Advances in Brief

Potentiation of the Antiangiogenic Ability of Linomide by Androgen Ablation Involves Down-Regulation of Vascular Endothelial Growth Factor in Human Androgen-responsive Prostatic Cancers

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Abstract

Linomide is a p.o. active antiangiogenic agent that has been demonstrated to be effective in suppressing the in vivo growth of rat and human prostatic cancer xenografts. The present studies were conducted to determine whether the angiogenic molecules, vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) and basic fibroblast growth factor (bFGF) are expressed in vitro by DU-145, PC-3, ThU-PR!, and LnCaP human prostate cancer cell lines and whether Linomide inhibits the secretion of these angiogenic molecules. Additionally, two different androgen-responsive human prostatic cancer xenograft models (i.e., PC-82 and A-2) were used to determine whether androgen ablation-induced reduction in tumor growth is associated with a reduction in tumor VEGF and/or bFGF levels. These studies demonstrated that both VEGF and bFGF proteins are expressed to different degrees in the human prostatic cancer cell lines. The secretion of VEGF but not bFGF is up-regulated by hypoxia. Linomide is unable to inhibit either basal or hypoxia-induced secretion of VEGF. Linomide also has no effect on secreted bFGF levels. Castration inhibited tumor VEGF but had no effect on bFGF levels in both the androgen-responsive PC-82 and A-2 human prostatic cancers when grown in severe combined immunodeficient mice.

Introduction

Although prostatic cancers are initially responsive to androgen ablation therapy, they usually progress to an androgen-independent state (1). Once this occurs, the disease is rarely curative due to lack of effective chemotherapeutic agents to target the androgen-independent cells (2). Thus, an alternative approach for the growth of androgen-independent prostate cancer cells is needed. Folkman and colleagues (3, 4) demonstrated that angiogenesis is a prerequisite for expansion of solid tumors beyond 1–3 mm3 and have suggested that angiogenesis is often activated during the early, preneoplastic stages in the development of a tumor. Thus, inhibition of angiogenesis should be highly effective in inhibiting even the growth of androgen-independent prostatic cancer cells. Tumor angiogenesis is controlled by a number of positive and negative regulators of angiogenesis elaborated by tumor cells and tumor-associated host cells (4–6). These include aFGF3 and bFGF, platelet-derived growth factor, TNF-α, and VEGF/VPF. A number of studies from this laboratory have demonstrated that Linomide, a quinoline-3-carboxamide, has antitumor effects against a series of androgen-dependent and -independent rat prostate cancers as well as human prostate adenocarcinoma xenografts through its ability to inhibit tumor angiogenesis (7, 8). Linomide’s antiangiogenic abilities involve a reduction in the number of tumor blood vessels and a subsequent reduction in tumor blood flow (9). Linomide also inhibits endothelial cell chemotactic migration and invasion (9). Additional studies have demonstrated that Linomide inhibits the number of tumor-associated macrophages and also the ability of tumor-associated macrophages to synthesize and secrete the angiogenic molecule TNF-α (10, 11). More recent studies demonstrated that Linomide can inhibit the angiogenic response induced by VEGF/VPF, aFGF, bFGF, and TNF-α (12).

Androgen ablation induces the programmed death of androgen-dependent normal (13) and malignant prostatic cells (14). Subsequent studies demonstrated that when androgen ablation is combined with Linomide treatment, it potentiates the growth inhibition of the androgen-responsive Dunning PAP and G prostatic cancer sublines (15). This combinatorial approach is accompanied by a further reduction in tumor blood vessel density induced by either castration or Linomide monotherapy (15). It was also demonstrated that as long as the animals continued to receive Linomide plus androgen ablation, the growth of the tumors remained suppressed. However, when Linomide is discontinued, the tumors begin to regrow (15). These observations demonstrate that androgen ablation can inhibit the growth of certain androgen-responsive prostatic cancers indirectly via inhibition of angiogenesis in addition to directly inducing programmed death of androgen-dependent prostatic cancer cells.

Studies performed on other hormone-responsive organs such as the uterus and pituitary have demonstrated that estrogens can stimulate angiogenesis via up-regulating the expression of the angiogenic factor, VEGF (16–18). Several reports indicate that VEGF is highly expressed in hormone-responsive tumors such as breast and ovarian carcinomas (19, 20). These observations coupled with our observation that androgen ablation decreases the blood vessel density within androgen responsive rat prostatic cancers (15) suggest that androgens may play a role in angiogenesis via regulating VEGF levels. Thus, this study was undertaken to determine whether VEGF and bFGF are expressed as the human prostate cancer cell lines and whether Linomide can regulate the secretion of these angiogenic molecules in vitro. Additionally, androgen responsive human prostate cancer xenograft models were used to determine whether androgen ablation induced
reduction in tumor growth is associated with reduction in tumor VEGF and bFGF levels and whether androgen ablation potentiation of Linomide’s tumor growth inhibition is accompanied by a further decrease in the levels of these angiogenic molecules.

Materials and Methods

Animals. Specific pathogen free SCID mice used in these studies were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). They were housed in groups of five per individual microisolater cage placed in well-ventilated rooms under controlled light (12-h light/12-h dark, lights set at 0700 h), temperature (21—22°C), and humidity (50%). Food (Picolab Rodent Diet 20; PMI Feeds, Inc., St. Louis, MO) and water were available ad libitum.

Reagents. Linomide (N-phenylmethyl-1,2-dihydro-4-hydroxyl-1-methyl-2-oxo-quinoline-3-carboxamide) is a water-soluble low molecular weight compound (M, 308,000) that was generously provided by Pharmacia and Upjohn (Land, Sweden). Cobalt chloride was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ).

Tumors. The origin and the characteristics of the androgen-responsive human PC-32 tumors have been described elsewhere (21). A-2 tumor tissue was from a bone metastatic lesion obtained at autopsy from a patient who had failed androgen ablation therapy. The A-2 tumor was originally implanted directly from the patient into an athymic nude mouse. This tumor was then passaged in SCID mice and was used as passages 3 and 4 in experiments described in this study. The A-2 tumor is androgen responsive as demonstrated by the slowing of its growth rate following castration. To produce tumors in SCID mice, solid trocar pieces of 5 mg of PC-82 or A-2 tumor tissue were placed in the flank region under Metofane anesthesia. Following the development of 1 cm² tumors, mice were randomized into control and treatment groups (castration, 325 μmol of Linomide/kg/day in the drinking water and castration + Linomide). Castration was performed via a scrotal route using sterile techniques under Metofane anesthesia. At various intervals following tumor inoculation, the individual tumor dimensions were serially measured using calibrated microcalipers. Tumor volumes in cm³ were calculated using the formula \( V = \frac{4}{3} \pi r^3 \), where \( r \) is the radius of the tumor. For the PC-82 studies, after 23 days of treatment, the animals were sacrificed, tumors harvested, fixed in 10% buffered formalin, paraffin embedded, and tissues sections prepared.

Cell Culture. The origins and characteristic of human PC-3, TSU-PR1, DU-145, and LnCaP prostate cancer cell lines have been described previously (21). These cells were maintained by in vitro culture in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 IU/ml penicillin G/streptomycin sulfate (Sigma Chemical Co., St. Louis, MO) at 37°C in a humidified atmosphere containing 5% CO₂ in air. Previous studies demonstrated that a minimum of 325 μM Linomide is required to inhibit RAW cell TNF-α secretion in vitro (11). Therefore, for all of the in vitro studies, 325 μM Linomide was used.

<table>
<thead>
<tr>
<th>Human prostate cancer lines</th>
<th>VEGF⁶ (ng/10⁶ cells/24 h)</th>
<th>bFGF⁶ (pg/10⁶ cells/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU-145</td>
<td>10.0 ± 0.03¹</td>
<td>3.93 ± 0.20</td>
</tr>
<tr>
<td>TSU-PR1</td>
<td>0.52 ± 0.04</td>
<td>2.54 ± 0.46</td>
</tr>
<tr>
<td>LnCaP</td>
<td>1.86 ± 0.07</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PC-3</td>
<td>1.13 ± 0.04</td>
<td>4.38 ± 0.25</td>
</tr>
</tbody>
</table>

* Three observations/group.
* Six observations/group.
* Values are means ± SE.
* ND, not determined.

Sample Preparation. The PC-3, TSU-PR1, DU-145, and LnCaP cells were plated at a density of 2 × 10⁵ cells/60-mm dish. One-half of the plates for each type of cell were exposed to Linomide (325 μM) for 24 h, and the conditioned medium was centrifuged at 1000 x g for 10 min to remove cellular debris, aliquoted, and stored at −70°C until assayed for VEGF and bFGF. Upon removal of the conditioned medium, the cells were trypsinized and an aliquoted cell suspension was used to count the total number of cells present using a Coulter electronic particle counter (Coulter Electronics, Inc., Hialeah, FL). As controls, conditioned medium was similarly obtained from cells not exposed to Linomide. To determine tumor cytokine levels, tumors harvested following treatment were homogenized in normal saline, centrifuged at 1000 x g for 20 min to remove cellular debris, aliquoted, and stored at −70°C until assayed for VEGF and bFGF.

VEGF and bFGF ELISA. Samples stored at −70°C were thawed, diluted appropriately, and VEGF and bFGF concentrations were determined using the ELISA following the protocol provided by the manufacturer (R & D Systems, Inc., Minneapolis, MN). Both of these ELISAs have been demonstrated by the manufacturer to be specific for human homologues and not to recognize the murine homologues. Results of the conditioned media were expressed as amount of VEGF and bFGF/10⁶ cells/24 h whereas those of the tumors were expressed as amount of VEGF and bFGF/g of tissue.

Statistical Analysis. Data are presented as means ± SE. The experimental data for statistical significance were analyzed using the one-way ANOVA followed by Newman-Keuls multiple comparison. All statistics were calculated using the True Epistat 3.0 statistical program (Epistat Services, Richardson, TX).

Results and Discussion

Effect of Hypoxia and Linomide on Secretion of the Angiogenic Factors VEGF and bFGF by Human Prostate Carcinoma Cell Lines. To determine whether VEGF and bFGF proteins are secreted by the human prostate cancer cell lines and whether their secretion is inhibited by Linomide, PC-3, DU-145, TSU-PR1, and LnCaP human carcinoma cells were used as models. These cells were exposed to medium alone or medium containing 325 μM Linomide for 24 h, and the conditioned medium was assayed for VEGF and bFGF. As shown in Table 1, DU-145 secrete the highest amount of VEGF followed by LnCaP, PC-3, and TSU-PR1. bFGF secretion is highest in PC-3 cells followed by DU-145 and TSU-PR1. LnCaP cells do not secrete detectable amounts of bFGF. Linomide does not inhibit the secretion of either VEGF or bFGF by these cells in vitro.

Several in vitro experiments have demonstrated that hypoxia induces a number of cell types to up-regulate VEGF/VPF mRNA (23—25). Expression of the VEGF gene has been found to be up-regulated by the hypoxia-like state induced by exposure to 100 μM CoCl₂ (26). Since TSU-PR1 cells secreted moderate amounts of VEGF and bFGF compared to the other three cell lines tested, the ability of hypoxia to induce VEGF secretion was tested in this cell line by treating them with medium alone or medium containing 100 μM CoCl₂. The ability of Linomide to inhibit hypoxia-induced VEGF and bFGF secretion was also tested by treating the cells with 325 μM Linomide or CoCl₂ + Linomide. After 24 h, the conditioned medium

Table 1 Effect of Linomide (325 μM) on the in vitro secretion of VEGF and bFGF by human prostate cancer cell lines

<table>
<thead>
<tr>
<th>Human prostate cancer lines</th>
<th>VEGF (ng/10⁶ cells/24 h)</th>
<th>bFGF (pg/10⁶ cells/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.0 ± 0.03</td>
<td>3.93 ± 0.20</td>
</tr>
<tr>
<td>Linomide</td>
<td>3.29 ± 0.10</td>
<td>2.54 ± 0.46</td>
</tr>
</tbody>
</table>

* Three observations/group.
* Six observations/group.
* Values are means ± SE.
* ND, not determined.

Table 2 Effect of Linomide (325 μM) on hypoxia (CoCl₂) induced VEGF and bFGF secretion by TSU cells in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VEGF (ng/10⁶ cells/24 h)</th>
<th>bFGF (pg/10⁶ cells/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.69 ± 0.02</td>
<td>2.54 ± 0.46</td>
</tr>
<tr>
<td>Linomide</td>
<td>0.67 ± 0.29</td>
<td>1.97 ± 0.02</td>
</tr>
<tr>
<td>Hypoxia control (CoCl₂, 100 μM)</td>
<td>1.73 ± 0.04</td>
<td>1.48 ± 0.30</td>
</tr>
<tr>
<td>Hypoxia + Linomide</td>
<td>1.74 ± 0.15</td>
<td>1.43 ± 0.11</td>
</tr>
</tbody>
</table>

* Three observations/group.
* Six observations/group.
* Values are means ± SE.
* P < 0.05 compared with VEGF control.

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was collected and assayed for VEGF and bFGF levels. Table 2 demonstrates that CoCl₂ induced a 2.5-fold increase in secreted VEGF protein by these cells and that Linomide is unable to inhibit either the basal or hypoxia-induced secretion. However, bFGF secretion was neither induced by hypoxia nor regulated by Linomide in this prostate cancer cell line. The inability of hypoxia to up-regulate bFGF in our study is consistent with other studies which demonstrated that hypoxia does not induce aFGF, bFGF, or PDGF in rat glioma cells (27). The fact that only VEGF is up-regulated by hypoxia in many tumor model systems and in cardiac myocytes (25, 28) suggests that VEGF may be the central mediator of hypoxia-induced angiogenesis.

**Effect of Castration and Linomide Alone and in Combination on Tumor Growth and VEGF and bFGF Levels in AndrogenSensitive Human Prostatic Cancers.** Since our previous studies demonstrated that castration decreases blood vessel density in androgen-responsive Dunning rat prostatic cancer sublines (15), we determined whether this decrease in blood vessel density is due to an inhibition of tumor VEGF and/or bFGF levels. To test this, SCID mice were inoculated with PC-82 human prostatic cancers since previous studies demonstrated that this cancer undergoes regression following androgen ablation due to the induction of programed death of androgen-dependent cancer cells within the tumor (14). When the tumors were ~1.0 cm³ (i.e., 52 days after inoculation), animals were randomized into a control group of untreated mice and three other groups, of which one was castrated, a second was treated daily with p.o. Linomide (325 mol/kg/day), and the third was treated daily with Linomide starting at the time of castration. After 23 days of treatment, the animals were sacrificed, tumor volume was determined, and tumors were harvested for determination of the volume of cancer cells present and tumor VEGF and bFGF levels. Control tumors expressed 10 times more VEGF protein compared to bFGF (Table 3). Castration induced a >80% reduction in tumor VEGF levels and a greater than 50% reduction in the volume of cancer cells (Table 3). Morphologically, tumors from castrated animals had smaller percentages of total tumor volume occupied by cancer cells and higher numbers of cancer cells undergoing apoptosis (Ref. 14; Fig. 1, A versus B). Daily Linomide treatment did not reduce either VEGF or bFGF levels within PC-82 tumors, although it did cause a reduction in the volume of cancer cells (Table 3). Morphologically, tumors from Linomide-treated animals had a smaller percentage of tumor volume occupied by cancer cells (Fig. 1, A versus C). By combining castration with daily Linomide, there was a further reduction in the volume of PC-82 cancer cells (Table 3). This antitumor potentiation in the combination treatment (P < 0.05 compared to either treatment alone) was achieved without any potentiation in the decrease of tumor VEGF levels in-

**Table 3 Effect of castration and daily p.o. Linomide (325 µmol/kg/day) on VEGF and bFGF levels and PC-82 cancer volume in SCID mice**

<table>
<thead>
<tr>
<th>23 Days of treatment</th>
<th>VEGF (ng/g tissue)</th>
<th>bFGF (ng/g tissue)</th>
<th>Volume of cancer cells²</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>105 ± 14b (4)</td>
<td>10 ± 2 (4)</td>
<td>2.05 ± 0.18 (5)</td>
</tr>
<tr>
<td>Castration</td>
<td>14 ± 6² (3)</td>
<td>7 ± 2 (3)</td>
<td>0.55 ± 0.06b (5)</td>
</tr>
<tr>
<td>Linomide</td>
<td>68 ± 14 (3)</td>
<td>8 ± 3 (3)</td>
<td>0.47 ± 0.08⁴ (5)</td>
</tr>
<tr>
<td>Castration + Linomide</td>
<td>17 ± 3³ (3)</td>
<td>9 ± 1 (3)</td>
<td>0.15 ± 0.01⁵ (5)</td>
</tr>
</tbody>
</table>

² Volume of cancer cells was 0.90 ± 0.12 cm³ at time 0.

b Values are means ± SE; numbers in parentheses, number of observations/treatment group.

⁴ p < 0.05 compared with control.

⁵ p < 0.05 compared with castration or Linomide alone.
duced by castration alone (Table 3). Morphologically, tumors from the combined treatment animals had the lowest (P < 0.05 compared to either treatment alone) percentage of total tumor volume occupied by cancer cells (Fig. 1, A versus D).

To test whether castration-induced reduction in VEGF was unique to the androgen-responsive PC-82 human prostatic cancers, VEGF levels were also determined using the androgen-responsive A-2 human prostatic cancer xenograft model. SCID mice were inoculated with A-2 tumor and allowed to go untreated until the tumors were 1 cm³. Animals were then randomized into a control and castrated group. In the A-2 tumor model, castration does not induce the programmed death of the cancer cells, but it does decrease the percentage of cells proliferating. The A-2 prostatic cancer cells are thus not androgen dependent, but androgen sensitive.4 Due to this sensitivity, castration slows the growth of A-2 tumors but does not induce regression. This slowing of the growth rate following castration is associated with a 50% decrease in tumor VEGF levels (92 ± 13 ng/g tumor tissue in control versus 48 ± 11 ng/g tumor tissue 4 weeks following castration) with no change in bFGF levels (18 ± 1 ng/g tumor tissue in control versus 21 ± 2 ng/g tumor tissue 4 weeks following castration). While this article was being reviewed, we have demonstrated that androgens regulate secretion of VEGF in vitro by LnCaP cells and that LnCaP xenografts grown in SCID mice also respond to castration by inhibiting the tumor VEGF content.5 This was associated with a slowing of the tumor growth.5

Conclusion. Androgen-responsive prostatic cancers invariably progress to become resistant to androgen ablation due to the heterogenous presence of both androgen-dependent and -independent prostatic cancer cells within these cancers (2). Previously, we have demonstrated that androgen ablation directly induces the programmed death of androgen-dependent prostatic cancer cells (14). In the present study, we have demonstrated that androgen ablation also decreases the ability of androgen-responsive human prostatic cancers to produce the angiogenic factor, VEGF, without decreasing bFGF levels. Once produced, VEGF-induced angiogenesis can support the growth of both androgen-dependent and -independent prostate cancer cells within the same tumor. Thus, androgen ablation via its inhibition of VEGF-induced angiogenesis can indirectly inhibit the growth of both androgen-dependent and -independent prostatic cancer cells. Linomide inhibits bFGF-, VEGF-, and TNF-α-induced angiogenesis (12). It also inhibits the secretion of the angiogenic factor, TNF-α, by tumor-infiltrating macrophages (10), but it does not decrease either bFGF or VEGF release by prostatic cancer cells (present study). Due to their complementary mechanisms, combining Linomide with androgen ablation potentiates the antitumor efficacy against both rodent (15) and human androgen-responsive prostatic cancers (present study). Thus, human clinical trials to test the potentiation between combination of androgen ablation plus Linomide appear warranted.

Acknowledgments

The in vivo studies were performed with the expert assistance of John C. Lamb, whose help is greatly appreciated. Linomide was generously supplied by Pharmacia and Upjohn through the help of Drs. Beryl-Harly Asp and Gunnar Hedlund.

References


LOCALIZING A CHROMOSOME 11 TUMOR SUPPRESSOR GENE

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