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Advances in Brief

CWR22: Androgen-dependent Xenograft Model Derived from a Primary Human Prostatic Carcinoma[1]

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Abstract

The long-term propagation of primary human prostate cancer (PCA) *in vivo* or *in vitro* has been rare. Most such PCAs are phenotypically different from most PCAs in humans; *i.e.*, they make little prostate specific antigen and respond little, if at all, to androgen deprivation. A serially transplantable, primary human PCA, designated CWR22, exhibits a clonal cytogenetic aberration, causes high elevations of prostate specific antigen in the peripheral blood of nude mice, and is unusually responsive to androgen deprivation as compared with other xenografts. Studies of mRNA from CWR22 have demonstrated the expression of prostate specific antigen and the epidermal growth factor receptor family including *erbB*1/epidermal growth factor receptor, *erbB2/neu*, and *erbB*3, but not *erbB*4. A ligand for these receptors, the *neu* differentiation factor, is also expressed.

Introduction

PCA[3] is the most commonly diagnosed carcinoma and the second most common cause of cancer deaths in men in the United States (1). Based on recent American Cancer Society estimates, the number of deaths from PCA is increasing at a rate in excess of 8% annually (1). The medical therapy of PCA is limited, and there are no therapeutic models of PCA *in vitro* or *in vivo* that have resulted in the development of superior methods of medical therapy in the past decade. Currently, the most commonly used pharmacological agents in the treatment of PCA are hormonal. More than half a century after the discovery of the effect of hormonal manipulation on PCA, after acknowledging the beneficial palliative effects of hormonal therapy, a leader in this area (2) discussed the "...weak evidence that endocrine treatment prolongs survival and...the evident side effects..." Of critical importance, he (2) points out that "...reports of apparent cure of PCA by endocrine management based on necropsy findings are rare in the urological literature." The development of more effective models might facilitate the development and testing of new pharmacological and biological approaches to the therapy of PCA. There are fewer than a dozen well characterized serially transplantable xenografts of PCA and even fewer such xenografts derived from primary PCAs. A very small number of these xenografts, without exception derived from metastases, has been reported to cause elevations of PSA in the blood of nude mice (3, 4). Recently, we (5) reported the development of four, new, serially transplantable xenografts of primary, human PCAs. We now describe CWR22 which we believe has characteristics that suggest that it is exceptional among the currently available models for the study of human PCA. Among these characteristics, it causes elevations of PSA in the blood of nude mice that are high and responds dramatically to androgen deprivation. We believe that the general availability of this xenograft will offer a unique opportunity for the investigation of experimental therapy and the control of growth in PCA.

Materials and Methods

The serially transplantable primary PCA xenograft, CWR22, initially was transplanted as minced tumor (5). More recently, it has been transplanted by the injection of cell suspensions that are prepared with a method described previously for the dissociation of human prostatic tissues (6). Nude mice were used at 4–8 weeks of age and were obtained from the Athymic Animal Facility of the Case Western Reserve University Cancer Center. Animals were housed singly and given food *ad libitum*. Before injection of tumor and at 3-month intervals thereafter, 12.5-mg sustained-release testosterone pellets (Innovative Research of America, Toledo, OH) were placed s.c. in each male animal by trocar.

Fresh cells were injected into mice after tumors were digested with Pronase. Stored cells were frozen at 1–2°C/min in medium with 8% dimethyl sulfoxide and 20% calf serum, thawed with agitation in a water bath at 37°C, washed with RPMI 1640 (GIBCO, Gaithersburg, MD) with 20% calf serum (GIBCO), filtered through a single layer of Nitex with a 100 μm porosity (Tetko, Inc., Briarcliff Manor, NY), counted in hemocytometer chambers, and injected as described (7). Except as noted, experiments were carried out with fresh cells.

All animals for multisite injection experiments were anesthetized by the i.p. injection of 1.9 mg of ketamine, 0.38 mg of xylazine, and 0.06 mg of acepromazine in 0.22 ml of saline. A midline incision was made from the xyphoid process to the pubis. Animals were given injections of cells in 0.05 ml of Matrigel (Collaborative Research, Bedford, MA) through a 26-gauge needle in up to four sites per animal as detailed below. Injection sites included the spleen, prostate, epididymal fat pad, seminal vesicles, testis, bone (vertebral body via an anterior approach), lung, i.p. cavity, s.c. tissue, and the liver (Table 1). A localized bleb within the target organ was used as a sign of successful injection.

Mice were anesthetized with ether, blood was obtained by cardiac puncture, and animals were killed by cervical dislocation. Tumor for the study of RNA was obtained rapidly, sliced, and frozen in the vapor phase of a nitrogen freezer at −195°C. Blood serum was stored over liquid nitrogen. PSA was assayed by a slight modification of the method described previously (8).

Total RNA was extracted from CWR22 with TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) and a protocol supplied by the manufacturer. The first strand complementary DNA synthesis was performed in a 50-μl reaction containing 50 mM Tris-HCl (pH 8.5), 30 mM KCl, 1 mM dithiothreitol, 8 mM $MgCl_2$, 1 mM concentrations of each deoxynucleotide triphosphate, 40 units avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Indianapolis, IN), 20 μg of total RNA, and 1 μg of oligo$(dT)_{12-18}$. The complementary DNA product was amplified directly in a 100-μl reaction containing 10 mM Tris-HCl (pH 9.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, and 50 μM concentrations of each deoxynucleotide triphosphate for 30 cycles, with a cycle profile of 50 s at 95°C, 2 min at 60°C, and 2 min at 72°C, followed by a 7-min extension at 72°C. PCR products were

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[3] The abbreviations used are: PCA, prostate cancer; PSA, prostate-specific antigen; NDF, *neu* differentiation factor.

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Table 1 *Growth of CWR22 after transplantation to various sites*

Organ	No. of tumors/no. of sites injected: Frozen 10^6 cells injected	Frozen 10^5 cells injected	Fresh (10^4 cells injected)
s.c.	2/16	1/3	4/4
Bone and muscle	2 /5	0/1	3/3
Testicle	1 /5	0/1	2/3
Abdominal cavity	0 /4	0/1	0/2
Lung	0 /4	0/1	1/2
Epididymal fat pad	2 /3	0/0	1/2
Prostate	0 /3	0/0	0/1
Spleen	0 /3	0/0	0/2
Seminal vesicle	0 /5	0/1	0/2
Liver	0 /5	0/1	1/2
Kidney	0 /0	0/0	0/1

analyzed by 4% polyacrylamide gel electrophoresis. The primers used are:

*erbB*1/epidermal growth factor receptor:
sense 5′CAGCGCTACCTTGTCATTCAG3′
antisense 5′TCATACTATCCTCCGTGGTCA3′
erbB2/neu: sense 5′CGCTTTGTGGTCATCCAGAATG3′
antisense 5′TCGTGTTCACACTGGCACGTC3′
erbB3: sense 5′TGGCCCGAGACCCACCACGGTATCTG3′
antisense 5′AGTTACGTTCTCTGGGCATTAGCCTT3′
erbB4: sense 5′AGTTTTCAAGGATGGCTCGAGACCCTC3′
antisense 5′AGCTTACACCACAGTATTCCGGTGTCT3′
PSA: sense 5′CCACTTGTCTGTAATGGTGTG3′
antisense 5′TCCCCAGGACACAGAGAGGA3′
Transforming growth factor-α
sense 5′CGCTCTGGGTATTGTGTTGG3′
antisense 5′GGTCCGCTGATTTCTTCTCT3′
NDF: sense 5′GAAAGAGATGAAGAGTCAGGAG3′
antisense 5′GCATTGCACAAGTATACTTGAG3′

Cytogenetic analysis was accomplished by preparing cell suspension cultures for immediate harvest from the xenografts. Single cells and small clusters of cells were pooled by centrifuging the transport medium and discarding the supernatant. The tumor was mechanically dissociated and added to the existing cell pellet. Following gravity sedimentation of the large chunks of tissue, the supernatant was harvested directly and made up to 10 ml. Colcemid was added (50 μl of a 10-μg/ml stock solution) for 30 min, and the cells were exposed to a hypotonic solution (0.75 M KCl) for 13 min. Following this treatment, the preparations were fixed for 15 min with a 3:1 methanol:acetic acid fixative three successive times. Banded metaphases were prepared by trypsin pretreatment followed by Giemsa staining to yield GTG bands (9).

Results

Because the yield of cells from enzymatically dissociated primary human PCAs is not high (10), all primary carcinomas resected from patients have been injected into mice as minced tissue, not cell suspensions. One hundred eight nude mice received injections of minced tissue in experiments done for other purposes. One half of these animals received tissue in Matrigel; the other half received tissue in culture medium. There was no difference between these two vehicles for the injection of minced tissue with respect to either rate of successful transplantation or rate of growth by the tumor. In contrast, Matrigel enhanced the tumorigenicity of cells given as cell suspensions. When CWR22 cells were injected in Matrigel, 1000 cells usually produced tumors (Fig. 1); and larger numbers of fresh cells have always produced tumors (some data from animals that received >3000 cells all of which developed tumors are omitted from Fig. 1 because of space). In contrast, 1 million cells or more are required to transplant CWR22 when culture medium is used as the vehicle. The use of cells stored over liquid nitrogen requires more cells for successful transplantation; however, the comparison between medium and Matrigel was not carried out in great detail for frozen cells because (*a*) fresh cells form tumors more rapidly and at lower doses than frozen cells and (*b*) these experiments are expensive.

Several preliminary experiments with small numbers of animals show CWR22 to be very sensitive to hormonal manipulation. In one experiment, minced CWR22 was injected into 3 female and 3 male mice. All three males were killed 7–8 weeks after transplantation with tumors that were at least 8 mm in smallest dimension. In contrast, none of the three females had tumors after observation for 5 months. In a subsequent experiment, 2 males and 2 females were given injections of 1.1 million cells, more than 1000 times a tumorigenic dose. Both males developed large tumors in less than 2 months; no female developed a tumor during 6 months.

In two experiments with a total of 14 animals with established tumors, orchiectomy and simultaneous resection of the sustained-release testosterone pellets have resulted in PSAs that have declined from several hundred ng/ml to very low levels. In the oldest of these two experiments, 4 months after orchiectomy, the animal with the highest PSA in his peripheral blood exhibited a decline in PSA to 0.6 ng/ml; seven of nine animals exhibited declines of PSA levels that were within the normal range for our mice (<0.25 ng/ml). The lowest levels were reached 1–4 months after orchiectomy. In the other experiment that is 2 months old, PSA levels are still falling. In every case, tumors have shrunk to less than 50% of their volume prior to orchiectomy. In these two experiments that are still in progress, tumors have continued to regress more than 4 months and 2 months, respectively, following orchiectomy.

In a series of experiments, animals were killed when their tumors were various sizes in order to permit us to relate PSA determined from 50 μl of serum obtained from the tail veins or from cardiac puncture to the weights of tumors taken from the animals at death. Between the lowest weight tested and tumors of 0.8 g, the relationship between PSA in the serum and the weights of tumors was linear (Fig. 2). Above 0.8 g, PSA is markedly elevated but not linearly related to the weight of the tumor. The slope of the graph suggests that one can

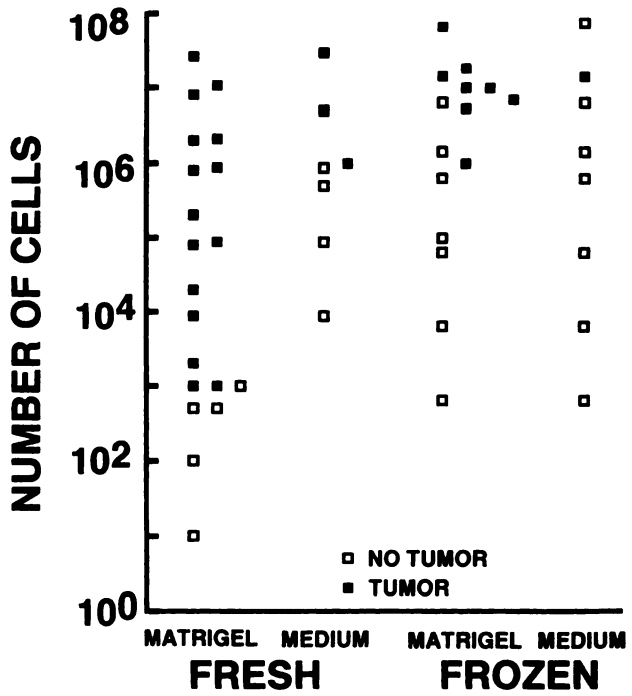

Fig. 1. Numbers of different kinds of cells required to produce tumors during 6 months of observation. Animals that did (■) and did not (□) develop tumors are shown in columns that are separated according to the kinds of cells injected and the vehicles in which cells were injected.

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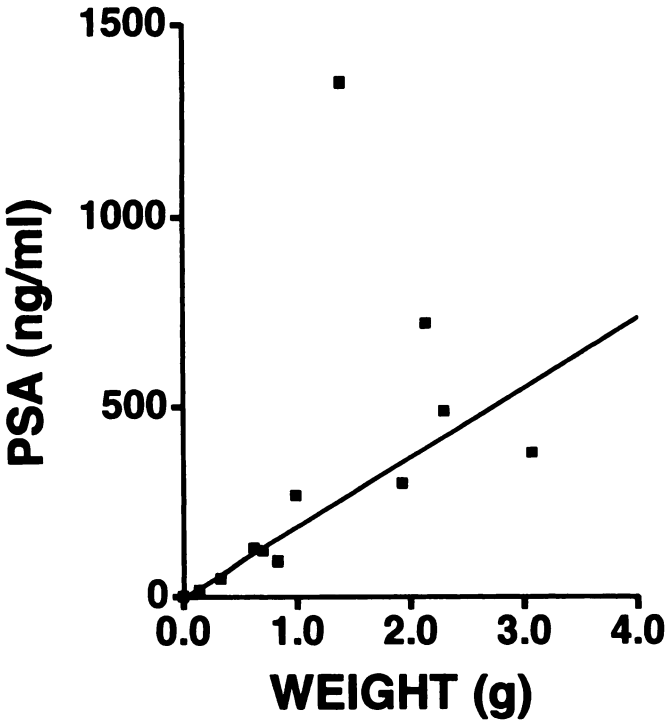

Fig. 2. Prostate-specific antigen in mouse serum was linearly related to the sizes of tumors up to 0.8 g in these mice and continues to increase in larger tumors.

detect 1–2 mg of tumor with 50 μl of serum from the tail vein; however, we have not rigorously tested this relationship or proved its linearity for tumors in the relevant range, *i.e.*, <50 mg.

The cytogenetic analysis of CWR22 included the examination of 20 cells that came from several different xenografts. Two distinct lines were observed. Both lines were missing chromosome 2 in every cell; had additional chromosomes 7, 8, and 12; contained an additional isochromosome for 1q in each cell; and had a derived chromosome 4 with an extra piece on the q arm. In addition, in 13 of the 20 cells, there was an unidentified marker chromosome [49,XY,+i(1)(q10),−2,add(4)(q35),+7,+8,+12[7]/50,XY,+i(1)(q10),−2,add(4)(q35),+7,+8,+12,+mar[13]].

As a first step in characterizing possible oncogenic signals involved in the growth of CWR22, we have analyzed the expression of the epidermal growth factor receptor/*erbB* family. We designed specific reverse transcription-polymerase chain reaction primers and applied them to the amplification of mRNA sequences from CWR22. Bands corresponding to *erbB*1 (11), *erbB*2 (12), and *erbB*3 (13) are present (Fig. 3). No *erbB*4 (14) was detected. Ligands for the *erbB* receptor family were also examined to identify possible autocrine growth loops. No specific band for transforming growth factor α, the ligand for *erbB*1, was detected. By contrast, the expression of NDF, the common ligand for *erbB*2, *erbB*3, and *erbB*4 (14, 15), was demonstrated. As a control for the xenograft RNA, primers for PSA were included. The expression of PSA was observed (Fig. 3).

Because of the potential importance of orthotopic transplantation, 8 pilot experiments (3–6 mice/experiment) were carried out in which 2–4 sites/mouse were injected with 1 million or 100,000 cells recovered from the nitrogen freezer or 10,000 cells fresh (Table 1). In earlier experiments, all animals in the experiment were killed when s.c. tumors developed in animals that had been given s.c. injections. In the later experiments, animals were killed when PSA in tail vein blood indicated the presence of tumors >5 mm in diameter. Six of 33 animals died unexpectedly, 4 less than 1 week following surgery; one, 39 days after surgery, and one, 61 days after surgery. In three of these six animals, abscesses were identified. Another had pneumonia, and two had no pathology identified at autopsy. These six animals have been omitted from the analysis of data, since they did not live sufficiently long to permit conclusions and did not live until they satisfied the criteria for killing animals in particular experiments as described above. In addition, 6 animals received injections at only 1 site each: 3 into the vertebrae anteriorly through the laparotomy incision; 3 into the testicle. Data from the 27 animals given injections in a total of 78 sites and 6 animals given injections in only one site each are summarized in Table 1. In the case of tumor that grew from injections of cells into the vertebral bodies, in several instances histological sections revealed the anterior defect in the bone that had been made for the injection of cells into bone at surgery. In two cases, neoplastic cells were proved histologically to be in the bone where they were injected; however, in no case did tumor appear to alter the morphology of the bone at the site of its location or invade the bone and/or marrow. In contrast, it grew luxuriantly in the surrounding skeletal muscle that was apposed to the vertebral column. The data (Table 1) suggest that, when cells are injected in Matrigel, (*a*) the s.c. site supports the growth of CWR22 as effectively as any site except possibly muscle and (*b*) the mouse prostate is a less effective site for the growth of CWR22.

Discussion

To our knowledge, the response of CWR22 to orchiectomy is greater than those reported for other xenografts as assessed by duration of regression, physical reduction in size of tumor, and fall in PSA in the nude mouse. In addition, CWR22 has the least deviated set of chromosomal aberrations reported to date in xenografts derived from primary human PCAs.

Three of the four *erbB* family receptors are expressed in CWR22. *erbB*1 and *erbB*2 have been implicated in the development of PCA. The presence of the *erbB*3 transcript in PCA, however, has not been reported previously. The fact that CWR22 also expresses NDF, the ligand for *erbB*2, *erbB*3, and *erbB*4, suggests that the *erbB* receptor family may

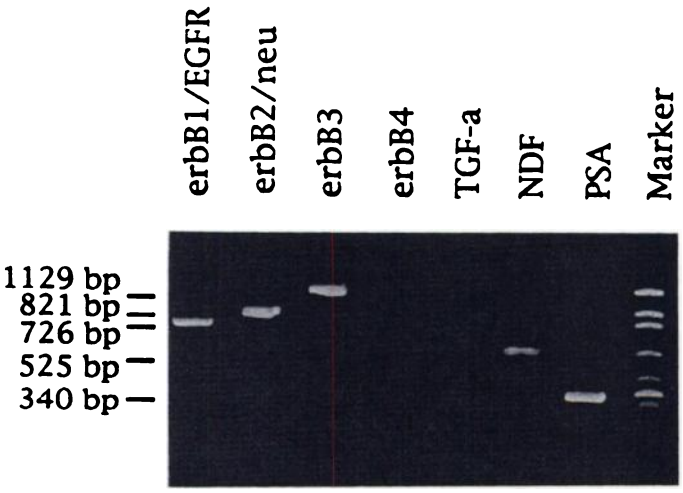

Fig. 3. Reverse transcriptase-polymerase chain reaction analysis for the expression of the epidermal growth factor receptor (*EGFR*)/*erbB* family members and their ligands. The primers of the receptors are derived from the COOH-terminal regions unique to each molecule. Control experiments based on Southern hybridizations to individual probes verified the expected specificity of the priming reactions. The sequences of the individual primers are listed in "Materials and Methods." The expected sizes for the polymerase chain reaction products are: *erbB*1, 726 base pairs (bp); erbB2, 821 base pairs (bp); erbB3, 1129 base pairs; erbB4, 1013 base pairs; transforming growth factor α (TGF-α), 680 base pairs; NDF, 525 base pairs; and PSA, 340 base pairs. The molecular size marker is *Hinc*II-digested ϕX174 replicative form DNA. The abilities of *erbB*4 and transforming growth factor α primers to initiate polymerase chain reactions were verified in similar experiments with mRNA from a glioma cell line, PL-1, which is known to express *erbB*4 and transforming growth factor α.

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play an important role in the growth of this neoplasm. The potential significance of this ligand can be tested in CWR22 not only by following the size of the tumor but also by assessing the very high levels of PSA produced by this tumor. Chung (16) has shown that the growth of the LNCaP PCA cell line as a xenograft is enhanced by the addition of medium conditioned by fibroblasts. Chung *et al.* (17) also showed that the anchorage-independent growth of LNCaP is stimulated *in vitro* by basic fibroblast growth factor, hepatocyte growth factor, and nerve growth factor, but not by a variety of other tested growth factors. This kind of approach should facilitate the investigation of the importance of growth factors for the growth of human PCA cells *in vivo*.

In 1988, Csapo *et al.* (3) demonstrated that some PCA xenografts produce detectable levels of PSA in the blood of nude mice. Gleave *et al.* (4) reported that the volume of LNCaP xenografts is closely correlated with the PSA levels in the blood of mice with xenografts. They found that: "Following castration, serum PSA levels decrease rapidly up to 8-fold and increase up to 20-fold following androgen supplementation without detectable castration-induced tumor cell death or concomitant changes in tumor volume." Other studies (18) had shown that the production of PSA by LNCaP was influenced not just by the number of neoplastic cells but also by androgens and other factors. The factors that may influence the fall in serum PSA after orchiectomies are carried out in mice that bear CWR22 are being investigated in greater detail; however, in contrast to LNCaP, orchiectomy is followed by a very marked shrinkage in the volume of CWR22 that is maintained for >4 months in experiments that are in progress. Instead of the 8-fold drop in PSA that is seen after orchiectomy in animals bearing LNCaP (4), animals with CWR22 tumors of 1 g or more usually show a 100–1000-fold fall in serum PSA.

As reviewed in detail recently (19), except for the observation that a peptide from laminin partially inhibits the enhanced tumorigenicity of cells injected in Matrigel, our understanding of the mechanism of action of Matrigel is very limited. Matrigel is an extract of a transplantable tumor that is available in the original form and as a "growth factor reduced" Matrigel. In comparing these two forms of this product, we have not found that they have any differences that affect the transplantation of our tumors. To our knowledge, this report is the first to compare minced tumor and cell suspensions in testing the efficacy of Matrigel for enhancing tumorigenicity. Matrigel does not affect the chance for success in the transplantation of minced tissue from CWR22 or from CWR31 and CWR91, the other two xenografts that we have tested sufficiently to warrant comment. Why Matrigel should enhance markedly the tumorigenicity of cell suspensions while not affecting the tumorigenicity of minced solid tumor is unclear. We have not been able to detect evidence that tumors that have been transplanted serially in Matrigel are more or less tumorigenic than tumors that have been passaged serially in culture medium.

The availability of a serially transplantable primary PCA that causes marked elevations of PSA in the blood of nude mice, regresses after orchiectomy, has a limited number of well defined chromosomal alterations, and expresses several members of the epidermal growth factor family provides a tool that has not been available previously. For studies that require DNA from neoplastic human prostatic epithelial cells, such as comparative genomic hybridization (20), the fact that the stromal cells in these human PCAs are from the mouse offers real advantages. If there are unique features of human PCAs that are responsible for their relative refractoriness to most tested chemotherapeutic and biological approaches to therapy, models like CWR22 should facilitate the investigation of the mechanisms that afford this kind of resistance.

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