ET-1 Expression and Growth Inhibition of Prostate Cancer Cells: A Retinoid Target with Novel Specificity

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Abstract

Endothelin-1 (ET-1) is not only a potent vasoconstrictor but also serves as an important growth stimulator in various cancers, including breast, cervix, pancreatic, and prostate cancer. This suggests that blockage of ET-1 production may suppress tumor growth and possibly metastasis. We observed that certain synthetic retinoids, and all-trans-retinoic acid can repress LNCaP prostate cancer cell growth in vitro. In addition, these retinoid compounds counteracted exogenous ET-1-induced growth stimulation. Retinoid-dependent growth retardation of LNCaP cells coincided with suppression of ET-1 gene expression to a level undetectable by reverse transcription-PCR. Contrarily, the androgen-insensitive DU145 cells were refractory to retinoid treatment. To investigate the underlying mechanisms of the cell-specific response to retinoids, we transfected ET-1 promoter constructs containing wild-type or mutated AP-1 or GATA-2 site into prostate cancer cells. Distinct regulations of ET-1 promoter activity were found; in LNCaP cells, both binding sites are essential for optimal promoter activation, whereas in DU145 cells, additional promoter sequences and/or transcriptional factors seem to be involved. Furthermore, several anti-AP-1 selective retinoids failed to repress ET-1 promoter activity and to exhibit a cell growth-inhibitory effect on LNCaP cells, suggesting that different retinoid structural configurations are required for the inhibition of an AP-1 complex versus an AP-1/GATA-2 complex.

Introduction

The potential of retinoids for cancer prevention and progression is well accepted and is of special interest for prostate cancer because of the relatively slow progression of this disease and because of a possible association between the increased incidence of prostate cancer and vitamin A deficiency. Several studies have demonstrated the ability of retinoids to inhibit prostate cancer cell growth and invasion (1, 2). Retinoic action is mediated by its nuclear receptors, of which two classes have been identified: the RARs and RXRs (3). Each class has α, β, and γ subtypes, which are encoded by separate genes. Prostate cancer cells express retinoid receptor subtypes that function as mediators of retinoid signals (4).

ET-1 is a potent vasoactive peptide synthesized in vascular endothelial cells and also in other nonvascular tissues (5, 6). In recent studies, ET-1 has been identified in several different types of human cancer cells, stimulating growth of neoplastic cells as well as of surrounding tissues (6–8). Clinically, a 2.6 times higher level of ET-1 was found in the plasma of patients with advanced prostate cancer compared with healthy males (9). These observations suggest a role for ET-1 in the regulation and promotion of prostate tumor growth. This growth promotion may result from the ability of ET to act synergistically with various growth factors, such as epidermal growth factor, platelet-derived growth factor, insulin-like growth factor, basic fibroblast growth factor, transforming growth factor, and others, potentiating cellular transformation and/or proliferation (10). Therefore, eliminating the production of ET-1 and blocking the cross-talk of ET-1 with other growth factors may result in suppression of tumor growth and the progression of prostate cancer. In this study, we investigated the potential of synthetic retinoids in suppressing ET-1 production. Here we show that only certain classes of retinoids were effective ET-1 inhibitors. The growth-inhibitory effects observed with retinoids correlate with retinoid-induced ET-1 suppression.

Although the regulation of ET-1 production by cancer cells is presently unknown, several lines of evidence suggest that ET-1 is mainly regulated at the transcriptional level involving AP-1 and GATA-2 binding sites within its promoter region (11). To investigate the mechanisms of ET-1 expression in prostate cancer cells, we used ET-1 reporter plasmids to demonstrate that both sites are essential for ET-1 transcription. Interestingly, several anti-AP-1 compounds found previously to inhibit AP-1-mediated transcription were inactive in inhibiting the AP-1/GATA-2 complex. Together, we report for the first time that the AP-1/GATA-2 complex represents a novel retinoid target specificity distinct from simple AP-1 sites.

Materials and Methods

Cell Culture. LNCaP and DU145 cells were obtained from American Type Culture Collection and cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal bovine serum (JRH Biosciences, Kansas, MO), 1-glutamine, penicillin, and streptomycin.

Retinoids. All-trans-RA was obtained from Sigma Chemical Co. (St. Louis, MO). SR compounds (SR11246, SR11256, SR11220, SR11238, and SR11302) were provided by Dr. M. I. Dawson. CD compounds (CD 270, CD 271, CD 436, CD 437, CD 2809, and AM80) were obtained from CIRD Geldarmon (San Diego, CA) and MAXIA Pharmaceuticals, Inc. (San Diego, CA).

Growth Assays. To determine the inhibitory effect of retinoids on cell growth, prostate cancer cells were seeded in six-well plates in RPMI 1640 with 10% charcoal-treated fetal bovine serum, and different retinoids at concentrations ranging from 0 to 2 μM were added. The cultures were refed every 2 days during the 6-day period of the experiment. Cells were then trypsinized and counted. To measure the mitogenic effect of exogenous ET-1 and the retinoid inhibitory effect on ET-1-induced LNCaP cell growth, LNCaP cells were seeded in six-well plates in RPMI 1640 with 10% serum and were incubated for 24 h. Cells were then rinsed with PBS and refed with RPMI supplemented with 1% ITS (Life Technologies, Inc., Gaithersburg, MD). Different concentrations of exogenous ET-1 (Sigma, St. Louis, MO) were incubated with or without retinoids (1 μM). To analyze the dose response of retinoids on LNCaP cells, the prostate cancer cells were also cultured in the presence of 10 nM of exogenous ET-1 with different concentrations of retinoids. The cultures were refed every 2 days during the 4-day period of the experiment.

RT-PCR Analysis. Total RNA was isolated from prostate cancer cells (LNCaP and DU145) by guanidine thiocyanate method. RT-PCR was performed using the following primers for endothelin-1 gene: sense primer, 5'-TGCCTGTGCTGCCTTTGATGATAAGAC-3'; and antisense primer, 5'-GGTCACATAAAGGCTCTGAGGGGCTCT-3'. Thirty cycles of PCR were run at conditions as follow: 95°C, 1 min; 56°C, 1 min; 72°C, 1.5 min.
c-Jun were provided by Dr. I. M. Verma (Salk Institute, San Diego, CA) and Dr. M. Karin (Department of Pharmacology and Biology, University of California, San Diego, CA), respectively.

Transfection and CAT Assays. Prostate cancer cells (LNCaP and DU145) were seeded 48 h before transfection. Transient transfection was carried out using lipofectin (Life Technologies, Inc.), following the manufacturer's instructions. Cells were refed with 5% charcoal-treated fetal bovine serum and then harvested 48 h after transfection. A plasmid CMV-β-gal, which encodes a β-gal gene driven by the cytomegalovirus promoter, was included in all transfections allowing normalization for differences in transfection efficiency. The CAT and β-gal assays were performed using CAT ELISA kit (Boehringer Mannheim, Indianapolis, IN) and chlorophenol red-β-D-galactopyranoside (Boehringer Mannheim). CAT activity was normalized according to β-gal activity.

Results

Retinoid-induced Growth Inhibition in Prostate Cancer Cells Correlates with ET-1 Repression. all-trans RA and several synthetic retinoids including SR11256, SR11246, CD 270, and CD 2809 (12-14) displayed a dose-dependent growth inhibition of LNCaP cells when applied for a 6-day period (Fig. 1A and data not shown), whereas the inactive control retinoid CD 436 did not show significant effects. In contrast, these active retinoids showed only marginal growth inhibition in DU145 cells (Fig. 1B). This suggests that the observed growth inhibition is specific and not due to "general toxicity" of the active retinoids. Interestingly, retinoid-induced growth inhibition in LNCaP cells was accompanied by a down-regulation of ET-1 expression. Using reverse transcription-PCR, ET-1 message was no longer detectable in cells treated with 2 μM of all-trans RA and several synthetic retinoids (including SR11256, SR11246, CD 270, and CD 2809), whereas the expression of a control gene (GAPDH) was unaffected (Fig. 2A and Table 1). Some receptor subtype-specific retinoids such as CD 271 (an RARβ,γ selective retinoid) and CD 437 (an RARγ selective compound; Ref. 15), and AM80 (an RARα selective retinoid; Ref. 16) were weak inhibitors of ET-1 activity (Table 1). In general, ET-1 suppression correlated well with the growth-inhibitory effects of retinoids in LNCaP cells. All retinoids tested were inactive in suppressing ET-1 transcription in DU145 cells (Fig. 2A and Table 1), which were resistant to retinoids. However, apoptosis-inducing compounds such as CD 271 and CD 437 are the exceptions. Apparently, both inhibit growth of the cell lines through ET-1-independent pathways that involve induction of DNA fragmentation. Because the ET-1 promoter contains an AP-1 site that is important for its activity (17), we analyzed several so-called "AP-1-selective" retinoids (SR11220, SR11238, and SR11302), which are inactive as transcriptional activators but are able to inhibit AP-1-mediated activity (1). Somewhat surprisingly, several anti-AP-1 selective retinoids failed to inhibit ET-1 transcription (Table 1) and cell growth (data not shown).

Retinoids Reduce Growth Stimulatory Effect of ET-1. To determine whether the retinoid-induced ET-1 repression can indeed account for the observed growth inhibition, LNCaP cells were cultivated for 4 days in serum-free RPMI 1640 in the presence of different concentrations of ET-1 peptide containing 1 μM of retinoids. The growth-stimulating effects of the exogenous ET-1 were obliterated by the addition of retinoids (Fig. 2, B and C). These data suggest that retinoids do not only inhibit ET-1 transcription but also the ET-1 signaling pathway itself (18).

Retinoids Modulate ET-1 Expression at the Transcriptional Level via Cell-Specific Mechanisms. To further analyze regulatory mechanisms of ET-1 inhibition by retinoids, we transfected an ET-1 promoter-reporter construct (17) into LNCaP cells. In this study, c-Jun, c-Fos, and GATA-2 expression plasmids were cotransfected to achieve optimal ET-1 promoter activity. Compared with cells cultured in the
absence of retinoids, almost one-half of the ET-1 promoter activity was
repressed in cells treated with all-trans RA, SR11256, and SR11246, but
not with CD 436 (Fig. 3). Because retinoids were able to repress ET-1
promoter activity without cotransfection of retinoid receptors, this
suggests that sufficient endogenous retinoid receptors were present to
mediate retinoid actions. Consistent with our RT-PCR results, anti-AP-1
compounds (SR11220 and SR11238) were unable to suppress the activity
of transfected ET-1 promoter construct in LNCaP cells (Fig. 3). This
suggests that an AP-1 site when located adjacent to a GATA-2 site as in
ET-1 promoter responds differently to retinoids than a single AP-1 site.
The apoptosis-inducing compound, CD 437, which failed to inhibit
dogenous ET-1 expression, also did not inhibit ET-1 promoter activity
(Fig. 3). This is consistent with the observation by Lu et al. (19) that
apoptosis-inducing retinoids do not inhibit AP-1 activity.

Analysis of ET-1 gene regulation in vascular endothelial cells (11, 20)
that previously revealed that GATA-2 and AP-1 sites are essential for
ET-1 promoter function (17). In our preliminary transfection studies
using increasing amounts of c-Jun, c-Fos, and GATA-2 expression vec-
tors, the ET-1 promoter activity reached a plateau (data not shown).
Transfection of optimal concentrations of either c-Jun/c-Fos or GATA-2
expression plasmids resulted in a small increase in wild-type ET-1
promoter activity in LNCaP cells (Fig. 4A, columns 2, and 3). However,
cotransfection of both c-Jun/c-Fos and GATA-2 expression plasmids
leads to a substantial increase in ET-1 promoter activity (Fig. 4A, column
4). Thus, the transcription factors c-Jun/c-Fos and GATA-2 synergisti-
cally contribute to the ET-1 promoter activity. To further demonstrate
this, we used ET-1 promoter constructs containing a mutated AP-1
(mutAP1; Fig. 4B) or GATA-2 site (mutGATA; Fig. 4C). Mutations in
either site decreased promoter activity (column 1 in Fig. 4, B and C) when
compared with that of the wild-type promoter construct (Fig. 4A, column
1), presumably due to the reduced affinities of the mutated DNA binding
sites for the transcription factors. Cotransfection of either c-Jun/c-Fos or
GATA-2 expression plasmids with the mutAP1 or mutGATA, respec-
tively (columns 2 and 3 in Fig. 4B and C), only led to small increases in
transcription, whereas a combination of both AP-1 and GATA-2 led to a
somewhat stronger increase (column 4 in Fig. 4, B and C). These data
indicate that both intact AP-1 and GATA-2 binding sites are required for
optimal ET-1 promoter activation in LNCaP cells. Further experiments
using an ET-1 promoter with mutations in both sites (mutAPmutGATA)
confirmed that both sequences are essential for ET-1 promoter activity
in LNCaP cells, because the double mutant showed very low activity, even
when AP-1 and GATA-2 transcription factors were cotransfected (Fig. 4D).
Retinoids did not lead to further repression of the mutant ET-1
promoter constructs (data not shown), indicating that the AP-1/GATA-2
sites are essential for retinoid repression.

To investigate a possible cell-specific regulation of the ET-1 gene,
transfection experiments with the ET-1 promoter constructs were per-
formed in DU145 cells. Compared with LNCaP cells, DU145 cells

Table 1 ET-1 repression by synthetic retinoids in LNCaP cells

<table>
<thead>
<tr>
<th>Retinoids</th>
<th>Structure</th>
<th>Name</th>
<th>Reference</th>
<th>Specificity</th>
<th>ET-1 repression</th>
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<tr>
<td>all-trans RA</td>
<td>[Image]</td>
<td>CO(_2)H</td>
<td></td>
<td>RAR(\alpha,\beta,\gamma)</td>
<td>+++, complete repression</td>
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<td></td>
<td></td>
<td></td>
<td>SR11256</td>
<td>13</td>
<td>RAR(\alpha,\beta,\gamma)</td>
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<td>SR11246</td>
<td>14</td>
<td>RXR</td>
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<td></td>
<td>CD 2809</td>
<td>RXR</td>
<td>+++, complete repression</td>
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<tr>
<td></td>
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<td></td>
<td>CD 270</td>
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<td></td>
<td>SR11302</td>
<td>1</td>
<td>Anti AP-1</td>
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* Relative degree of ET-1 repression by retinoids: +++, complete repression (message no longer detectable by RT-PCR); ++, poor repression; +, no repression.

displayed higher basal level activity of the wild-type as well as the single mutant promoters (column 1 in Fig. 4). In addition, cotransfection of c-Jun/c-Fos led to a strong induction of promoter activity in wild-type and single mutant-transfected cells (column 2 in Fig. 4, A–C). In contrast, transfection of the GATA-2 expression vector did not change the activity of these promoters significantly (column 3 in Fig. 4, A–C). The strong responses of the wild-type and single mutant promoters to cotransfected c-Jun/c-Fos in DU145 cells appear to be due to low levels of endogenous c-Jun/c-Fos in these cells.\(^3\) Cotransfection of c-Jun/c-Fos and GATA-2 transcription factors led to the highest level of transcription of wild-type and single mutant promoters (column 4 in Fig. 4, A–C). These data

\(^3\) Unpublished results.
ET-1 EXPRESSION IN PROSTATE CANCER CELLS

Fig. 3. Retinoids repress the ET-1 promoter in vitro in LNCaP cells. LNCaP cells were transfected with an ET-1 promoter reporter construct along with c-Fos, c-Jun, and GATA-2 expression vectors. A constitutive ß-gal vector was included in each experiment to control for differences in efficiency of transfection. Cells were grown in RPMI 1640 containing 5% charcoal-treated fetal bovine serum with or without retinoids (2 µM). Cell lysates were prepared for CAT assays after 18 h of incubation. CAT activity was normalized according to ß-galactosidase activity. Data represent means of two independent experiments in quadruplicate: bars, SD. *, statistically significant reduction in CAT activity compared with that of control (no ligand) by Student’s t test (P < 0.05).

suggest that in DU145 cells, the ET-1 promoter can also be induced by the AP-1/GATA-2 complex. When the double mutant construct (mutAP-mutGATA2) was analyzed in DU145 cells, a significant reduction in basal activity relatively insensitive to either c-Jun/c-Fos or GATA-2 cotransfection was observed. However, cotransfection of both c-Jun/c-Fos and GATA-2 led to a substantial activation of the double mutant promoter. This suggests that additional factors and/or sequences may complement the double mutant defect in the presence of c-Jun/c-Fos and GATA-2 in DU145 cells. This difference in the regulation of ET-1 expression in DU145 cells versus LNCaP cells may account for the retinoid resistance in DU145 cells.

Discussion

In the present study, we show that all-trans RA and certain synthetic retinoids can suppress ET-1, a mitogenic growth factor produced by prostate cancer cells, and inhibit cell growth in vitro. We also demonstrate that retinoids can inhibit exogenous ET-1-induced cell growth (Fig. 3, B and C). It has been shown that ET-1 can potentiate the proliferative activities of several growth factors including platelet-derived growth factor, epidermal growth factor, basic fibroblast growth factor, and insulin-like growth factor in prostate cancer cells (9). ET-1 enhances mitogenesis through at least two pathways that utilize protein kinase C and tyrosine kinases as major downstream effectors. Therefore, treatment with retinoids may not only result in the disruption of the synergy arising from the interaction of ET-1 and various growth factors, but in addition, retinoids are likely to interfere with the signal transduction pathway of other growth factors.

LNCaP cell transfection experiments using ET-1 promoter constructs led to maximal ET-1 transcription with the combination of the transcription factors AP-1 (c-Jun/c-Fos) and GATA-2 (Fig. 4A). This is consistent with previous studies in endothelial cells where formation of an optimal transactivation unit between AP-1 and GATA-2 transcription factors leads to a synergistic effect (17). We confirmed the importance of the AP-1 or GATA-2 binding sites with mutant promoters. Mutations in the AP-1 or GATA-2 sites only reduced
promoter activity, whereas a combination of the mutants almost completely shut down the transcription promoter (Fig. 4D).

LNCaP and DU145 prostate cancer cells are different in several characteristics including isolation origins, proliferation rates, prostate-specific antigen expression, androgen dependency and metabolism, as well as expression of growth factors and their receptors (21, 22). Not surprisingly, ET-1 production also varies in these established prostate cancer cell lines. DU145 cells produce the highest levels of the ET-1 peptide among all prostate cancer cell lines tested (9), suggesting a distinct regulatory system of ET-1 synthesis.

Our cotransfection data suggest that the wild-type ET-1 promoter construct is more active in DU145 cells than in LNCaP cells. In addition, the ET-1 promoter constructs in DU145 cells, including the single mutant promoters (mutAP1 and mutGATA), responded strongly to cotransfected AP-1 but not to GATA-2. It is therefore conceivable that DU145 cells are deficient in AP-1 factor but contain sufficient amounts of GATA-2 and possibly other factors for ET-1 promoter activation. Indeed, we have found that DU145 cells are very low in AP-1 DNA binding activity because of low levels of endogenous c-Jun/c-Fos. Thus, addition of exogenous AP-1 factor allows for optimal formation of the AP-1/GATA-2 activation complex, whereas addition of GATA-2 cannot overcome the lack of endogenous AP-1 activity. When both AP-1 and GATA-2 were added in DU145 cells, an increase in ET-1 activity was still observed in the double mutant promoter (mutAPmutGATA). This suggests that additional factors or sites in the ET-1 promoter region may be important for ET-1 regulation in DU145 cells. In fact, two forms of preproET-1 cDNA have been isolated from human placenta, indicating two promoter elements controlling the transcription of mRNA-1 and mRNA-2 (23). Our data suggest that different mechanisms controlling ET-1 gene expression may exist in the two different cell types.

Results from transfection experiments with ET promoter construct in retinoid-treated LNCaP cells clearly suggest that a specific retinoic signal transduction pathway is involved in the suppression of ET-1 (Fig. 3). Some types of synthetic retinoids, SR11256 and CD 270 (RAR panagonist) or SR11246 and CD2809 (RXR selective compound; Refs. 13 and 14), efficiently inhibit ET-1 expression, whereas other compounds including the RARa selective retinoid AM80 and RAR β, selective compounds, were ineffective. Retinoid activities are mediated by specific nuclear receptors. In the presence of ligands, receptors undergo a conformational change that allows them to recognize and interact with other compounds of the regulatory apparatus. Because the synthetic anti-AP-1 retinoids (SR11220 and SR11238) did not alter ET-1 expression, it becomes apparent that the AP-1/GATA-2 complex and the simple AP-1 complex respond differently to retinoids. This implies that a different receptor configuration is required when interacting with an AP-1 complex or AP-1/GATA-2 complex.

Excessive ET production has been noted in various diseases (7, 8, 24, 25). ET-1 therefore provides a possible therapeutic target for selective retinoids. Our finding that specific structural requirements are necessary for effective ET-1 repression suggests that highly selective retinoids, such as RXR selective compounds that inhibit ET-1 could represent desirable compounds for the treatment of prostate cancer and possibly other diseases in which ET-1 overexpression contributes to a pathological situation.

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