Inhibitory Effects of Castration in an Orthotopic Model of Androgen-Independent Prostate Cancer Can Be Mimicked and Enhanced by Angiogenesis Inhibition

Peter Hammarsten,[1] Sofia Halin,[1] Pernilla Wikström,[1] Roger Henriksson,[2] Stina Häggström Rudolfsson,[3] and Anders Bergh[1]

Abstract

Purpose: Today, the most important treatment of advanced prostate cancer is castration; unfortunately, however, the long-term effect of this therapy is insufficient. Recent studies suggest that castration-induced prostate involution could be caused by primary effects in the prostate vasculature; therefore, we examined if antivascular treatments could mimic the effects of castration.

Experimental Design: Androgen-independent AT-1 prostate cancer cells were grown inside the ventral prostate in adult rats. Tumor-bearing animals were treated with an inhibitor of vascular endothelial growth factor receptor 2 and epidermal growth factor receptor signaling, *N*-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine (ZD6474, AstraZeneca, Södertälje, Sweden), and short-term effects (after 3 days) were compared with those induced by castration.

Results: Castration caused decreased vascular density in the normal tissue surrounding the tumor and consequently increased tumor hypoxia and apoptosis, and moderately decreased tumor growth. ZD6474 treatment resulted in decreased tumor vascular density accompanied by increased tumor hypoxia, apoptosis, and decreased tumor growth, suggesting that castration and antiangiogenic therapy work through similar mechanisms. Interestingly, castration or ZD6474 alone worked by reducing vascular density in the surrounding normal tissue and ZD6474 also in the tumor. Combined treatment with castration + ZD6474 was more effective than castration and ZD6474 alone in inducing tumor hypoxia, apoptosis, necrosis, and decreasing tumor vascular density.

Conclusion: These findings show that a drug that targets the vasculature in the tumor and in the surrounding ventral prostate lobe could mimic and even enhance the effects of castration. Our present findings thus suggest that castration + ZD6474 could be a particularly effective way to treat prostate tumors.

Recent studies suggest that castration, the principal treatment for advanced prostate cancer, may work by targeting the prostate stroma and particularly the vasculature (1–15). The magnitude of prostate tumor regression following castration may be related to the magnitude of the vascular response (4, 5, 7, 10).

To examine the role of primary vascular changes (10) and altered epithelial support from stroma-derived growth factors during castration (16–19), we have developed an experimental model where androgen-independent prostate cancer cells grow in the normal androgen-dependent ventral prostate in rats (20). In this context, castration caused tumor cell apoptosis and transiently delayed tumor growth by acting principally on the vasculature in the surrounding normal prostate (20). As this effect of castration on cells lacking functional androgen receptors is apparently mediated through the vasculature, we asked whether it could be mimicked by antiangiogenic therapy. For this purpose, we chose the angiogenesis inhibitor *N*-(4-bromo-2-fluorophenyl)-6-methoxy-7-[(1-methylpiperidin-4-yl)methoxy]quinazolin-4-amine (ZD6474), a tyrosine kinase inhibitor that prevents vascular endothelial growth factor receptor 2 (VEGFR2), epithelial growth factor receptor (EGFR), and RET (REarranged during Transfection) kinase signaling (21). The rationale for inhibiting VEGF signaling is obvious, as VEGF is one of the most important factors regulating the prostate vasculature (see above) and various inhibitors of VEGF have been shown to reduce the growth of prostate tumors (22–24). Additional inhibition of EGFR signaling is probably also of importance, as we have recently observed that selective

Authors' Affiliations: Departments of [1]Medical Biosciences, Pathology; [2]Radiation Sciences, Oncology; and [3]Surgical and Perioperative Sciences, Urology, Umeå University, Umeå, Sweden

Received 7/31/06; revised 10/1/06; accepted 10/5/06.

Grant support: Swedish Cancer Society project no. 4716 and the Cancer Research Foundation in Northern Sweden.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: The authors declare that they have no competing financial interests. R. Henriksson is a part-time medical advisor for AstraZeneca, manufacturer of ZD6474.

Requests for reprints: Anders Bergh, Department of Medical Biosciences, Pathology, Umeå University, 901 87 Umeå, Sweden. Phone: 46-90-7851530; Fax: 46-90-7852829; E-mail: anders.bergh@medbio.umu.se.

©2006 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-06-1895

Downloaded from clincancerres.aacrjournals.org on April 16, 2021. © 2006 American Association for Cancer Research.

inhibition of EGFR signaling [with the tyrosine kinase inhibitor gefitinib (Iressa, ZD1839)] is able to enhance the effect of castration and inhibit testosterone-stimulated prostate growth in rats by acting on epithelial and endothelial EGFR (25).

Materials and Methods

Animals and treatments. Fast-growing, androgen-insensitive, anaplastic, and low metastatic Dunning rat AT-1 prostate cancer cells (kindly donated by J. Isaacs, Johns Hopkins Oncology Center, Baltimore, MD) were grown in culture as previously described (26). Adult male Copenhagen rats (Charles River, Germany; bred in our laboratory) were anesthetized with pentobarbital (50 mg/kg), and an incision was made in the lower abdomen to expose the ventral prostate lobes. AT-1 cells (2×10^3 in 50 μL RPMI 1640) were carefully injected into one lobe of the ventral prostate using a Hamilton syringe as described earlier (20). The contralateral ventral prostate lobe and dorsolateral prostate served as controls and were not injected. The rats were divided into five weight-matched groups. The first group was castrated via a scrotal incision on day 7 after tumor cell injection and sacrificed 3 days later ($n = 18$). The second group was also castrated first, and, in addition, treated with ZD6474 (50 mg/kg, dissolved in 1% Tween 80, provided by AstraZeneca, Södertälje, Sweden) orally daily from day 7 and sacrificed 3 days later ($n = 13$). The third group was only treated with ZD6474 for 3 days from day 7 and sacrificed at day 10 ($n = 13$). The fourth and fifth groups were left untreated and sacrificed at day 7 ($n = 6$) and day 10 ($n = 18$), respectively. One hour before sacrifice, the animals were injected with bromodeoxyuridine (50 mg/kg, Sigma-Aldrich, St. Louis, MO) to label proliferating cells and with the tissue hypoxia marker pimonidazole that stain tissues with a pO_2 <10 mm Hg (hypoxyprobe 100 mg/kg, Chemicon, Temecula, CA; ref. 27). The animals were anesthetized and perfusion fixed in 4% paraformaldehyde as described earlier (1, 11). The ventral prostate and dorsolateral prostate, kidneys, liver, and lungs were removed and weighed. The perfusion-fixed tissues were also fixed by immersion for another 24 h, dehydrated, and paraffin embedded for morphologic analysis. The local ethical committee for animal research approved all of the animal work.

Morphologic analysis. First, multiple sections were cut through the prostate lobes to locate the tumor tissue. One 5-μm-thick section, through the central part of the tumor and surrounding normal prostate lobe, was immunohistochemically stained using primary antibodies against activated caspase-3 (Cell Signalling Technology, Danvers, MA), bromodeoxyuridine (DAKO, Stockholm, Sweden), factor VIII–related antigen (DAKO), and hypoxyprobe (Chemicon) as described earlier (1, 11, 20, 27, 28). The slides were counterstained with Meyer's hematoxylin (Sigma-Aldrich) solution and mounted. To determine apoptosis, the fraction of active caspase-3–positive tumor cells was assessed by counting 1,000 cells. For proliferation, the fraction of bromodeoxyuridine-stained cells was determined in 500 cells. Using a 121-point square lattice mounted in the eyepiece of a light microscope, the volume density of factor VIII–related antigen–stained blood vessels was determined using a stereological method as earlier described (1, 11, 20). The volume densities of tumor and normal prostate tissues in the ventral prostate (using multiple sections through different parts of the lobe), as well as the volume density of necrotic and hypoxyprobe-stained tumor tissue (using central sections through the tumor), were determined in a similar way on Mayer's H&E (Sigma Diagnostics, St. Louis, MO)–stained sections by counting the number of grid intersection falling on each tissue compartment (1, 20). The weights of the total tumor and normal prostate tissues, as well as the weight of viable tumor tissue, were then estimated by multiplying the volume density with prostate weight. The sections were measured in a random order and without prior knowledge of treatment.

In vitro *studies.* Dunning rat AT-1 prostate cancer cells were grown in culture as previously described (26). Cells were harvested and plated in a volume of 100 μL at 5,000 per well in microtiter plates. ZD6474 was dissolved in 100% DMSO (Sigma, Stockholm, Sweden) to a concentration of 10 mmol/L. The stock solution was then diluted in cell culture medium to achieve different working concentrations of ZD6474 (0-30 μmol/L), and each working concentration had 0.3% DMSO. Cells were cultured until cell growth was exponential before ZD6474 was added to the medium. Plates were incubated at 37°C for 72 h. To quantify the cytotoxic effect of ZD6474, fluorescein diacetate (Sigma-Aldrich) was added in a fluorometric microculture cytotoxicity assay (29). Cells were washed with PBS. PBS (100 μL) containing 10 mg/L fluorescein diacetate was added to each well, and plates were incubated in 37°C for 50 min, followed by fluorescence determination using 485 and 538 nm for excitation and emission, respectively.

RNA preparation and real-time quantitative reverse transcription-PCR. Total RNA was prepared from Dunning rat AT-1 prostate cancer cells using TRIzol reagent (Invitrogen, Stockholm, Sweden) according to the manufacturer's instructions. Five hundred nanograms of RNA were reversed transcribed with Superscript II reverse transcriptase (Invitrogen) and the cDNA was diluted 4-fold before PCR amplification.

Real-time PCR for EGFR and VEGFR2 was done using the LightCycler SYBR Green I technology (Roche Diagnostics, Bromma, Sweden). Reactions were done in a 20-μL volume with 0.5 μmol/L primers and 3 mmol/L $MgCl_2$. Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics). Primers for EGFR were designed by the nucleotide sequence relative to EGFR Genbank accession no. NM031507 (forward 5′-TTGCCGAGCCCTACAG-3′ and reverse 5′-AACGGTATAGCTCCGATCC-3′) and resulted in a product of 248 bp. Primers for VEGFR2 were designed by the nucleotide sequence relative to VEGFR2 Genbank accession no. X61656 (forward 5′-CTCTTGGCCGTGGTGCCT-3′ and reverse 5′-CGTTCAACGACAGAACAGTTAAC-3′) and resulted in a product of 71 bp. The PCR reaction was initiated with a 10-min enzyme activation step at 95°C followed by 45 cycles of 95°C denaturation for 15 s, 60°C annealing for 10 s, and 72°C extension for 10 s. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis. Each experimental sample was run in duplicates and negative controls were always run in parallel. The quantification data were analyzed with the LightCycler analysis Software 3.5.3 (Roche Diagnostics).

Western blot. For Western blotting, protein samples were electrophoresed on 7.5% SDS-polyacrylamide gels under reducing conditions and fractionated proteins were electrophoretically transferred onto Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences, Uppsala, Sweden). Membranes were blocked in 5% dry milk and 0.05% Tween 20 in PBS before incubation with sheep polyclonal antibody against EGFR (Abcam, Cambridge, United Kingdom), mouse monoclonal antibody against VEGFR2 (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit polyclonal antibody against actin (Sigma-Aldrich). After incubation with peroxidase-conjugated secondary antibodies (Amersham Biosciences), proteins were detected using enhanced chemiluminescence detection system (Amersham Biosciences). Molecular sizes of protein bands were determined by parallel electrophoresis of molecular weight markers (Bio-Rad Laboratories AB, Sundbyberg, Sweden).

Statistical analysis. Values are presented as means ± SD of 6 to 18 observations. The Student's t test was used for comparison between groups. $P < 0.05$ was considered significant. Statistical analysis was done using the statistical software Statistica 6.0 (StatSoft, Tulsa, OK). For curve estimation, quadratic regression was used. For this purpose, the statistical software SPSS 14.0 (SPSS, Inc., Chicago, IL) for Windows was used.

Results

Effects of castration in an orthotopic model of androgen-independent prostate cancer. A small number of AT-1 tumor

Downloaded from clincancerres.aacrjournals.org on April 16, 2021. © 2006 American Association for Cancer Research.

cells were injected into one of the ventral prostate lobes of immunocompetent rats. The contralateral ventral prostate lobe served as a control and was not injected. The AT-1 cells rapidly established a small tumor that was surrounded by normal ventral prostate tissue at 7 and 10 days after injection as earlier described (20). Tumor weight showed a 4.2-fold increase from day 7 to 10 ($P = 0.04$; Table 1). The total weight of normal ventral prostate tissue (left + right lobe) was apparently unaffected by the rapidly growing tumor during the study period (Table 2).

At day 10, castration decreased the viable tumor weight (total tumor weight − tumor necrosis weight) significantly (mean 29 mg) compared with that in intact animals (mean 62 mg, $P = 0.02$; Table 1). At this time point, viable tumor weight was only 47% of that in the noncastrated animals and not significantly larger than 3 days earlier (mean 15 mg), suggesting that the androgen-independent AT-1 tumor responds to castration when it is growing inside the androgen-dependent ventral prostate lobe. Apoptosis (activated caspase-3 labeling) was significantly increased 3 days after castration in both the ipsilateral (Table 2) and contralateral ventral prostate (not shown) and in the tumor itself (Fig. 1; Table 1). The magnitude of the peak apoptotic response at day 3 was, however, considerably larger in the surrounding ipsilateral (15-fold, $P < 0.000001$) ventral prostate than in the tumor (2.7-fold, $P = 0.0004$). Cell proliferation (bromodeoxyuridine labeling) in the tumor tended to be slightly lower in the castrated group than in the intact group ($P = 0.05$; Table 1).

In noncastrated controls, vascular densities in normal ventral prostate tissue in the tumor-bearing ipsilateral lobe (Table 2) increased with time (see ref. 20 for details), indicating that the tumor stimulates its blood supply through the surrounding normal tissue. After castration, the vascular density in the ventral prostate tissue surrounding the tumor was significantly decreased at day 3 (Table 2), but no vascular response was seen in the tumor (Fig. 1; Table 1). Previous studies have shown that the magnitude of the decrease in blood flow in the ventral prostate is sufficient to cause tissue hypoxia (14) and cell death (9, 10). Decreased blood supply may therefore induce apoptosis in the normal surrounding tissue (9) and in the tumor. In line with this, staining with hypoxyprobe showed increased tumor hypoxia 3 days after castration ($P = 0.001$; Fig. 1; Table 1). Tumor necrosis also seemed to be increased after castration ($P = 0.06$; Table 1).

Effects of ZD6474* in vitro *and in the orthotopic AT-1 tumor model. To examine whether AT-1 cells could respond directly to ZD6474 treatment, we first examined if AT-1 cells express VEGFR2 and EGFR. Using reverse transcription-PCR (data not shown) and Western blotting, we found that AT-1 cells express VEGFR2 and EGFR (Fig. 2). The ability of ZD6474 to directly inhibit cell growth was examined *in vitro* by incubation of AT-1 cells for 72 h with ZD6474 at different concentrations. ZD6474 significantly and dose dependently inhibited the growth of AT-1 cells. The IC_{50} for inhibition of AT-1 was 3.1 μmol/L (Fig. 3).

Treatment of tumor-bearing rats with ZD6474 reduced the tumor weight to 35% of that in the intact controls ($P = 0.03$). Thus, viable tumor weight at sacrifice (mean 22 mg) was not significantly increased compared with that 3 days earlier (mean 15 mg; Table 1). The main reason of the decreased tumor growth was a 2.6-fold increase ($P = 0.0006$) in tumor cell apoptosis (Fig 1; Table 1). In contrast, tumor cell proliferation was unaffected. Vascular densities in the ipsilateral ventral prostate lobe and in the tumor were decreased to 81% ($P = 0.01$) and 66% ($P = 0.002$) of that in intact controls, respectively (Fig. 1; Tables 1 and 2), and the hypoxic fraction of the tumors was apparently increased ($P = 0.05$; Fig. 1; Table 1). Vascular density in the contralateral ventral prostate lobe was not significantly affected by ZD6474 treatment ($P = 0.08$; data not shown).

When the effects of ZD6474 were compared with castration, it was found that both of these treatments had a similar inhibitory effect on tumor growth (average viable tumor weight was 22 mg in the ZD6474 and 29 mg in the castrated group), although some differences were observed (Table 1). ZD6474 decreased vascular density in the tumor compared with castration ($P = 0.00004$), but tumor cell proliferation was larger in ZD6474-treated animals than in castrated animals ($P = 0.02$). Tumor cell apoptosis, hypoxic cell fraction, and necrosis were similar in both groups. The major difference between these two treatments was the response in the normal ventral prostate tissue. Castration resulted in prostate glandular and vascular involution (see above), whereas ZD6474 had almost no effect on the normal tissue, except the slight decrease in vascular density in the tumor-bearing ventral prostate lobe ($P = 0.01$; Table 2).

Table 1. Effects of castration and ZD6474 treatment in orthotopic AT-1 tumors

	Intact day 7* ($n = 6$)	Intact day 10* ($n = 15$)	Castrated day 10* ($n = 18$)	ZD6474 day 10* ($n = 13$)	ZD6474 + castrated day 10* ($n = 13$)
Tumor weight (mg)	15 ± 4.0	64 ± 54†	31 ± 25‡	22 ± 27‡	21 ± 20‡
Tumor cell apoptosis (%)	0.44 ± 0.14	0.49 ± 0.28	1.3 ± 0.76‡	1.3 ± 0.51‡	1.9 ± 0.59‡§‖
Tumor cell proliferation (%)	38 ± 7.6	32 ± 8.4	26 ± 7.6	35 ± 6.5§	32 ± 3.8
Volume density of blood vessels (%)	2.5 ± 0.29	3.6 ± 0.80†	3.7 ± 0.66	2.4 ± 0.4‡§	1.9 ± 0.41‡§‖
Tumor necrosis fraction (%)	0.82 ± 0.93	2.3 ± 2.8	4.8 ± 4.2	3.7 ± 2.9	9.5 ± 4.1‡§‖
Tumor hypoxia fraction (%)	NA	56 ± 17	77 ± 16‡	73 ± 26	91 ± 3.4‡§‖

Abbreviation: NA, not analyzed.
*Days after AT-1 tumor cell inoculation. Therapy with castration and/or ZD6474 was given from day 7. For details, see Materials and Methods. Values are presented as means ± SD.
†Significantly different than in controls at day 7, $P < 0.05$.
‡Significantly different than in controls at day 10, $P < 0.05$.
§Significantly different than in castrated animals at day 10, $P < 0.05$.
‖Significantly different than in ZD6474-treated animals at day 10, $P < 0.05$.

Downloaded from clincancerres.aacrjournals.org on April 16, 2021. © 2006 American Association for Cancer Research.

Table 2. Effects of castration and ZD6474 treatment in the ipsilateral ventral prostate lobe inoculated with AT-1 tumor cells

	Intact day 7* (*n* = 6)	Intact day 10* (*n* = 10-15)	Castrated day 10* (*n* = 14-18)	ZD6474 day 10 (*n* = 8-13)	ZD6474 + castrated day 10* (*n* = 8-13)
Ventral prostate weight (both lobes, mg)	251 ± 79	263 ± 59	191 ± 58†	242 ± 58‡	169 ± 21†§
Apoptosis index (tumor-bearing lobe, %)	0.051 ± 0.0027	0.17 ± 0.05‖	2.6 ± 0.93†	0.15 ± 0.076‡	2.4 ± 0.54†§
Proliferation index (tumor-bearing lobe, %)	1.4 ± 0.59	1.9 ± 0.94	2.7 ± 0.71†	1.7 ± 0.78‡	2.9 ± 0.63†§
Volume density of blood vessels (tumor-bearing lobe, %)	1.4 ± 0.54	1.8 ± 0.32‖	1.4 ± 0.24†	1.5 ± 0.27†	1.1 ± 0.21†‡§
Apoptosis index (tumor-embedded glands, %)	NA	0.76 ± 0.48	2.4 ± 0.98†	1.5 ± 0.72†‡	2.1 ± 0.50†§

*Days after AT-1 tumor cell inoculation. Therapy with castration and/or ZD6474 was given from day 7. For details, see Materials and Methods. Values are presented as means ± SD.
†Significantly different than in controls at day 10, $P < 0.05$.
‡Significantly different than in castrated animals at day 10, $P < 0.05$.
§Significantly different than in ZD6474-treated animals at day 10, $P < 0.05$.
‖Significantly different than in controls at day 7, $P < 0.05$.

When AT-1 tumors grow orthotopically in the ventral prostate, tumor cells occasionally grow around normal glands. In such glands, epithelial cell apoptosis was increased after treatment with ZD6474 ($P = 0.03$) but not to the same extent as after castration ($P = 0.046$; Table 2).

Effects of combined castration and ZD6474 treatment. The most pronounced antitumor effects were observed when ZD6474 treatment and castration were combined (Table 1). This treatment resulted in a 4-fold increase in tumor cell apoptosis versus that in intact controls ($P < 0.000001$). The

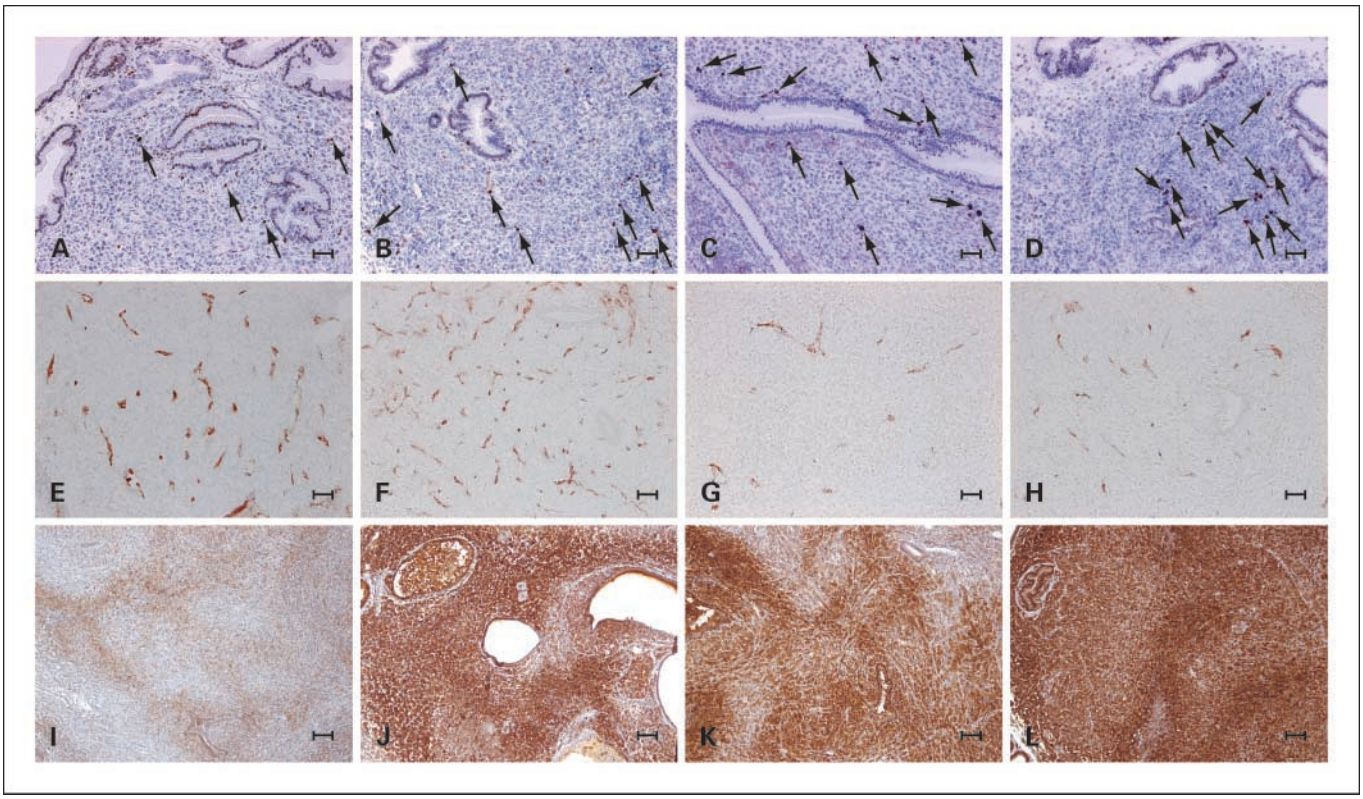

Fig. 1. AT-1 tumor sections from untreated (*A, E*, and *I*), 3-d castrated (*B, F*, and *J*), 3-d treated with ZD6474 (*C, G*, and *K*), and 3-d castrated + ZD6474-treated rats (*D, H*, and *L*). Bars, 40 μm (*A-D* and *E-H*) and 80 μm (*I-L*). *A* to *D,* apoptotic index (active caspase-3). Animals 3-d castrated and 3-d treated with ZD6474 had higher tumor cell apoptosis (*arrows,* apoptotic tumor cells) compared with those untreated. Animals 3-d castrated + ZD6474-treated had higher tumor cell apoptosis compared with those untreated, 3-d castrated, and 3-d treated with ZD6474. *E* to *H,* vascular density (factor VIII – related antigen). Animals 3-d treated with ZD6474 and 3-d castrated + ZD6474-treated had lower vascular density compared with those that are untreated and 3-d castrated. Three-day castrated + ZD6474-treated rats also had lower vascular density compared with those 3-d treated with ZD6474. *I* to *L,* pimonidazole staining (tumor hypoxia). Three-day castrated rats had increased fraction of hypoxic cells (*brown stained*) compared with untreated animals. Three-day castrated + ZD6474-treated rats had increased fraction of hypoxic cells compared with those that are intact, 3-d castrated, and 3-d treated with ZD6474. Three days of treatment with ZD6474 tended to increase the fraction of hypoxic cells compared with untreated animals.

Downloaded from clincancerres.aacrjournals.org on April 16, 2021. © 2006 American Association for Cancer Research.

tumor vascular density was decreased to 53% of that in intact control (P = 0.000007). Combined treatment increased the tumor necrosis fraction 4-fold (P = 0.00001) and the hypoxic tumor cell fraction 1.6-fold (P < 0.000001). Total viable tumor weight in this group (mean 19 mg) was only 30% of that in intact controls (mean 62 mg; P = 0.01).

The combination of ZD6474 + castration was significantly more effective in decreasing tumor vascular density (P = 0.000001) and in increasing tumor cell apoptosis (P = 0.04), tumor hypoxic cell fraction (P = 0.006), and tumor necrosis (P = 0.005) versus castration alone; however, these cellular changes did not result in a significantly reduced tumor weight during the short time period studied (P = 0.21).

Discussion

We have recently developed a model where androgen receptor–negative Dunning AT-1 rat prostate cancer cells are grown orthotopically within the normal prostate (20). Using this model, we have shown that castration can induce cell death among totally androgen-independent AT-1 tumor cells. The effect is apparently caused by reduced blood supply through the surrounding normal prostate tissue, resulting in tumor cell hypoxia and death (20). We now extend these findings by showing that a drug that targets the vasculature in the tumor and in the surrounding ventral prostate lobe could partly mimic and even enhance the effects of castration. Our findings are thereby in line with previous findings which suggest that castration induced cell death among prostate tumor cells are related to primary effects in the surrounding stroma (18, 19, 30) and, particularly, in prostate blood vessels (10, 28).

Treatment with ZD6474, a potent inhibitor of VEGFR2 and EGFR (21), decreased the volume density of blood vessels in the tumor and in the surrounding ventral prostate lobe. This suggests that prostate blood vessels are dependent on signaling through VEGFR2, and also likely EGFR, present on prostate blood vessels (11, 21, 25). These effects were anticipated as previous studies have shown that this compound slows growth of s.c. transplanted human prostate cancer cell lines, such as the PC3 and LnCaP, principally by inhibiting angiogenesis (31–33). Direct effect mediated by the VEGFR2 and EGFR present on the AT-1 cells (and possibly also RET kinase; ref. 34) are, however, also possible, as has been discussed when treating glioma (35). However, the peak plasma concentration of ZD6474 in rats after oral

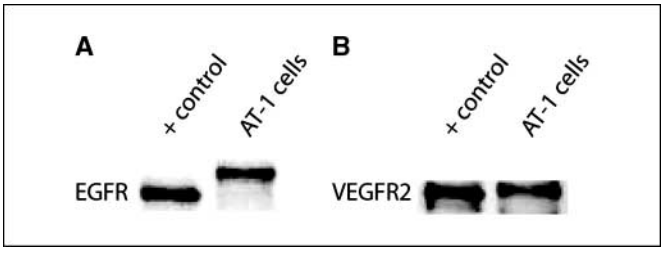

Fig. 2. Western blot results of EGFR and VEGFR2 by 7.5% SDS-PAGE under reducing conditions. *A,* EGFR (M_r 170,000) expression in AT-1 tumor cells. The EGFR expressed in the AT-1 tumor cells gave a somewhat larger band, which has previously been seen in other studies (37). This could possibly be due to differences in glycosylation. *B,* VEGFR2 (M_r 170,000) expression in AT-1 tumor cells. Rat brain, M_r 170,000, served as a positive control (*A* and *B*). The experiment was repeated twice using different rat brain samples and AT-1 tumor cells with similar results (data not shown).

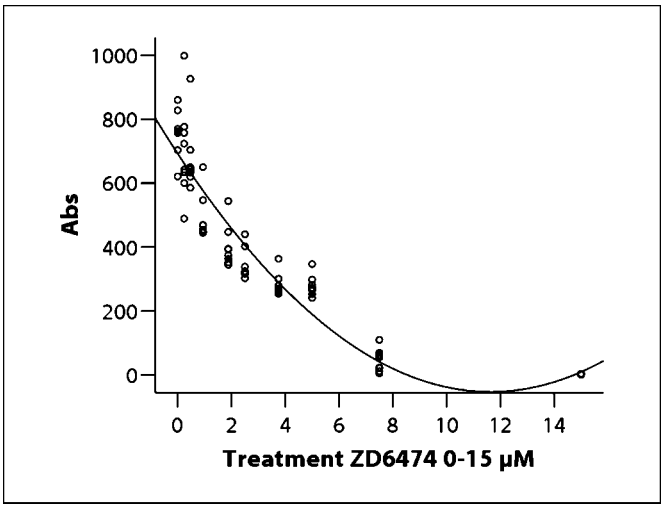

Fig. 3. Dose-dependent inhibition of ZD6474 on growth of AT-1 tumor cells measured by fluorometric microculture cytotoxicity assay with an IC_{50} of 3.1 μmol/L. Abs, absorbance.

administration of 50 mg/kg is ~0.5 μmol/L.[4] The IC_{50} for ZD6474 to inhibit proliferation in AT-1 cells is 3.1 μmol/L (this study); for endothelial cells, the IC_{50} is 0.06 μmol/L (33). These IC_{50} values and the peak plasma concentration at the doses examined (50 mg/kg) suggest that ZD6474 acts mainly by vascular effects *in vivo* (31–33).

ZD6474 induced vascular involution, which resulted in increased tumor cell hypoxia and apoptotic cell death of the same magnitude as accomplished by castration. Castration, in this orthotopic model, works as an antivascular treatment that targets the vasculature in the surrounding tissue (10, 20) but leaves the tumor vasculature unaffected. The reason why the tumor vasculature was morphologically unaffected after castration is probably related to the previous observation that AT-1 cells secrete angiogenic factors like VEGF independently of androgens (5). On the other hand, ZD6474 targets the tumor vasculature directly by inhibiting effects of locally produced VEGF and EGF. Both treatments, by slightly different mechanisms, thus induce the same effects on tumor growth. As ZD6474 and castration apparently works through slightly different mechanisms, it was not surprising to observe that the combination of ZD6474 and castration was more effective than castration alone in increasing tumor cell apoptosis, tumor hypoxic cell fraction, and necrosis.

Thus, our present findings suggest that castration + ZD6474 could be an effective way to treat androgen-insensitive prostate tumors or perhaps clinically more relevant tumors composed of a mixture of more and less androgen-responsive cells (36). In contrast to our findings in the orthotopic model, ZD6474 was more effective than castration in inhibiting growth of s.c. transplanted, androgen-sensitive human LnCaP cells (32). This discrepancy is, however, anticipated as the castration response in s.c. LnCaP tumors may be suboptimal as such tumors are not supported by an androgen-regulated stroma and vasculature. A castration response in the surrounding normal tissue and in the tumor stroma may also explain why the combination of castration and ZD6474 was

[4] R. Henriksson, et al., unpublished observations.

Downloaded from clincancerres.aacrjournals.org on April 16, 2021. © 2006 American Association for Cancer Research.

more effective than ZD6474 alone in our orthotopic model, whereas castration had no additive effect to ZD6474 in a s.c. LnCaP tumor (32).

When AT-1 cells are growing in the ventral prostate lobe, they sometimes encircle normal prostate glands (20). Androgen-independent stroma and vasculature then surround normal androgen-dependent cells. By analyzing how such epithelial cells respond to castration and other treatments, one can elucidate the relative importance of direct effects on epithelial cells and indirect effects mediated by the microenvironment. Interestingly, treatment with ZD6474 induced apoptosis in such epithelial cells probably by its antivascular effects in both the surrounding normal ventral prostate tissue and in the tumor, but the apoptosis response was not as high as after castration. The response to castration in androgen receptor–positive epithelial cells may, thus, in addition to tissue hypoxia and decreased growth factor support from the stroma (17, 18, 30), also involve direct effects on epithelial cell, such as reduced androgen stimulation and responsiveness to locally produced antiapoptotic factors like insulin-like growth factor-I (30).

Acknowledgments

We thank Pernilla Andersson, Elisabeth Dahlberg, Birgitta Ekbolm, Åsa Skytt, and Sigrid Kilter for skillful technical assistance; and AstraZeneca, Sweden, for ZD6474.

References

1. Franck-Lissbrant I, Haggstrom S, Damber JE, Bergh A. Testosterone stimulates angiogenesis and vascular regrowth in the ventral prostate in castrated adult rats. Endocrinology 1998;139:451–6.
2. Frost GI, Lustgarten J, Dudouet B, Nyberg L, Hartley-Asp B, Borgstrom P. Novel syngeneic pseudo-orthotopic prostate cancer model: vascular, mitotic and apoptotic responses to castration. Microvasc Res 2005;69:1–9.
3. Haggstrom S, Lissbrant IF, Bergh A, Damber JE. Testosterone induces vascular endothelial growth factor synthesis in the ventral prostate in castrated rats. J Urol 1999;161:1620–5.
4. Haggstrom S, Stattin P, Wikstrom P, Bergh A, Damber JE. Castration-induced reduction of vascular endothelial growth factor expression in benign human prostate tissue is lost in advanced prostate cancer. BJU Int 2001;88:110–6.
5. Haggstrom S, Wikstrom P, Bergh A, Damber JE. Expression of vascular endothelial growth factor and its receptors in the rat ventral prostate and Dunning R3327 PAP adenocarcinoma before and after castration. Prostate 1998;36:71–9.
6. Hansen-Algenstaedt N, Stoll BR, Padera TP, et al. Tumor oxygenation in hormone-dependent tumors during vascular endothelial growth factor receptor-2 blockade, hormone ablation, and chemotherapy. Cancer Res 2000;60:4556–60.
7. Jain RK, Safabakhsh N, Sckell A, et al. Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: role of vascular endothelial growth factor. Proc Natl Acad Sci U S A 1998;95:10820–5.
8. Joseph IB, Nelson JB, Denmeade SR, Isaacs JT. Androgens regulate vascular endothelial growth factor content in normal and malignant prostatic tissue. Clin Cancer Res 1997;3:2507–11.
9. Lekas E, Engstrand C, Bergh A, Damber JE. Transient ischemia induces apoptosis in the ventral prostate of the rat. Urol Res 1999;27:174–9.
10. Lekas E, Johansson M, Widmark A, Bergh A, Damber JE. Decrement of blood flow precedes the involution of the ventral prostate in the rat after castration. Urol Res 1997;25:309–14.
11. Lissbrant IF, Hammarsten P, Lissbrant E, Ferrara N, Rudolfsson SH, Bergh A. Neutralizing VEGF bioactivity with a soluble chimeric VEGF-receptor protein flt(1-3)IgG inhibits testosterone-stimulated prostate growth in castrated mice. Prostate 2004;58:57–65.
12. Shabisgh A, Tanji N, D'Agati V, et al. Early effects of castration on the vascular system of the rat ventral prostate gland. Endocrinology 1999;140:1920–6.
13. Shabsigh A, Chang DT, Heitjan DF, et al. Rapid reduction in blood flow to the rat ventral prostate gland after castration: preliminary evidence that androgens influence prostate size by regulating blood flow to the prostate gland and prostatic endothelial cell survival. Prostate 1998;36:201–6.
14. Shabsigh A, Ghafar MA, de la Taille A, et al. Biomarker analysis demonstrates a hypoxic environment in the castrated rat ventral prostate gland. J Cell Biochem 2001;81:437–44.
15. Stewart RJ, Panigrahy D, Flynn E, Folkman J. Vascular endothelial growth factor expression and tumor angiogenesis are regulated by androgens in hormone responsive human prostate carcinoma: evidence for androgen dependent destabilization of vascular endothelial growth factor transcripts. J Urol 2001;165:688–93.
16. Gao J, Arnold JT, Isaacs JT. Conversion from a paracrine to an autocrine mechanism of androgen-stimulated growth during malignant transformation of prostatic epithelial cells. Cancer Res 2001;61:5038–44.
17. Kurita T, Wang YZ, Donjacour AA, et al. Paracrine regulation of apoptosis by steroid hormones in the male and female reproductive system. Cell Death Differ 2001;8:192–200.
18. Ohlson N, Bergh A, Stattin P, Wikström P. Reduction in local IGF-1 synthesis is of importance for castration-induced regression of normal and malignant human prostate tissue. Prostate. In press 2006.
19. Wang Y, Sudilovsky D, Zhang B, et al. A human prostatic epithelial model of hormonal carcinogenesis. Cancer Res 2001;61:6064–72.
20. Halin S, Hammarsten P, Wikström P, Bergh A. Androgen-insensitive prostate cancer cells respond to castration treatment when growing in an androgen-dependent prostate environment. Prostate. In press 2006.
21. Ryan AJ, Wedge SR. ZD6474—a novel inhibitor of VEGFR and EGFR tyrosine kinase activity. Br J Cancer 2005;92 Suppl 1:S6–13.
22. Borgstrom P, Bourdon MA, Hillan KJ, Sriramarao P, Ferrara N. Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors *in vivo*. Prostate 1998;35:1–10.
23. Melnyk O, Zimmerman M, Kim KJ, Shuman M. Neutralizing anti-vascular endothelial growth factor antibody inhibits further growth of established prostate cancer and metastases in a pre-clinical model. J Urol 1999;161:960–3.
24. Sweeney P, Karashima T, Kim SJ, et al. Anti-vascular endothelial growth factor receptor 2 antibody reduces tumorigenicity and metastasis in orthotopic prostate cancer xenografts via induction of endothelial cell apoptosis and reduction of endothelial cell matrix metalloproteinase type 9 production. Clin Cancer Res 2002;8:2714–24.
25. Hammarsten P, Rudolfsson S, Henriksson R, Wikström P, Bergh A. Inhibition of the epidermal growth factor receptor enhances castration induced prostate involution and reduces testosterone-stimulated prostate growth in adult rats. Prostate. In press 2006.
26. Isaacs JT, Isaacs WB, Feitz WF, Scheres J. Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostatic cancers. Prostate 1986;9:261–81.
27. Raleigh JA, Chou SC, Bono EL, Thrall DE, Varia MA. Semiquantitative immunohistochemical analysis for hypoxia in human tumors. Int J Radiat Oncol Biol Phys 2001;49:569–74.
28. Johansson A, Rudolfsson SH, Wikstrom P, Bergh A. Altered levels of angiopoietin 1 and tie 2 are associated with androgen-regulated vascular regression and growth in the ventral prostate in adult mice and rats. Endocrinology 2005;146:3463–70.
29. Larsson R, Kristensen J, Sandberg C, Nygren P. Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia, using a fluorometric microculture cytotoxicity assay (FMCA). Int J Cancer 1992;50:177–85.
30. Ohlson N, Bergh A, Persson ML, Wikstrom P. Castration rapidly decreases local insulin-like growth factor-1 levels and inhibits its effects in the ventral prostate in mice. Prostate 2006;66:1687–97.
31. Checkley D, Tessier JJ, Kendrew J, Waterton JC, Wedge SR. Use of dynamic contrast-enhanced MRI to evaluate acute treatment with ZD6474, a VEGF signalling inhibitor, in PC-3 prostate tumours. Br J Cancer 2003;89:1889–95.
32. Nicholson B, Gulding K, Conaway M, Wedge SR, Theodorescu D. Combination antiangiogenic and androgen deprivation therapy for prostate cancer: a promising therapeutic approach. Clin Cancer Res 2004;10:8728–34.
33. Wedge SR, Ogilvie DJ, Dukes M, et al. ZD6474 inhibits vascular endothelial growth factor signaling, angiogenesis, and tumor growth following oral administration. Cancer Res 2002;62:4645–55.
34. Dawson DM, Lawrence EG, MacLennan GT, et al. Altered expression of RET proto-oncogene product in prostatic intraepithelial neoplasia and prostate cancer. J Natl Cancer Inst 1998;90:519–23.
35. Sandstrom M, Johansson M, Andersson U, Bergh A, Bergenheim AT, Henriksson R. The tyrosine kinase inhibitor ZD6474 inhibits tumour growth in an intracerebral rat glioma model. Br J Cancer 2004;91:1174–80.
36. Ohlson N, Wikstrom P, Stattin P, Bergh A. Cell proliferation and apoptosis in prostate tumors and adjacent non-malignant prostate tissue in patients at different time-points after castration treatment. Prostate 2005;62:307–15.
37. Boldrup L, Coates PJ, Hedberg Y, Sjostrom B, Dahlqvist A, Nylander K. Expression of p63, COX-2, EGFR and β-catenin in smokers and patients with squamous cell carcinoma of the head and neck reveal variations in non-neoplastic tissue and no obvious changes in smokers. Int J Oncol 2005;27:1661–7.

Downloaded from clincancerres.aacrjournals.org on April 16, 2021. © 2006 American Association for Cancer Research.

AACR American Association for Cancer Research

Clinical Cancer Research

Inhibitory Effects of Castration in an Orthotopic Model of Androgen-Independent Prostate Cancer Can Be Mimicked and Enhanced by Angiogenesis Inhibition

Peter Hammarsten, Sofia Halin, Pernilla Wikström, et al.

Clin Cancer Res 2006;12:7431-7436.

Updated version Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/24/7431

Cited articles This article cites 34 articles, 8 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/24/7431.full#ref-list-1

Citing articles This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/12/24/7431.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/12/24/7431.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC)
Rightslink site.

Downloaded from clincancerres.aacrjournals.org on April 16, 2021. © 2006 American Association for Cancer Research.