Development of an Androgen Receptor-null Model for Identifying the Initiation Site for Androgen Stimulation of Proliferation and Suppression of Programmed (Apoptotic) Death of PC-82 Human Prostate Cancer Cells

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ABSTRACT

Whether androgen regulates the proliferation and survival of androgen-responsive prostate cancer cells directly or indirectly via a paracrine pathway initiated in androgen receptor (AR)-expressing stromal cells is unknown. To resolve this issue, female mice heterozygous for the testicular feminized male loss of function mutation in their X-linked AR genes were crossed to T cell-defective homozygous male nude mice. Using a PCR-based restriction enzyme digestion method, the resulting AR/tfm, Nu/nu F1 hybrid females were identified and back-crossed to homozygous male nude mice to produce AR-null male nude mice lacking both AR and T-cell function. Androgen-responsive PC-82 human prostate cancers were xenografted into these AR-null versus AR-wild-type male nude mice. In both backgrounds, the cancer cells did not grow in nonandrogenized hosts. In contrast, PC-82 prostate cancer cells grew with identical characteristics (i.e., take rate, morphology, PSA expression, growth rate, and percentage of cell proliferating or dying) in androgenized hosts of both backgrounds. Likewise, in both backgrounds, androgen ablation of mice bearing growing PC-82 cancers resulted in the inhibition of proliferation and activation of programmed (apoptotic) cell death of the cancer cells. These results demonstrate that both the androgen-stimulated proliferation and the suppression of programmed cell death of PC-82 human prostate cancer cells are initiated by the AR pathway directly within these cancer cells themselves and do not involve initiation by AR-expressing stromal cells in a paracrine manner.

INTRODUCTION

Androgens are major growth and survival factors for prostate cancer cells (1). Androgens function via binding to their cognate nuclear receptors inducing conformational changes in the occupied AR. This allows the interaction of the occupied AR with additional nuclear proteins to produce transcriptional complexes, which can activate or repress specific gene expression by binding to the androgen-responsive elements present in the promoter regions in a series of androgen regulated genes (1). It is the regulation of the expression of these genes that both stimulates prostate cell proliferation and inhibits prostate cell death (1).

AR is nearly universally expressed within the nucleus of prostate cancer cells in both primary and metastatic sites in untreated and androgen ablation-failing patients (2). This suggests that AR is required for the progression of prostate cancer to its lethal metastatic stage. Comparative genomic and fluorescence in situ hybridization analyses have demonstrated that amplification of the Xq11–q13 region, where the AR gene is located, occurs in 30% of recurrent prostate cancer cells but not in specimens from the same patients prior to androgen ablation therapy (3, 4). This suggests that in approximately 1/3 of the patients, failure of androgen ablation therapy may be caused by clonal outgrowth of prostate cancer cells with increased AR expression, which enables them both to survive and to continue growth despite low concentrations of serum androgens. In addition to amplification, mutations in the AR gene can cause AR dysfunction, including alterations of AR specificity, binding affinity, and expression (5, 6). AR mutations occur in a low frequency in primary prostate cancer cells (7). In contrast, malignant cells in the distant metastases of androgen ablation-failing patients often contain AR mutations (8).

If the initiation site of androgen-stimulated proliferation and suppression of PCD is within the nucleus of these prostate cancer cells, then studying the consequences of these molecular aberrations in the AR gene within pure populations of prostate cancer cells in vitro should be useful for developing new therapeutic approaches both for prevention and treatment of prostate cancer. In contrast to such a direct mechanism for androgen action, there are data demonstrating, however, that androgen effects during prostate development can be indirect, involving a paracrine interaction initiated within the nucleus of stromal cells, inducing these stromal cells to secrete soluble growth factors to which the epithelial cells respond. For example, Cunha and Lung (9) isolated the murine embryonic UGS, from the anlage of which the prostate develops, and separated into its mesenchymal and epithelial components (9). By recombining the UGS mesenchyme (i.e., stroma) with a variety of epithelial cell types, Cunha et al. (10) demonstrated that the recombinants always grew in tissue morphologically resembling prostate if transplanted into androgenized animals but remained morphologically simple without the development of glandular structures if the host lacked sufficient systemic androgen levels. Cunha and Lung (9) further demonstrated that UGS epithelial cells from male mice inheriting the tfm loss of function germ line mutation in the AR gene and thus lacking functional AR could still be induced to grow and undergo prostate organogenesis in the presence of adequate systemic androgen, when recombined with UGS mesenchymal cells obtained from a non-tfm male mouse possessing wild-type functional AR (9). In contrast, UGS epithelial cells obtained from a non-tfm male mouse expressing functional AR could not be induced to grow or undergo prostate organogenesis when recombined with UGS mesenchymal cells from a tfm male mouse lacking functional AR, even in the presence of adequate systemic androgen (9).

In addition to these developmental studies, Habib and co-workers (11) have demonstrated that when adult human prostate epithelial cells are cultured without stromal cells, these cells stop expressing AR, PSA, and 5α-reductase activity, and their in vitro growth is androgen insensitive (11). In contrast, Bayne et al. has demonstrated that when such adult human prostate epithelial cells are cocultured with prostate stromal cells which were physically separated by a micro-porous membrane preventing their physical contact, the epithelial cells retain AR, PSA, and 5α-reductase activity, and their growth is androgen responsive (12). Whether this androgen-sensitive growth is due to the diffusion of soluble factors from the stromal cells that maintain AR expression in the prostate epithelial cells, thus allowing the epithelial cells to directly respond to androgen or whether such...
soluble stromal factors function indirectly as paracrine growth factors without the requirement of AR initiation in the epithelial cells is unknown.

Likewise, it is unknown whether or not androgen-stimulated growth of prostate cancer cells is a paracrine pathway initiated in supporting tumor stromal cells. To address this latter issue, PC-82 human prostate cancer xenograft is useful as an appropriate model system. PC-82 human prostate cancer cells are serially passageable as xenografts only in androgenized male nude mice, they do not grow in androgen- ablated hosts (13, 14). PC-82 cells express glandular cytokeratins (i.e., they are epithelial; Ref. 15), AR (16), PSA (17), and prostate-specific acid phosphatase (18). Androgen ablation of nude mice bearing growing PC-82 cancers results in the inhibition of proliferation and activation of programmed (apoptotic) death of the prostate cancer cells (19). Thus, PC-82 is a useful model to identify the site of initiation of androgen action by prostate cancer cells. To do this, AR-null male nude mice were generated that lack both systemic expression of AR and T-cell function. If the androgen-dependent growth of PC-82 human prostate cancer cells requires a paracrine pathway initiated by functional AR expressed in stromal cells, then PC-82 cancer cells should not grow when inoculated into these AR-null male nude mice. To test for this possibility the growth characteristics of PC-82 prostate cancer cells inoculated in AR-null versus AR wild-type male nude mice with and without androgen stimulation were compared.

MATERIALS AND METHODS

Animals. Male AR wild-type homozygous nude mice (i.e., /nu/ at the Nude locus and /Y/ at the AR locus) were obtained from Harlan Sprague Dawley. C57 black female mice heterozygous for the tfm mutation in their AR gene but homozygous for wild-type at the Nude locus (i.e., /Y/, Nu/Na) were commercially obtained from The Jackson Laboratory. For breeding, one or two of these AYtfn, Nu/Nu female mice were caged with one male nude mouse. All of the F1 hybrids were Nu/nu for the nude mutation. The phenotypic male F1 hybrids were removed, and each of the female F1 hybrids had 1 cm of tail cut off and immediately frozen on dry ice. The tail tissue was used to extract the genomic DNA of each mouse using an AutoGen 540 automated DNA extractor from Integrated Separation Systems (Natick, MA). Genomic DNA was extracted using phenol-chloroform reagents and a protocol provided by the manufacturer. The genomic DNA of each F1 hybrid female mouse was then dissolved in H2O and used as the template DNA for a PCR-based restriction enzyme digestion method (described below) to determine the status of the AR gene in each mouse. F1 hybrid female mice heterozygous for the tfm mutation in the AR gene, as well as the Nude gene (AR/Y, Nu/nu), were selected and used for further breeding. These F1 hybrids were bred to the male homozygous nude mice (AY, Nu/nu). Among the F2 hybrids from this second crossing, all animals that had hair (i.e., Nu/nu at the nude gene locus) were removed as soon as recognizable. Among the hairless nude pups (nu/nu at their nude gene locus), the phenotypic males were removed. All of the F2 hybrid nude mice that were phenotypic females had their genotype at the AR locus determined using the PCR method described below. The AR-null male nude mice (tfm/Y, nu/nu) were selected for inoculation experiments. All of these AR-null male nude mice had their peritoneum opened under Methoxyflurane anesthesia (Mallinckrodt Veterinary, Inc., Mundelein, IL) to confirm their genotype by identifying the undescended testes which are characteristic of the male mice with tfm mutation in their AR genes (20). These undescended testes were then removed from all of the AR-null male nude mice to ablate endogenous androgen production. The peritoneum and skin was then sutured, and the mice were allowed to recover. AR wild-type male nude mice (AR/Y, nu/nu) were androgen ablated via castration using a scrotal route under Methoxyflurane anesthesia as described previously (19). Exogenous androgen replacement (i.e., androgenization) was performed under Methoxyflurane anesthesia by implanting a 1-cm long polydimethylsiloxane (Silastic) sealed capsule packed with testosterone into androgen-ablated mice s.c. in the flank as described (19).

PCR-based Restriction Endonuclease Digestion for Detection of tfm Mutation. Two oligonucleotide primers were designed to amplify a 166-bp fragment of the AR gene from base 1034 to base 1200 (according to the AR gene cDNA sequence published in GenBank, MMANDREC; accession no. X53779). Primer one is complimentary to bases 1034–1053 of the antisense strand of the AR gene: cgcgtcttctgctgct. Primer two is complimentary to bases 1200–1181 of the AR gene: tgcgtagcaagggttc. PCR was performed using a PCR kit from Perkin-Elmer (Foster City, CA). Each PCR mix (in a total volume of 50 µl) contained the following: 50 ng of mouse genomic DNA, 0.2 mM dNTP, 0.4 µM each primer, 1 × PCR buffer with MgCl2 (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, and 0.001% gelatin), and 2.5 units of Taq polymerase. The reaction conditions were 95°C for 5 min; 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min; and 72°C for 8 min. The PCR product was purified using the QIAquick PCR purification kit from Qiagen (Chatsworth, CA). The purified PCR product was then digested with MwoI (New England Biolabs, Inc., Beverly, MA) with 250 µl of 30 pg of BglII (New England Biolabs, Inc.) in 25 µl of distilled water at 37°C for 30 min. The digested PCR product was then analyzed on a 2% agarose gel (FMC Bioproducts, Rockland, ME).

PC-82 Inoculation. Donor PC-82 tumors were microwaved with sterile scalpels, and the mince was filtered using tissue sieves (E-C Apparatus Corp.) to remove large fragments of the connective tissue. The mince, composed of small clumps of malignant cell organoid (i.e., 10–50 cells), was washed with PBS and mixed with Matrigel (Collaborative Biomedical Products, Bedford, MA) at 4°C at a ratio of 100 µg of tissue per 1 ml of Matrigel. Two hundred µl of this tumor mince-Matrigel mixture were then injected s.c. in the flank of mice using an 18 gauge sterile needle. To inoculate single cells, PC-82 tumor mince was prepared as described above and then processed according to a protocol described previously (21). Briefly, the mince was first incubated in 50 ml of 1 mM DTt (in PBS) for 30 min with gentle rotation of 30 cycles per min at 37°C and then allowed to settle without centrifugation. The supernatant fraction was discarded. Ten ml of dissociation solution, containing 1% DNase type I (Sigma), and 0.28% collagenase type I (Sigma) in RPMI 1640 (Life Technologies, Inc.) with 10% FBS (Life Technologies, Inc.) were added to the mince and rotated at 37°C for 30 min. The mince was allowed to settle for 5 min, the supernatant fraction collected, and another 10 ml of dissociation solution were added to the settled mince for another 30 min of rotation at 37°C, as before. A second supernatant was collected, as described, and combined with the first. The pooled supernatant was then passed through a series of tissue sieves with pore sizes ranging from 230 to 46 µm. The sieved cell suspension was then washed twice with RPMI 1640 containing 10% FBS, passed through a 25-gauge needle twice, and inoculated into tissue culture flasks in RPMI 1640 (containing 10% FBS) to allow selective attachment of the fibroblast cells overnight. The next morning, the unattached cells (floaters) were collected and were either mixed with Matrigel (107 cells/ml of Matrigel) or resuspended in PBS for injection into the mice. The tumor dimension in each animal was determined at various times using a micro-caliper and was used to calculate the tumor volume as described previously (19).

Quantitative Histological Analysis of PC-82 Tumor. PC-82 tumors were removed, formalin fixed, paraffin embedded, and thin sectioned. Tissue sections were stained with H&E for quantitative determination of the percentage of cancer cells dying (i.e., apoptotic). The identification of apoptotic cells was based on one or more of the following morphological criteria: (a) condensed nuclei; (b) crescent appearance of chromatin; (c) emargination of chromatin along nuclear membrane and nuclear fragmentation; or (d) presence of apoptotic bodies or phagocytosis by neighboring cells. For qualitative comparison, additional sections were stained using TUNEL to identify apoptotic cells as described previously (22). For quantitative analysis, apoptotic bodies were counted using morphological criteria in H&E-stained sections because in formalin-fixed tissues, this was more reproducible than identifying apoptotic cells using TUNEL assay. Additional sections were also immunocytochemically stained for Ki-67 expression using the MIB1 monoclonal antibody to identify the human cells that were in cell cycle as described previously (22). The percentage of PC-82 cells in cycle (Ki-67 positive) and cells undergoing apoptosis (by morphology criteria) were determined by randomly scoring 1000 cancer cells per stained tissue section. Additional sections were immunocytochemically stained for PSA, smooth muscle α-actin, desmin, and cytokeratin 8 using antibodies from DAKO Corp. (Carpinteria, CA). AR was detected immunocytochemically using a rabbit polyclonal antibody from Santa Cruz Biotechnology.
In Situ Hybridization Using Alu1/2 Probes. The reagents used in this procedure are purchased from Research Genetics (Huntsville, AL), unless otherwise indicated. In situ hybridization was performed according to the manufacturer’s protocol, with slight modifications. Briefly, the tissue sections were dewaxed in xylene (twice, 10 min each time) and rehydrated by being immersed in 100, 95, 90, and 80% ethanol for 1 min each and then in distilled water, twice, for 1 min each time. The tissue sections were then treated with Auto Blocker for 1 min at room temperature and washed three times with Universal Buffer at room temperature. The tissue sections were then treated with Pepsin for 3 min at 105°C. Pepsin was removed and the biotin-labeled Alu1/2 probes were added to the tissue sections. The tissue sections were incubated at 105°C for 5 min and then at 45°C for 1 h. The probes were then removed, and the tissue sections were washed in Post Hybe Wash for 5 min at 45°C. The tissue sections were then treated with Streptavidin HRP for 5 min at 50°C and then with Probe Lock for 10 s at room temperature. The tissue sections were then treated twice with Stable DAB at 50°C for 5 min each. The DAB was removed, and the tissue sections were washed twice with Auto Wash at room temperature. The tissue sections were then counter stained with Hematoxylin (CMS, Houston, TX) for 1 min. The tissue sections were mounted with Universal Mount.

Serum PSA Analysis. Serum PSA values were determined using a commercially available kit (i.e., Tandem R assay) from Hybritech (San Diego, CA).

Statistics. All of the values are presented as means ± SE. The number of samples in each value is indicated. Statistical analysis was performed by a one-way ANOVA with the Newman-Keuls test for multiple comparison.

RESULTS

Androgen Responsiveness of PC-82 Prostate Cancer Cells in AR Wild-Type Male Nude Mice. The PC-82 human prostate cancer cells were originally established from a radical prostatectomy specimen from a hormonally untreated patient (13). The PC-82 is serially passagable as xenografts in nude mice but has not been established as a continuously passagable in vitro cell line. Coinoculation of prostate cancer cells with Matrigel (i.e., the solubilized extracellular matrix from the EHS mouse sarcoma) enhances the initial take rate and subsequent growth in nude mice (23). Therefore, PC-82 tumors were minced, and the minces were coinoculated with Matrigel. The tumors that were thus produced in AR wild-type male nude mice were moderately differentiated adenocarcinomas composed of malignant prostate epithelial cells organized into glandular acini supported by prostate epithelial cells. The PC-82 minces that were coinoculated with Matrigel were then treated with Auto Blocker. The tissue sections were then treated twice with Probe Lock for 5 min at 50°C and then with Probe Lock for 10 s at room temperature. The tissue sections were then treated twice with Stable DAB at 50°C for 5 min each. The DAB was removed, and the tissue sections were washed twice with Auto Wash at room temperature. The tissue sections were then counter stained with Hematoxylin (CMS, Houston, TX) for 1 min. The tissue sections were mounted with Universal Mount.

Development of an AR-null Nude Mouse Model System. The tfm mutation in the mouse AR gene is caused by the deletion of one cytosine within a string of six cytosines at position 1139–1144 of the AR gene (cDNA), resulting in a frame-shift mutation (27, 28). This frame-shift mutation leads to low mRNA levels of the mutant gene and no functional AR protein in the tfm mice (27, 28). Female mice heterozygous for the tfm mutation and homozygous for the wild-type nude gene (Ar/tfm, Nu/Nu) were commercially obtained. These females were cross-bred to athymic male nude mice (Ar/Y, Nu/nu) to produce F1 hybrids that were heterozygous for the tfm and nude mutations (Ar/tfm, Nu/nu).

To identify these heterozygous F1 females (Ar/tfm, Nu/nu) from the other Ar/Ar, Nu/nu female or tfm/Y, Nu/nu male F1 hybrids, a PCR-based restriction digestion method was used to distinguish the mutant AR gene from the wild-type AR gene (Fig. 3). At the site of the tfm frame-shift mutation, there are originally six cytosine bases in the wild-type AR gene, making this 6-bp region a recognition site for the MwoI restriction endonuclease. The tfm frame shift mutation involves the loss of one of these cytosines, thereby destroying this MwoI restriction site. A pair of oligonucleotide primers was designed to amplify a 166-bp DNA segment flanking this mutation site using PCR. Restriction digestion by MwoI of this PCR product amplified from a wild-type AR gene produces two sizes of DNA pieces of 58 and 108 bp each. In contrast, because the PCR product amplified from the tfm mutant AR gene is resistant to MwoI digestion, it remains 166 bp in size. Separation of the MwoI digested PCR products on an agarose gel can thus reveal the genetic status of the mouse at the AR locus (Fig. 3). A homozygous wild-type mouse (Ar/Ar) produces the two smaller bands on the gel, a heterozygous mouse (Ar/tfm) produces all three bands on the gel, and a tfm mouse (tfm/Y) produces only the 166-bp band on the gel.

Using this PCR-based restriction digestion method, the Ar/tfm, Nu/nu F1 hybrid female mice were identified. These Ar/tfm, Nu/nu F1 females were then bred to homozygous male nude mice (Ar/Y, Nu/nu). Among the F2 hybrids, half were heterozygous for the nude mutation (i.e., Nu/nu) and had hair, whereas the other half were homozygous for the nude mutation (i.e., Nu/Nu) and were hairless. From the hairless F2 hybrid females, the tfm/Y, Nu/nu mice (i.e., AR-null male nude) were identified using the same PCR-based restriction digestion method. All of these AR-null male nude mice characteristically have undescended testes, which were surgically removed, to ablate endogenous androgen production.

The Growth of PC-82 Tumor Mincs in AR-null versus AR Wild-Type Male Nude Mice. PC-82 minces were coinoculated with Matrigel into either 17 castrated AR-null male nude mice or 11
Fig. 1. PC-82 cancer cells grown in AR wild-type and AR-null male nude mice. A, high-power (×200) histology of PC-82 cells in androgenized AR wild-type male nude mice during the initial establishment in Matrigel (i.e., 1 month postinoculation). B, low-power (×100) histology of a fully established (i.e., 12 weeks postinoculation) PC-82 tumor in AR wild-type male nude mice. C, low-power (×100) histology of the Ki-67 immunocytochemical staining of the PC-82 cells in AR wild-type male nude mice. Note the high level of Ki-67 positivity (brown nuclei) in epithelial cells. D, high-power (×200) immunocytochemical staining for AR in AR wild-type male nude mice. Arrows, mouse stromal cells, the nuclei of which are negative for AR, whereas the nuclei of the PC-82 cells are uniformly positive (i.e., brown) for AR staining. E, low-power (×100) in situ hybridization of PC-82 cells grown in AR wild-type male nude mice using human Alul/12 probes. The glandular epithelial cells are uniformly positive (i.e., brown), indicating human origin, whereas the supporting stromal cells are uniformly negative, indicating mouse origin. F, high-power (×200) histology of PC-82 cells in androgenized AR-null male nude mice during initial establishment in Matrigel. G, high-power (×200) of the Ki-67 immunocytochemical staining of the PC-82 cells in androgenized AR-null male nude mice. H, high-power (×200) TUNEL labeling PC-82 cells in androgenized AR-null male nude mice. Arrows, mitotic figures; arrows, TUNEL-labeled apoptotic bodies. I, TUNEL labeling of the PC-82 cells in androgen ablated AR-null male nude mice. Arrows indicate several TUNEL-positive (i.e., brown) apoptotic cells.
castrated AR wild-type male nude mice, each of which was androgenized by implantation of an exogenous testosterone time release capsule. The results demonstrated that PC-82 cancers grew in 94% (16 of 17) of the inoculation sites in androgenized AR-null male nude mice versus 100% (11 of 11) in androgenized AR wild-type male nude mice (Table 1). Mean tumor volume at 36 days postinoculation (i.e., 155 ± 58 mm³) was not significantly different between the androgenized AR-null male nude mice and the androgenized AR wild-type male nude mice (i.e., 194 ± 50 mm³; Table 1). At 36 days postinoculation, tumors from AR-null (n = 4) and AR wild-type (n = 5) hosts were harvested for histological analysis. The histology of the PC-82 cancers growing in androgenized AR-null male nude mice (Fig. 1F) is identical to that of the PC-82 cancers growing in androgenized AR wild-type male nude mice (Fig. 1A). In addition, the percentage of PC-82 cells proliferating (28.5 ± 1.5%; Fig. 1G) and dying (0.4 ± 0.05%; Fig. 1H) in androgenized AR-null male nude mice are not different from that of the PC-82 cells in androgenized AR wild-type male nude mice (Table 1). On the basis of take and growth rates, histology, and tumor cell kinetic parameters, there is no difference in the androgen-responsive growth of PC-82 cells in AR-null versus AR wild-type male nude mice.

At 36 days postinoculation, the remaining androgenized animals had their testosterone implants removed to determine the response of the PC-82 cells to androgen ablation in the AR-null (n = 12) male nude mice versus AR wild-type (n = 6) male nude mice. Tumor volumes were measured following androgen ablation. At 4 days post-androgen ablation, three tumors were harvested from AR-null and AR wild-type mice. These tumors were processed for histological analysis. These results demonstrated that after 4 days of androgen ablation, the percentage of proliferating PC-82 cells in AR-null and AR wild-type male nude mice decreased from values greater than 20% to 0.9 ± 0.1 and 0.3 ± 0.05%, respectively (Table 2). In addition, after 4 days of androgen ablation, the histologically detectable apoptotic bodies increased nearly 10-fold, from 0.4–0.5% (Table 1) to 3.7 ± 0.4% in the AR-null and 4.3 ± 0.4% in AR wild-type mice (Table 2). This increase in apoptotic death was confirmed by TUNEL labeling (Fig. 1I). Due to the essentially identical decrease in proliferation and increase in PCD, PC-82 tumor volumes were equally decreased in both types of hosts by 17 days post-androgen ablation [i.e., 67 ± 2.3% (n = 9) versus 51 ± 6.5% (n = 3) of original tumor volume in AR-null or AR wild-type mice, respectively; Table 2]. These results demonstrate that the PC-82 cancer cells grown in androgenized AR-null male nude mice respond to androgen ablation identically to cells grown in AR wild-type male nude mice.

To further test the androgen responsiveness of PC-82 cells in AR-null hosts, tumors from the first passage in the AR-null male nude mice were harvested and minced, and the minces were coinoculated with Matrigel into 12 additional castrated AR-null male nude mice and 12 castrated AR wild-type male nude mice. Half of each of these second-passage hosts were androgenized with an exogenous testosterone capsule. No tumors grew in any of the castrated animals not given exogenous testosterone replacement in either the AR-null (n = 6) or AR wild-type male nude mice (n = 6). In contrast, PC-82 cells grew in 100% (6 of 6) of both the androgenized AR-null and AR wild-type male hosts.

The histology of PC-82 cells from the second passage was identical in both types of androgenized hosts and was identical to those presented in Fig. 1, A and B. A PC-82 tumor from this second passage was harvested from an androgenized AR-null host and passaged into 14 castrated AR-null and AR wild-type male nude mice. Twelve of each type of these castrated hosts were androgenized. As previously observed for the first two passages, third-passage PC-82 cancers did not grow when inoculated into nonandrogenized castrated male hosts. These third-passage PC-82 cancers did grow, however, in 67% (8 of 12) of the androgenized AR-null male nude mice versus 58% (7 of 12) of the androgenized AR wild-type male nude mice.

Growth of Single PC-82 Cells in AR-null versus AR Wild-Type Male Nude Mice. By serially passaging minces of PC-82 cancers in AR-null male nude mice, there should be minimal, if any, AR wild-type stromal cells derived from the original passage in AR wild-type male nude mice present in these third passage tumors in AR-null hosts. To eliminate even the slight possibility of carryover of such AR wild-type stromal cells during serial passaging in AR-null mice, a PC-82 cancer from the third serial passage in androgenized AR-null male nude mice was enzymatically dissociated, and the dissociated cells were inoculated into AR-null male hosts. To ensure that the response to androgen ablation in AR-null male nude mice was not due to androgenized AR wild-type male nude mice that might have been included in the dissociated cells, these dissociated cells were further passaged into additional AR-null male nude mice. These third-passage PC-82 cancers did not grow when inoculated into nonandrogenized castrated male hosts. These third-passage PC-82 cancers did grow, however, in 67% (8 of 12) of the androgenized AR-null male nude mice versus 58% (7 of 12) of the androgenized AR wild-type male nude mice.

Table 2 PC-82 response to androgen ablation in AR wild-type versus AR-null male nude mice

<table>
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<tr>
<th>Type of host</th>
<th>4 days of androgen ablation</th>
<th>17 days of androgen ablation, relative tumor volume (%)</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>0.9 ± 0.1</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>AR-null</td>
<td>0.9 ± 0.1</td>
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epithelial cells grew in 50% of the androgenized AR-null (3 of 6) and in the androgenized AR wild-type (1 of 2) male nude mice.

PC-82 cancers that grow up in the androgenized hosts were harvested and processed for histology analysis, as well as for determination of percent of cancer cells proliferating or dying. The histology of the PC-82 cancers grown from single cell mixtures with Matrigel was identical in both the AR-null and the AR wild-type male nude mice, and was not different from the histology of the PC-82 cancer grown from minced tissue mixed in Matrigel (see, for example, Fig. 1, A and B). The percentage of PC-82 cells proliferating (i.e., 25%) or dying (i.e., 0.4%) also were not different from the previous results.

**Growth of PC-82 Single Cells without Coinoculation with Matrigel in AR-null or AR Wild-Type Male Nude Mice.** In all previous studies, PC-82 minces or single cell suspensions were coinoculated with Matrigel. Matrigel is solubilized from the extracellular matrix isolated from Engelbreth-Holm-Swarm mouse sarcomas grown in mice (29). Soluble Matrigel solidifies above 4°C and contains a variety of growth factors (i.e., epidermal growth factor, basic fibroblast growth factor, transforming growth factor β, platelet-derived growth factor, and so forth), as well as fibrillar material (i.e., collagen type IV, laminin, and entectin) and heparan sulfate proteoglycans (30). The extracellular matrix is an important determinant of the response of cells to a variety of peptide growth factor (e.g., basic fibroblast growth factor) via interaction between the cells, growth factors, and heparan sulfate proteoglycans (31). This raised the possibility that the coinoculation of a preformed extracellular matrix might obviate a requirement for an androgen-dependent production of specific extracellular matrix components by androgen-responsive stromal cells. To test for such an androgen-dependent stromal requirement, single cell suspensions of PC-82 cells were inoculated without Matrigel into four androgenized AR-null and four AR wild-type male nude mice. Although the time required for initial detection of tumor growth (i.e., 10 weeks) in both the AR-null and AR wild-type male nude mice was twice that required in androgenized hosts coinoculated with Matrigel (i.e., 5 weeks), 50% (2 of 4) of the inoculations eventually produced continuous PC-82 growth regardless of host. On the basis of histology, PSA expression, growth rate, and percentages of cells proliferating and dying, these PC-82 cells had characteristics identical to those cells growing after coinoculation with Matrigel. These results demonstrate that although Matrigel coinoculation does enhance the initial growth rate, PC-82 prostate cancer cells still initiate an androgen-responsive stimulation of proliferation and inhibition of cell death without Matrigel coinoculation in both AR-null and AR wild-type male nude mice.

**DISCUSSION**

By introducing the tfm mutation into the nude background, an AR-null male nude mouse was created in which normal host mouse cells do not express functional AR and in which human xenografts are not immunologically rejected. Using such AR-null male nude mice, it is possible to test whether the initiation site of androgen action in androgen-responsive human prostate cancer cells is within the host stromal cells or the prostate cancer cells themselves. If such androgen action is via a paracrine pathway initiated by androgen occupancy of AR within the stromal cells, no growth should occur when AR-expressing androgen-responsive human prostate cancer cells are inoculated into such androgenized AR-null male nude mice. In contrast, if androgen action is initiated via either an intracrine or autocrine pathway, then growth should occur when AR-expressing androgen-responsive prostate cancer cells are inoculated into such androgenized AR-null male nude mice. Using the PC-82 human prostate cancer cells as a model, the present studies demonstrated that an identical
androgen-responsive stimulation of proliferation and inhibition of death of these PC-82 cells occurs consistently in both AR-null as well as AR wild-type male nude mice. In addition, once established in such androgenized AR-null hosts, the growing PC-82 cancer cells are just as androgen-dependent as they are in AR wild-type hosts, as demonstrated by their identical response to androgen ablation (i.e., decreased cell proliferation, increased cell death, and regression). These results demonstrated that the initiation site of androgen action is within these PC-82 cells themselves and does not require the expression of functional AR by supportive tumor stromal cells (i.e., the cancer cells are themselves directly androgen dependent).

These results do not demonstrate, however, that stromal cells are not required for the growth of such androgen-dependent prostate cancer cells. Indeed, such in vivo growth requires the development of a blood supply and an adequate microenvironment, both of which would be provided by the stromal cells. PC-82 prostate cancer cells growing in either AR-null or AR wild-type male nude mice induce a well-developed angiogenic response (Fig. 1, A and F). In previous studies, this angiogenic response has been demonstrated to be via the androgen-stimulated productions by PC-82 cells of the potent angiogenic factor, vascular endothelial growth factor (32). Thus, there is a paracrine interaction between the androgen-induced secretion of vascular endothelial growth factor by PC-82 cells and the migration and proliferation of stromal endothelial cells to produce new blood vessels. This androgen-induced paracrine interaction, however, is initiated by the occupancy of functional AR by androgen within the nucleus of these cancer cells and not the stromal cells.

The growth of another androgen-responsive prostate cancer cell line, the rat R-3327G, was tested in this AR-null versus AR wild-type male nude mice system. Whereas the maximal take and growth rate of G prostate cancers requires the treatment of castrated hosts with exogenous androgen replacement, the magnitude of their androgen responsiveness was identical in both the AR-null and AR wild-type male nude mice. Combining these results with those of the PC-82 provided evidence that the androgen responsiveness of prostate cancers is initiated by the occupancy of the AR within the cancer cells themselves, not within the host stromal cells. Because prostate cancer cells may acquire the independence to stromal signals during neoplastic transformation, these results may not hold true for the androgen responsiveness of normal adult prostate epithelial cells. Therefore, it is important to test the androgen responsiveness of normal adult prostate epithelial cells in this AR-null male nude mouse model. Presently, these experiments are being performed using normal epithelial cells from adult rat ventral prostate and normal human prostate tissues.

Regardless of the outcome of the studies using normal prostate epithelial cells, the results of the present study have significant basic and translational implications for prostate cancer. There is a growing body of literature demonstrating a variety of molecular changes in the AR gene during the progression of prostate cancer. If the initiation of androgen action had been due to the interaction of androgen with the AR in the nucleus of tumor stromal cells, then these molecular changes in the AR gene of prostate cancer cells, although reflecting the genetic instability of these cancer cells, would not affect the sensitivity of these cells to androgen ablation. Thus, studying the consequences of these changes in pure populations of cancer cells in vitro would provide little therapeutically useful information. In contrast, as demonstrated by the data in the present study, the AR in the PC-82 androgen-dependent prostate cancer cells is itself the initial site of androgen action responsible for their androgen sensitive growth.

Thus, studying the consequences of the molecular aberration in the AR gene within pure populations of prostate cancer cells in vitro should be very useful for developing new therapeutic approaches both for prevention and treatment of prostate cancers.

In order for this to be possible, it will be critical to establish androgen-dependent prostate cancer cells in long-term in vitro culture. Presently, there are only a few androgen-sensitive and no androgen-dependent in vitro human prostate cancer cell lines. The LNCAP is the most widely used in vitro human prostate cancer cell line, and while it expresses both AR and PSA, it is not androgen dependent [i.e., it does not undergo programmed (apoptotic) cell death following androgen ablation (33)], and its AR is mutated, changing the ligand specificity (34). In contrast, the human PC-82 prostate cancer cells express AR and PSA and are highly androgen dependent [i.e., they undergo programmed (apoptotic) death following androgen ablation (present study and Ref. 19)]. Thus, it would be of great value if these cells could be established as a permanent in vitro cell line. Presently, this is being attempted. Using such an in vitro PC-82 cell line, it should be possible to resolve whether the androgen dependence of these cells involves autocrine secretion of peptide growth and survival factors that trigger the response externally on the plasma membrane or whether an entirely internal intracrine pathway is involved.

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