conditioned medium was analyzed for u-PA activity by SDS-PAGE zymography. Two major bands of u-PA with Mr of ~54,000 (high molecular weight u-PA) and ~33,000 (low molecular weight u-PA) were detected. Plasminogen-dependent catalytic activity of these bands could be specifically inhibited with antibody to u-PA, confirming that these bands represent u-PA. A 48-h treatment with 1.0 μM RA reduced u-PA activity in conditioned medium to 51.6% of control. A 50% reduction in free u-PA antigen level, as compared to control, was further demonstrated at 1.0 μM RA by Western blot analysis and densitometry. These results show that RA can decrease the net extracellular urokinase activity produced by prostatic carcinoma cells. It is proposed that these effects of RA may have important implications not only in the chemoprevention of prostate cancer, by inhibition of promotion of prostatic intraepithelial neoplasia to invasive carcinoma, but also in tumor progression during invasion and metastasis, by decreasing extracellular matrix degradation, as shown in our accompanying article (M. M. Webber and A. Waghray, Clin. Cancer Res., 1: 755–761, 1995).

INTRODUCTION
Prostate cancer, excluding nonmelanoma skin cancer, is the most common cancer in adult men in the United States (1–3). The American Cancer Society estimated that 200,000 new cases and 38,000 deaths from prostate cancer will occur in the United States in 1994 (2). The incidence of such cancers increases with age and about 80% are diagnosed in men over the age of 60 years. Both incidence and mortality from prostate cancer are increasing (3). Because of the increasing life span and an aging population in the United States, prostate cancer is a major health concern today (4). Based on autopsy studies, approximately 11 million men in the United States, older than 45–50 years, have histologically identifiable carcinoma of the prostate, and it has been predicted that 1 in 10 men will develop prostate cancer in his lifetime (3, 5). A primary cause of death from prostate cancer is invasion and metastasis. One of the first steps in progression to cancer is degradation of the basement membrane. Proteases intervene at the transition from in situ to invasive carcinoma where local dissolution of the basement membrane occurs. The major extracellular matrix-degrading enzymes in neoplasia comprise the serine proteases, (PAs)3 and plasmin, and a family of structurally related metalloproteases. A correlation between metastatic potential and increased expression of these proteases has been demonstrated (6). The PA-mediated proteolysis involves activation of the zymogen plasminogen by PA to plasmin, a trypsin-like enzyme with broad substrate specificity.

There are two types of PAs, the t-PA is found in most normal tissues and in blood where it is involved in fibrinolysis. The u-PA is involved in the degradative events involving extracellular proteolysis during tissue remodeling, involution, wound healing, and cancer. u-PA, a glycoprotein, is secreted as a single-chain proenzyme. The active form of u-PA is a two-chain, Mr 54,000 molecule consisting of a light A chain representing the NH2 terminus containing the receptor-binding domain and a single kringle unit, and a heavy B chain which contains the catalytic domain (7). The catalytic domain is conserved in all serine proteases including trypsin, plasmin, and kallikrein. Two active, two-chain forms of u-PA have been identified, the high Mr ~54,000 and the low Mr ~33,000, the latter composed primarily of the B chain.

Prostatic epithelium intrinsically secretes both t-PA and u-PA. Prostatic PAs are contributed to the seminal fluid as part of the prostatic secretion, of which u-PA is a major component and is involved in the liquefaction of semen (8). Our hypothesis is that because of this high u-PA expression, prostatic intraepithelial neoplasia and prostatic carcinomas (PCas) have an inherent advantage and predilection for progression to invasion and metastasis. A change in normal homeostasis and regulation of u-PA occurring even in very small foci of malignant cells,

3 The abbreviations used are: PA, plasminogen activator; EGF, epidermal growth factor; EtOH, absolute ethanol; PCa, prostatic carcinoma; PIN, prostatic intraepithelial neoplasia; RA, all-trans retinoic acid; SF-CM, serum free-conditioned medium; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; CM, conditioned medium.
such as PIN, could result in invasion. Prostatic carcinomas express more u-PA activity than benign tumors and most of this activity represents the $M_r \sim 54,000$ u-PA (9). u-PA levels also show a correlation with bony metastases (10, 11). PA activity in several human prostatic carcinoma cell lines has been correlated with the aggressiveness of the cell type (12).

Although PA activity has been recognized in prostatic tissue, the regulation and role of different PAs in extracellular matrix degradation during tumor progression has not been studied extensively in prostate cancer. Hormones, growth factors, and tumor promoters can modulate the expression and secretion of PAs. EGF, transforming growth factor $\beta$, basic fibroblast growth factor, platelet-derived growth factor, and cytokines (interleukin 1, interleukin 4, and tumor necrosis factor), can modulate u-PA synthesis; however, the effects of various factors may vary with the cell type (13). Estrogens and prolactin stimulate PA secretion in breast cancer cells while hydrocortisone inhibits it (14). A large variety of carcinoma cells express high levels of u-PA and a correlation between u-PA secretion, malignant transformation, and aggressiveness of human and animal cancers has been shown (15).

Our objectives were: (a) to compare levels of secreted u-PA by normal and malignant prostate cells, (b) to examine modulation of the net extracellular u-PA activity by RA, and (c) to determine possible usefulness of RA in inhibition of tumor progression involving invasion and metastasis in prostate cancer. Many retinoids are inhibitors of tumor promotion in vitro and in vivo (16, 17). Of special interest is the observation that retinoids inhibit invasion and metastasis (1, 18). Information is not available on the possible regulatory effects of RA on the extracellular u-PA activity in prostate cancer. Therefore, the present investigation examines the effects of RA on the net activity of u-PA secreted by DU-145 human prostatic carcinoma cells.

**MATERIALS AND METHODS**

**Materials.** The materials used were: human prostatic carcinoma cell line DU-145 HTB 81 from American Type Culture Collection; RPMI 1640 medium 320-1875AJ; antibiotic/antimycotic mixture 600-5240AG from GIBCO; fetal bovine serum. Cells were subcultured once per week. Normal prostatic epithelial cells were isolated from fresh surgical specimens according to methods developed in this laboratory (19). Cells were maintained in keratinocyte growth medium serum-free medium supplemented with 10 ng/ml EGF. Passage 1 and 2 cells were used as normal controls. EtOH was used as the vehicle for RA. The final concentration of the vehicle in the culture medium was 0.1%.

**Microplate Assay for Establishing Effects of RA on the Growth of DU-145 Cells.** Ten thousand cells were plated/well in a 96-well plate in 100 $\mu$l medium. RA at 2$\times$ the final concentration in 100 $\mu$l medium was added 24 h later. Medium containing RA was changed every 72 h. Test plates were recovered after a 10-day period and stained with the protein-binding dye methylene blue. Bound dye was released with 1% SDS, and absorbance measured at 620 nm with a Titertek microplate reader (20).

**Assays for Plasminogen Activators.** For the assays listed below for PAs, 500,000 cells were plated/60-mm culture dish in RPMI 1640 and 10% fetal bovine serum. Twenty-four h later, cells were washed twice with PBS, and serum-free RPMI 1640 medium containing RA was added. Samples of SF-CM from 48-h treated and untreated cells were collected and centrifuged at 2000 rpm for 5 min to remove cell debris. If not immediately used, medium samples were stored at $-75^\circ$C. For some experiments, the medium was concentrated using filter units with a molecular weight cutoff of 10,000.

**Chromogenic Substrate Assay for Plasminogen Activator Activity.** The u-PA enzyme activity was determined in SF-CM, using a synthetic substrate for u-PA by measuring the increase in absorbance of the free chromophore generated in comparison to the original substrate per unit time at 405 nm. At excess substrate concentration, the rate of absorbance increase, due to the amount of chromophore released, is linearly related to enzyme concentration (21).

**Gel Electrophoresis and Zymography.** SDS-PAGE zymography was performed as described by Heussen and Dowdle (22), using the indicated (see legends to Figs. 1, 2, and 4) concentrations of polyacrylamide in the separating gel into which the specific enzyme substrate plasminogen (12 $\mu$g/ml) along with 1% gelatin were incorporated. PAs have species specificity for plasminogen, hence human plasminogen was used. The volume of CM sample loaded per lane in the gel was based on a fixed cell number as indicated in the legends to Figs. 1, 2, and 4. Minigels were run at 200 V at 4°C for 45 min, then gently rocked in two changes of 2.5% Triton X-100 in distilled water for 1 h at room temperature to remove SDS, and incubated at 37°C for 18 h in Tris-HCl buffer (pH 7.6) to allow renaturing of the enzyme. The gels were stained with Coomassie blue and destained in methanol:acetic acid:water (3:1:6). The presence of enzyme activity is indicated by specific bands of lysis against a dark background.

**Inhibition of u-PA Activity in Zymograms by Specific Antibody.** To confirm the presence of u-PA, samples of SF-CM from DU-145 cells were incubated for 2 h with undiluted polyclonal antibody to u-PA prior to SDS-PAGE zymography as described above. This antibody also has anticalytic activity.

**Western Blot Analysis.** Effects of RA on the free u-PA antigen levels in the CM were examined using Western blots. The volume of the concentrated SF-CM loaded per lane in the gel was standardized against a fixed cell number of 12,000 cells.
Blots were stained using the primary antibody of u-PA (1:1000), goat anti-rabbit secondary antibody (1 :10,000), and avidin-biotin alkaline phosphatase.

RESULTS

u-PA Activity in CM from Malignant DU-145 Prostatic Cells Is Higher than in SF-CM from Normal Prostatic Epithelium. PA activity in 48-h CM from primary cultures of normal human prostatic epithelium was compared with that from DU-145 cells. Fig. 1A shows a control gel without plasminogen, which received a sample of CM from DU-145 cells similar to lane labeled as DU-145 in Fig. 1B. Absence of PA-mediated lysis in Fig. 1A confirms that PA activity is plasminogen dependent. The very light, thin band (Fig. 1A) represents gelatinase activity. Results in Fig. 1B show that DU-145 cells produce considerably higher levels of u-PA activity than the normal cells, as evident from the large zones of lysis at Mr, ~54,000 and ~33,000 (Fig. 1B). A clear Mr, ~74,000 t-PA band is shown by normal cells but not by DU-145 cells.

Antibody to u-PA Inhibits Mr, ~54,000 and ~33,000 u-PA Bands. To establish the identity of the lysis bands, an anticalytic antibody to u-PA was used to determine whether the respective PA activity in zymograms could be inhibited. In Fig. 2A, PA activity in SF-CM from DU-145 cultures is shown in the left lane (~Ab). The Mr, ~54,000 and ~33,000 bands were inhibited. Mixing the same amount of antibody with a 1:200 diluted sample of CM resulted in complete inhibition (Fig. 2B, +Ab), confirming that the Mr, ~54,000 and ~33,000 bands represent u-PA.

Effects of RA on the Growth of DU-145 Cells. Effects of RA at concentrations from 0.001 nM to 10 µM were tested in dose-response and time course experiments over a 10-day period. RA concentrations varying from 0.001 nM to 10 nM did not show any significant effect on growth (data not shown). However, when treated with RA at levels from 0.1 µM to 10 µM, significant growth inhibition was observed after 10 days of RA treatment (Fig. 3). It was important to first establish the effects of RA on growth so that samples of CM for zymography and chromogenic assays could be collected at a time when there was no growth inhibition. On the basis of these results, SF-CM samples were collected after 48 h of RA treatment (Fig. 3).
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Nevertheless, medium samples corresponding to a fixed cell number were used for testing u-PA activity in zymograms,chromogenic assays, and Western blot analysis.

**RA Causes a Decrease in Extracellular u-PA Activity.**

Triplicate cultures were placed in serum-free RPMI 1640 medium (untreated control), medium containing 0.1% ethanol (vehicle control), or 0.1, 0.5, or 1.0 μM RA. CM was collected after a 48-h treatment, and assayed for PA activity by SDS-PAGE zymography using a 10% polyacrylamide gel (Fig. 4). In untreated and ethanol-treated controls (Lanes DU-145 and ETOH, respectively), the large zone of lysis represents the high Mr =54,000 u-PA and the smaller ~33,000 u-PA bands in a dose-dependent manner. The urokinase/plasminogen-based zymography is a sensitive assay for detecting low levels of u-PA activity. However, if u-PA activity is very high in a test sample, effects of modulators of u-PA activity can be missed due to excessive lysis in the zymogram. Therefore, dilution of samples of SF-CM may be needed to detect the effect. The RA effect was quantified and confirmed by a chromogenic substrate assay.

**Decrease in u-PA Activity by RA Is Quantified by a Chromogenic Substrate Assay.** Using a specific, synthetic substrate for u-PA, u-PA activity was measured in CM from normal prostatic epithelium and from ethanol-treated 0.5 and 1.0 μM RA-treated DU-145 cell cultures. Results show (Fig. 5) a low level (21.1% of control) of u-PA activity in CM from cultures of normal prostatic epithelial cells as compared to a high level (100%) of activity from EtOH-treated control cultures. A dose-dependent decrease in u-PA activity to 66% and 51.6% of control was observed in SF-CM from cultures treated with 0.5 μM RA and 1.0 μM RA, respectively.

**RA also Reduces Extracellular, Free u-PA Antigen Levels.** Effects of RA on u-PA antigen levels in the CM were investigated. 2.5 million cells were plated/150-mm plate and treated as described earlier, with ethyl alcohol alone or with 0.5 or 1.0 μM RA. Concentrated CM was used for Western blots, which were stained with antibody to u-PA. Results show (Fig. 6) a wide band representing the high Mr =54,000 u-PA in CM from untreated and EtOH-treated cells, and a dose-dependent decrease in free u-PA antigen levels in CM from cells after treatment with 0.5 or 1.0 μM RA for 48 h. In a densitometric analysis of these u-PA bands, if ethanol control = 100%, then 0.5 μM RA = 82%, and 1.0 μM RA = 49.6% of control.

**DISCUSSION**

Malignant cells show anchorage independence, reduced intercellular and substrate adhesion, and loss of contact inhibi-
tion, which facilitate their increased mobility. However, normal cells in solid tissues are under the restraints of contact inhibition of movement and growth. An increase in extracellular plasminogen activator secretion can remove these constraints, mediate the altered tumor cell behavior, and favor the process of invasion and metastasis. In vivo, secreted prostatic PAs become a part of the semen, and their activity is plasminogen dependent. A correlation between u-PA secretion, malignant transformation, metastasis, and aggressiveness in animal and human breast, bladder, and lung tumors has also been demonstrated. Increased skeletal metastasis by rat prostate cancer cells, transfected with u-PA cDNA, is associated with overproduction of u-PA.

Retinoids are important in normal epithelial cell proliferation and differentiation but they can also reduce the invasive and metastatic potential of human and rodent epithelial tumor cells in vivo and in vitro, an effect associated with a decrease in PA and collagenase activity (1, 18, 24). Our objective was to determine whether RA could inhibit pericellular proteolysis and extracellular matrix degradation by reducing the net extracellular urokinase enzymatic activity. Treatment with 1.0 μM RA for 48 h caused a 50% reduction in extracellular u-PA enzymatic activity and in free u-PA antigen levels (Figs. 4–6). However, growth was not inhibited at this time period (Fig. 3). We propose that since normal prostatic epithelium, and by inference, early invasive prostatic carcinomas would intrinsically secrete u-PA, invasion may begin at an early stage. Our implication is that it may be possible to inhibit these degradative processes in vivo using retinoids (1).

The response of PAs to RA treatment differs in cells of different origin, e.g., 1 μM RA induced PA in cells of mesenchymal origin but decreased PA by 50% in human normal kidney epithelial cells (25). In F-9 mouse teratocarcinoma cells, RA induced differentiation and concomitantly suppressed u-PA expression (26). u-PA is involved not only in invasion and metastasis but also in tumor promotion in earlier stages of carcinogenesis. 12-O-tetradecanoylphorbol-13-acetate enhanced PA expression in DU-145 human prostate carcinoma cells (data not shown). In mouse skin carcinogenesis, retinoids inhibit phorbol ester-mediated tumor promotion (27). Inhibition of these promotional effects by retinoids may be of special significance. The net extracellular u-PA activity is determined not only by the amount of the enzyme itself, but also by its state of activation and the presence and levels of specific inhibitors of PA. The mechanisms of RA effects on extracellular u-PA activity and expression in human prostatic carcinoma cells are not known. Our preliminary results suggest that RA may not have a direct effect on u-PA expression; however, the decrease in extracellular u-PA activity may be the result of changes in the expression of transforming growth factor β and urokinase inhibitors.

A pronounced heterogeneity in the u-PA content in tissue sections from different parts of a tumor has been observed, with the most intensive staining being in the areas of invasive growth (28). Similar heterogeneity has been encountered in clonal populations derived from tumor cell lines in culture (29). The picture becomes even more complex when one considers hormone-responsive tissues. For example, estrogen-induced u-PA in estrogen receptor-positive breast cancer cells, but estrogen receptor-negative cells intrinsically secreted high u-PA levels and were highly invasive. Therefore, u-PA in breast cancer was considered to be a prognostic marker. This example further illustrates the variation in response to RA in hormone-responsive tissues. In estrogen-responsive cells, RA caused inhibition of PA production in the absence of estrogen, but increased PA production in its presence. On the other hand, the estrogen-resistant clone showed unresponsiveness to RA (23, 30).

The advantage of using heterogeneous tumor cell populations, such as DU-145 and most other tumor cell lines, is that they reflect the cellular heterogeneity of tumors in vivo. However, the disadvantage is that different clones within a cell population may respond differently to the test agents, resulting in variations in response under different culture conditions. Variability in PA secretion and response to RA has also been found among rhabdomyosarcoma and glioma cell lines (29, 31). We have observed similar variations in u-PA expression and response to RA in the DU-145 cell line, which consists of a very heterogeneous cell population. In some experiments, a decrease in u-PA activity was observed only at 10 μM RA. Therefore, it would not be surprising to find wide variations in secreted u-PA activity (29) and response to RA in different clones and cell lines under different culture conditions (13, 30).

The following facts about advanced prostatic carcinoma demonstrate the clinical relevance of our findings. In 80% of patients with disseminated prostatic carcinoma, a significant increase in plasma u-PA levels has been observed, and it was suggested that u-PA may be a reliable marker for prostatic metastatic disease (11). PCA patients have a latent tendency to bleed and have higher levels of fibrin-degrading enzymes in the plasma than in benign prostatic hyperplasia patients. The clinical picture is usually one of a patient with bony metastases and generalized bleeding. ε-Aminocaproic acid, a potent plasmin inhibitor, has been used to treat bleeding associated with PCAs (32). In about 20% of prostate cancer patients, fibrinolysis is associated with hemorrhagic manifestations. Extracts of tissue from metastases showed 2-fold more fibrinolytic activity than primary tumors (33). Huber et al. (34) have recently reported that plasma u-PA levels were indicative of colon cancer in 75.5% of the cases as compared to 51.5% for carcinoembryonic antigen, but together they gave the highest sensitivity value of
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90.9% for detection of colon cancer. u-PA could serve as a useful prognostic marker also for prostate cancer.

Retinoids can restore normal cell differentiation in a dysplastic epithelium (18). An example of such epithelium is the PIN or carcinoma in situ, which represents precancerous, dysplastic lesions, characterized by proliferation and anaplasia of cells (35). PIN also represents a continuum of morphological changes which progress toward increased cell proliferation, crowding, nuclear and nucleolar enlargement and heterogeneity, and finally to microinvasion of the basement membrane. There is evidence to suggest that prostatic carcinomas arise in foci of PIN (35). Because an increase in u-PA is associated with tumor promotion, PIN is an excellent target for chemoprevention. The most desirable effect of a chemopreventive agent would be to cause a regression of such preneoplastic lesions. Retinoids and protease inhibitors may accomplish this. A recent report shows that dietary N-(4-hydroxyphenyl)retinamide decreases tumor incidence of ras-myc-induced carcinomas in the mouse prostate reconstitution model (36).

Our proposition is that u-PA plays a key role in tumor progression from in situ carcinomas such as PIN to invasive tumors. The role of type IV collagenases in invasion and metastasis has been elegantly described by Liotta et al. (6). However, the procollagenases must be first activated to collagenases. This activation can not only be efficiently accomplished by plasmin generated from plasminogen by u-PA action, but a recent study reports that u-PA can directly activate type IV collagenase (37). These observations point to a key role of u-PA in initiating the enzymatic cascade at the cancer cell-extracellular matrix interface in the prostate, since we have observed that DU-145 cells express only trace gelatinase activity (Fig. 1A). Furthermore, ras and src oncogene expression is associated with increased u-PA production and inhibition of u-PA expression by antisense results in inhibition of lung colonization by NIH-3T3 cells transfected by EJ/vHa-ras (15, 38). Thus, transformation itself imparts the ability to secrete increased levels of u-PA. This evidence provides further support for our hypothesis that u-PA activity is critical in early invasion and plays a key role in the progression of PIN to invasive carcinoma. Measurement of u-PA along with prostate specific antigen levels in serum for early detection of prostate cancer may be very useful. Reduction of extracellular u-PA activity in advanced PIN by retinoids and protease inhibitors is an important area of investigation in cancer prevention and intervention, particularly when one considers the following observations. The incidence of histological prostate carcinoma is high and an estimated 11 million men older than 46–51 years may have latent carcinoma (3). PIN predates clinical carcinoma by about 5 years (35). Also, the incidence of latent carcinoma shows only small differences between different racial groups worldwide, e.g., 20.5% in Japan and 34.6% in the American white population (4). However, the incidence of clinical cancer is about 8 and 15 times higher in American white and black men, respectively, than in native Japanese men, suggesting that latent carcinomas progress to invasive cancer more frequently in American men than in native Japanese (4). Thus, this progression, spanning a period of 20–30 years, is an appropriate target for early intervention and prevention of prostatic carcinoma in American men.

Clonal evolution within epithelial neoplasms begins early in the neoplastic process, and regression of early intraepithelial lesions by retinoids has been reported. For example, remission of preneoplastic lesions such as oral leukoplasia in tobacco chewers occurs by intervention with β-carotene or vitamin A (17, 39). However, cancer prevention by retinoids is not a universal finding. In some cases, retinoids have been shown to promote skin cancer induction in animals (40). Since tumor cells must successfully complete a number of critical, sequential steps before invasion occurs and metastasis can be established, this implies that the inhibition of any one of these steps should lead to a decrease in invasion. We conclude that RA decreased the net extracellular urokinase activity and free u-PA antigen levels in cultures of DU-145 human prostatic carcinoma cells. The role of urokinase in extracellular matrix degradation and invasion and the effects of RA on these processes are examined in our accompanying article (1).

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