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Expression of the Coxsackie Adenovirus Receptor in Normal Prostate and in Primary and Metastatic Prostate Carcinoma: Potential Relevance to Gene Therapy[1]

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ABSTRACT

Adenovirus-based gene therapy may provide an alternative mode of treatment for prostate cancer, especially for late-stage and androgen-independent disease for which there is currently no effective treatment. Efficient adenovirus infection of target cells depends upon the presence of the coxsackie adenovirus cell surface receptor, CAR, which is the primary receptor for group C adenoviruses and is important for the attachment of adenovirus to the cell membrane. To evaluate the potential efficacy of adenoviral therapy for prostate cancer, we evaluated CAR expression in normal prostate tissue and in prostate carcinoma of increasing Gleason grades in paraffin-embedded, archival tissues using a polyclonal antibody raised against human CAR. Immunohistochemical analysis of benign prostate epithelia demonstrated intense luminal and lateral cell membrane staining. There was a statistically significant difference in CAR membrane expression with respect to Gleason score. In addition, metastatic prostate specimens demonstrated strong membrane staining for CAR. Adenovirus therapy may, therefore, provide an alternate modality in the treatment of prostate cancer and may be especially efficacious in the treatment of metastatic disease.

INTRODUCTION

The development of human adenoviral vectors for gene therapy and cancer therapy protocols has led to an increasing interest in the characterization of expression of CAR[3] in human tissues. A number of adenovirus-based gene therapy protocols for the treatment of prostate cancer are currently being developed and evaluated in clinical trials. Several of these trials involve replication-deficient adenoviral vectors to deliver cell cycle genes such as *p53* (1) and *p16* (2), cytokines such as interleukin 12 (2) or cytotoxic genes such as the *herpes simplex virus-thymidine kinase* gene (3–5). In an effort to target hormone-independent metastatic or locally recurrent prostate cancer, Koeneman *et al.* (6) have constructed an adenoviral vector in which the *herpes simplex virus-thymidine kinase* gene is driven by the osteocalcin tissue-specific promoter. Replication-competent adenoviral therapies for the treatment of prostate cancer are also in clinical trials. Calydon virus 787 is a human adenovirus genetically engineered to selectively replicate in prostate cells expressing prostate-specific antigen by placing the *E1a* and the E1b-55K genes under prostate-specific promoters (7, 8).

All of the adenoviral therapies described above rely on the ability of the virus to gain entry efficiently into the malignant target cells. This is dependent on the presence of CAR, the primary receptor for group C adenoviruses. The expression of CAR in malignancies is beginning to be defined. Marked variations in cell surface expression of CAR levels have been demonstrated using different cancer cell lines of the same tissue origin. Hemmi *et al.* (9) have examined primary melanoma cell lines in culture and found various levels of CAR expression. Similarly, Li *et al.* (10) found marked variation of CAR receptor levels in human bladder cancer cell lines. Variations have also been noted in primary and established glioma (11), lung and pancreatic (12), and head and neck (13) carcinoma cell lines. Okegawa *et al.* (14) examined three prostate cancer cell lines and found that CAR is down-regulated in the tumorigenic cell line PC3 as compared with DU145 and LNCaP. In all of these studies, a high cell surface density of CAR correlated with increased adenoviral attachment to the cell surface. The cell lines with the highest level of receptor expression demonstrated an increased susceptibility to infection with adenovirus, and those cells with the lowest receptor level were refractory to adenovirus infection, indicating that the level of CAR on the cell surface is an important factor in the efficacy of adenoviral therapy. To date, the cell surface receptor status of CAR in target tissues and human cancer tissues from patients undergoing adenoviral therapy has not been defined immunohistochemically because of a lack of an appropriate antibody that works in formalin-fixed, paraffin-embedded tissues. This has proven to be one of the limitations in the evaluation of adenoviral gene therapy.

To evaluate the potential efficacy of adenoviral therapy for prostate cancer, we examined the expression of CAR in normal prostate and prostate carcinoma using archival formalin-fixed, paraffin-embedded tissues. A polyclonal antibody was developed, and using antigen retrieval techniques, the expression of CAR was analyzed in normal prostate, low Gleason grade, intermediate Gleason grade, and high Gleason grade prostate carcinoma, as well as in metastatic and androgen-independent prostate cancer.

MATERIALS AND METHODS

Specimens. Formalin-fixed, paraffin-embedded archival tissue blocks of normal prostate, prostate carcinoma, and metastases were obtained from the University of California San Francisco Comprehensive Cancer Center Tissue Core. None of the patients, except those in the androgen-independent prostate cancer group, had undergone either hormone therapy or radiotherapy before the collection of the samples. Thirty-three histologically normal prostate samples were obtained at the time of cystoprostatectomy for bladder malignancy ($n = 11$) or radical prostatectomy for prostate cancer ($n = 22$). All tissues were sectioned in the fresh state. The resulting 4-mm-thick tissue slices were placed in 10% buffered formalin for fixation within 60 min of removal from the patient and then fixed for 4–12 h before routine histological processing. All tumor specimens ($n = 129$) were graded by GS criteria using H&E-stained sections at the time of immunohistochemical evaluation. The GS ranged from a low score of 5–6 ($n = 29$), an intermediate score of 7 ($n = 24$), and up to a high score of 8–10 ($n = 23$; Ref. 15). The pathological stages ranged from

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[3] The abbreviations used are: CAR, coxsackie adenovirus receptor; GS, Gleason score; AIPC, androgen-independent prostate carcinoma; CHO, Chinese hamster ovary.

pT_1 to pT_4. Formalin-fixed, paraffin-embedded tissue blocks of AIPC ($n = 20$) were obtained from patients who had undergone transurethral resection of the prostate, prostate biopsy, or surgical resection of a metastasis. All patient charts were reviewed to ensure that the patient had AIPC (defined as progressive disease despite androgen deprivation therapy) at the time tissue was collected. Metastatic specimens ($n = 10$) consisted of 4 bone metastases (2 of which were from patients with AIPC), 4 lymph node, 1 liver fine needle aspirate, and 1 lung fine-needle aspirate. The study was done under an approved Committee on Human Research protocol at the University of California, San Francisco.

Antibodies. A polyclonal antiserum to human CAR was produced by using the published human CAR sequence to search national databases (ONYX Pharmaceuticals, Richmond, CA). An expressed sequence tag that was predicted to encode >90% of the CAR open reading frame was identified and obtained from Genome Systems Inc. (accession number N25352, ID number 265680) and verified by sequencing. The nucleotide sequence encoding amino acid residues 15–366 was amplified by PCR using the 5′-AAACTGCAGGAAGAATACATGCCAATGGAACATATGAGTATCACTACTCCTGAAGA-3′ (forward primer) and 5′-AAAGGTACCTACTTATTCATATGGAGGCTCTATACTATAG-3′ (reverse primer) with an EE-tag added to the NH_2 terminus. The conditions of amplification were as follows: 95°C for 30 s, 58°C for 30 s, and 72°C for 30 min for 30 cycles. The 1.1-kb amplified product was cloned into the baculovirus vector, pBACsurf-1 (Novagen, Madison, WI), and expressed in Sf-9 cells (ATCC CRL-1711). The protein product was affinity purified over an anti-EE-tag column, and the EE-tagged purified CAR protein was used to immunize two rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, PA). The antisera from two rabbits was collected. One of these antibodies, CAR 72, was used for immunohistochemistry. The mouse monoclonal antibody to human CAR, RmcB, was used to stain fresh frozen tissue (16).

Immunohistochemistry. Routine H&E-stained sections were reviewed by a single pathologist for histological confirmation and Gleason scoring. Four-μm sections were cut from archival, formalin-fixed, paraffin-embedded normal prostate and prostate tumor specimens and mounted on Superfrost/Plus slides (Fisher Scientific). The slides were baked for 30 min at 60°C, deparaffinized in xylene, rehydrated, and placed in PBS. Slides were then incubated in 0.01% trypsin/PBS for 15 min at 37°C, washed in PBS, followed by distilled water. The sections were then microwaved for 10 min in 10 mM citrate buffer (pH 6.0) and cooled for 30 min back to room temperature. The tissue was blocked in 10% goat serum/PBS for 30 min at room temperature and subsequently incubated in primary polyclonal antibody CAR 72 diluted 1:7000 for 8–16 h at 4°C. After incubation, the slides were rinsed in PBS and incubated for 30 min at room temperature in secondary biotinylated goat antirabbit immunoglobulin diluted 1:200 (Vector Laboratories, Burlingame, CA). After rinsing in PBS, the tissues sections were treated with streptavidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 30 min at room temperature. The peroxidase reaction was developed using 0.0014 M diaminobenzidine tetrahydrochloride (Sigma) in 0.1% hydrogen peroxide/PBS for 5 min at room temperature. The sections were rinsed and counterstained with hematoxylin. The CAR polyclonal antibody was used with the peroxidase-antiperoxidase method to stain for CAR protein, and sites of immunoprecipitate formation were identified using light microscopy. All staining was performed within 7 days of slide preparation to prevent antigen degradation. Controls included preimmune rabbit serum, nonimmune rabbit serum, or rabbit IgG immunoglobulins in place of the CAR polyclonal primary antibody.

To compare the CAR staining patterns, fresh-frozen radical prostatectomy specimens were stained in parallel with RmcB at a dilution 1:25 and the rabbit polyclonal antibody CAR 72 using a dilution of 1:2000. Samples from 10 intermediate grade GS and 5 normal prostate specimens were cut in 6-μm sections, fixed in cold acetone for 10 min, air dried, and stored at −80°C until use. The sections were blocked in 10% normal goat serum and incubated in primary antibody overnight at 4°C. The slides were processed using the methodology described above. Controls for antibody staining included the following: cytospin preparations and paraffin cell buttons of CHO cells stably transfected with human CAR (16) were used as a positive control, and CHO cells (ATCC CCL-61) were used as a negative control. In addition, negative controls for antibody staining involved incubating control prostate tissue sections in preimmune sera as the primary antibody. Internal negative controls included infiltrative lymphocytes present in the tissue sections.

Statistical Methods. The association between CAR expression and tumor grade in prostate cancer tissue obtained from radical prostatectomy specimens was conducted in two stages. Because this was the first investigation of this receptor, initially the two extremes of tumor grade, normal tissue from patients with prostate cancer, and high GSs (8–10) were stained to determine whether a difference in membrane staining intensity could be recognized. Then, to further investigate the possible change in expression with tumor grade, tissue samples with GSs of 5 or 6 (low) and 7 (intermediate) were evaluated, resulting in a total of four subsets for analysis. It was hypothesized that 90% of normal specimens would have a staining intensity of 3+. A minimum of 20 samples in each of the four subsets was required to be able to detect at least a 40% difference in the proportion of samples with a high degree of staining for any of the pairwise comparisons. This assumed a power of 80% and a level of significance of 5% for a directional test with no adjustment for multiple comparisons. To confirm that what was being measured in normal tissue did not reflect undetectable disease, an additional control group of specimens from men without any prostate cancer was also analyzed. To fully evaluate the spectrum of prostate cancer, two additional subsets of tissue were analyzed for CAR membrane expression. A group of samples known to be androgen independent was stained. This subset was compared with the high-grade group as part of an exploratory analysis. In addition, specimens from patients with metastatic disease were also stained. Normal, prostate cancer and metastatic tissue specimens were analyzed for the degree of CAR expression on the cell surface and scored from 0 to 3+ on a categorical scale. The intensity of membrane staining for the normal and tumor specimens were grouped into four categories: no staining/background of negative controls (0), weak staining detectable above background (1+), moderate staining (2+), and intense staining (3+). In addition, tissue sections were semiquantitatively scored by estimating the percentage of cells with membrane staining. Cytoplasmic staining alone was scored as negative. Each section was scored independently by two individuals (D. S. and K. A. R.). The scores of intensity and the percentage of cells with membrane staining were concordant. Analyses to compare the distribution of each of these two factors by tumor grade were performed using a χ^2 statistic for CAR staining intensity and ANOVA methods for percentage of membrane staining. The corresponding tests performed when the subset sample size was small were Fisher's exact test and the nonparametric Kruskal-Wallis test.

RESULTS

Comparison of CAR Expression in CHO Cell Cytospins and in Fresh-Frozen Prostate Tissue Using RmcB and Rabbit Polyclonal CAR. The RmcB antibody has been characterized previously in fresh frozen tissue by immunohistochemistry (9) and by immunofluorescence (17) and specifically stains human CAR. Control cytospin preparations of CHO cells and CHO cells stably transfected with human CAR were stained with both RmcB and CAR 72. CHO cells with human CAR demonstrated intense 2–3+ cytoplasmic and membrane staining, whereas nontransfected CHO cells had 0–1+ background staining. Both CAR 72 and RmcB antibody staining patterns were identical (data not shown). To compare the membrane staining pattern of CAR 72 with RmcB in prostate tissue, 10 intermediate GS and 5 normal prostate fresh-frozen specimens from radical prostatectomies were stained in parallel with both antibodies. The CAR 72 and RmcB membrane staining patterns for CAR expression were comparable. The prostatic secretory epithelial cell membrane was preferentially stained. Luminal membrane (3+) staining as well as lateral membrane staining was observed using both antibodies. Basal cells exhibited strong cytoplasmic and membrane staining with both antibodies (data not shown).

Expression of CAR in Nonmalignant Prostate. A total of 22 histologically normal, paraffin-embedded prostate specimens from radical prostatectomies were examined by immunohistochemistry for CAR expression. To confirm that CAR staining in normal prostate

samples represented normal tissue and that the membrane staining response did not indicate undetectable disease, an additional control group (cystoprostatectomy specimens, $n = 11$) without any evidence of prostate cancer was also assessed for the degree of CAR staining. All samples stained with CAR 72 antibody demonstrated 2+ or 3+ membrane staining of the epithelial cells in the normal glands (Fig. 1*B*). Cytoplasmic staining was 0–1+. CAR staining was localized to the lateral aspect of the membrane where cell-cell contact occurred and to the luminal apices of the prostatic columnar epithelium. The luminal border consistently appeared more intense in staining as compared with the lateral aspect of the cell. Normal hyperplastic glands and the basal cells of benign glands consistently had increased cytoplasmic staining of 1–2+. Because there was no difference in the frequency of 3+ membrane staining between normal prostate tissue from patients with prostate cancer and the control group (91% *versus* 73%; $P = 0.30$), the information from the two normal subsets was pooled in this analysis and served as the control group for comparison with the tumor tissue. As controls, paraffin-embedded normal prostate

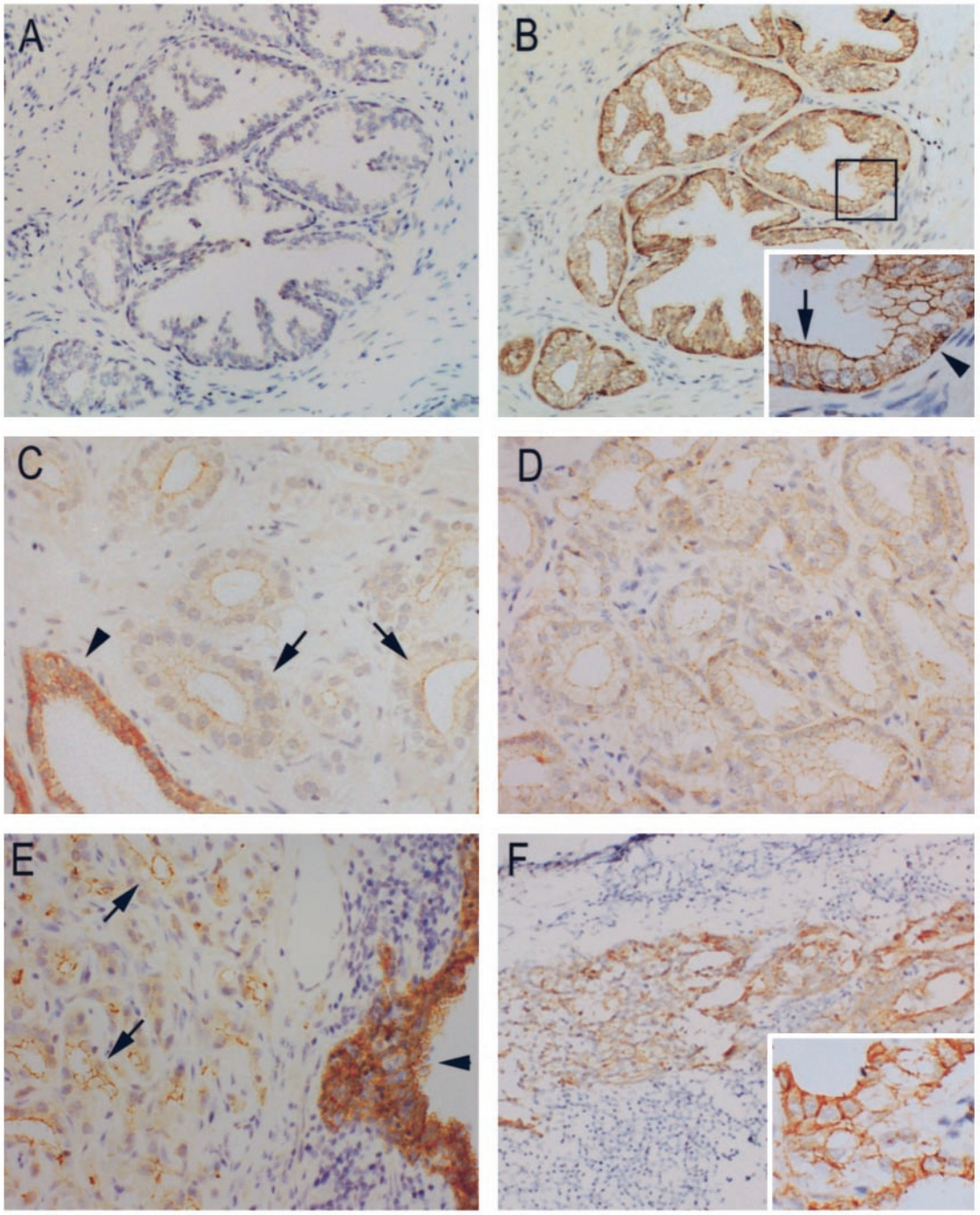

Fig. 1. Immunohistochemical staining of normal prostate tissue with preimmune serum (*A*) and immune serum (*B*) to CAR. Normal prostate glands have luminal border staining as well as cell-cell border staining (*arrow*). Basal cells demonstrate intense cytoplasmic staining (*arrowhead*). Low-grade prostatic carcinoma (*C*) demonstrates luminal and lateral cytoplasmic membrane staining (*arrows*) of decreased intensity as compared with the CAR staining of the normal gland (*arrowhead*). *D*, intermediate-grade prostatic adenocarcinoma. *E*, high-grade prostatic adenocarcinoma demonstrate diminished CAR staining (*arrows*) as compared with the normal gland (*arrowhead*). Lymphocytes are negative for CAR staining. *F*, lymph node metastasis has strong CAR cytoplasmic membrane staining.

Table 1 *Characteristics of samples*

	Tumor grade				
	Normal	Low	Intermediate	High	Androgen independent
	33 (26%)	29 (22%)	24 (19%)	23 (18%)	20 (15%)
Total GS					
5		1			
6		28			
7			24		
8				9	2
9				13	5
10				1	13
Primary GS					
3		29	17		
4			7	21	3
5				2	17
Mean age (yr)	56.8	57.4	60.9	61.0	
Age ≥70	0	2 (7%)	3 (14%)	1 (5%)	
Pathological stage					
T_1		1 (3%)			
T_2	9 (50%)	27 (93%)	14 (58%)	8 (35%)	
T_3	9 (50%)	1 (3%)	9 (38%)	13 (56%)	
T_4			1 (4%)	2 (9%)	

specimens from radical prostatectomies were also stained with preimmune rabbit serum (Fig. 1*A*), nonimmune rabbit serum, or rabbit IgG immunoglobulins in place of the CAR 72 polyclonal primary antibody. There was no staining (0+) observed.

Correlation between Decreased CAR Expression and Local Advancement of Tumor GS. A total of 129 paraffin-embedded tumor specimens were immunohistochemically stained with the rabbit polyclonal CAR 72 antibody. The overall mean age of the patients was 59 years of age, ranging from 44 to 75, at the time of radical prostatectomy for the specimens analyzed. There was a significant difference in mean age among the four subsets ($P = 0.047$), with patients having intermediate- or high-grade tumors being slightly older than the normal or low-grade subsets. There is a strong association between GS and pathological stage ($P < 0.0001$). The GS of these tumors ranged from well-differentiated (GS 5) to an undifferentiated GS of 10. Approximately one-third of the tumor samples had pathological stage T_3 or T_4 tumors, and all, except one, had intermediate- or high-grade tumors. Only 50% of the patients with normal tissue had tumors with pathological stage T_3-T_4 disease. The distributions of specimens by grade and presenting features are shown in Table 1. The CAR staining pattern was similar to that seen in normal prostate tissue with CAR localizing to the lateral aspect of the membrane where cell-cell contact occurs as well as the luminal surface of the prostatic columnar epithelium (Fig. 1, *C–E*). There is a significant difference in the proportion of specimens displaying 3+ staining with it occurring most often in normal tissue (85%; $P < 0.0001$). Compared with the high intensity staining of normal prostate, the intensity of CAR staining in low, intermediate, and high Gleason grade prostate carcinoma was significantly reduced (Table 2A). Some CAR membrane staining was detected in all but 1 androgen-independent specimen (GS 10). In comparing high-grade androgen-dependent and androgen-independent samples, there was a difference in the proportion of 3+ staining between the two groups (26% *versus* 0%; $P = 0.02$). There was no association between age and 3+ staining with specimens from 33% of patients <60 years of age and 29% of patients 60 years or more having 3+ membrane staining.

There was also a significant difference in the mean percentage of cells stained among normal and tumor samples ($P < 0.0001$), which is primarily attributable to the normal cells staining significantly higher than the other three subsets (Table 2B, Column 1). Even among those with staining intensity of 3+, a significant difference in the mean percentage of cells stained by grade was observed ($P < 0.0001$; Fig. 1*E*). Of the 16 specimens with <50% of the cells exhibiting membrane staining, 8 were androgen independent, 7 were high grade, and the other had a GS of 6. All specimens with membrane staining of 3+ demonstrated ≥70% of the cells stained with one exception. This was an androgen-dependent GS 4 + 5 patient with 40% of cells staining. Therefore, the association between the percentage of cells staining with CAR and the intensity of 3+ staining was usually recognized when at least 70% of the total cells were stained with CAR. Even when the sample was limited to cases with at least 70% of cells staining for CAR, there is a highly significant difference in the proportion of 3+ staining between normal and tumor specimens ($P = 0.0004$). There was no difference in the mean percentage of the cells stained between the high-grade androgen-dependent group and the androgen-independent group. These two groups demonstrated a high degree of heterogeneity in membrane staining ranging from diffuse 3+ CAR membrane staining to virtually no tumor cells expressing CAR.

Expression of CAR in Prostate Cancer Metastases. Among the 10 metastatic samples, all 4 bone metastases exhibited a high degree of CAR membrane staining. The 2 specimens from patients with

Table 2 *CAR membrane staining of normal prostate, prostate carcinoma, and metastatic disease*

A. Distribution of CAR staining intensity by tumor grade

Grade	Number	Intensity			
		0	1+	2+	3+
Normal	33			5	28 (85%)
Low	29		3	19	7 (24%)
Intermediate	24			13	11 (46%)
High	23		1	16	6 (26%)
Androgen independent	20	1	2	17	0
Metastatic disease	9				9 (100%)

B. Percentage of cells stained with CAR by tumor grade and intensity

	Total		3+ intensity		0–2+ intensity	
	No.	Mean % (range)	No.	Mean % (range)	No.	Mean % (range)
Normal	33	100% (100%)	28	100% (100%)	5	100% (100%)
Tumor grade						
Low	29	71% (40–100%)	7	80% (70–100%)	22	68% (40–100%)
Intermediate	24	77% (50–100%)	11	85% (70–100%)	13	70% (50–90%)
High	23	63% (5–100%)	6	83% (40–100%)	17	57% (0–100%)
Androgen independent	20	52% (0–100%)	0		20	52% (0–100%)
Metastatic disease	9	72% (10–100%)	9	72% (10–100%)		

AIPC had 3+ staining in 80 and 100% of the cells, whereas the metastases from the 2 patients without prior androgen manipulation had 3+ membrane staining in 50 and 100% of the cells. The 4 cases with lymph node metastases also had high CAR membrane staining in the majority of the cancer cells (Fig. 1*F*). The fine-needle aspirate from the liver metastasis had focally preserved 3+ membrane staining in 10% of the cells with 2+ cytoplasmic background in all of the cells. The fine-needle aspirate from the lung metastasis had diffuse cytoplasmic 2+ staining, which made it difficult to distinguish membrane staining. Thus, the proportion with 3+ CAR staining among the 9 evaluable metastatic samples was not different from the normal specimens (100% *versus* 85%; $P = 0.57$) but was higher than the tumor samples (metastatic disease *versus* 3 tumor grades; $P < 0.001$). A wide range of percentage of cells with membrane staining was observed among the metastatic samples, ranging from 10 to 100%, even with 3+ CAR intensity. There was no difference in the percentage of cells with membrane staining between tumor and metastatic specimens ($P = 0.23$), but a difference was observed when the metastatic tissue was compared with the normal tissue ($P < 0.001$). Thus, metastases have the same proportion of 3+ staining as normal tissue; however, the percentage of cells exhibiting CAR membrane staining was different.

DISCUSSION

Adenoviral gene therapy is an intense area of research and is being developed as a therapeutic modality for a variety of malignancies. Prostate cancer, which is the second leading cause of cancer-related deaths in men, is one such malignancy. Current therapies exist, but there is still a significant percentage of patients with local recurrent disease, androgen-independent disease, or metastatic disease for which there is no effective treatment. At present, there are several clinical trials for the treatment of prostate cancer using replication-deficient adenovirus vectors for transgene delivery and replication-competent adenoviruses for oncolytic therapy. Patients enrolled in these adenoviral trials are being treated with virus without the evaluation of CAR receptor status of their cancer before therapy. This has been a major limitation in the proper evaluation and subsequent stratification of patients.

CAR is the primary adenoviral receptor, and its presence is a determining factor in efficient adenoviral infection (16). CAR serves as the site for attachment of human adenovirus via the fiber protein to the target cell membrane and is found in a variety of cell types. Although its physiological function is currently unknown, it is believed to be important in cell-cell interaction and adhesion. Subsequent internalization of human adenovirus into the cell is enhanced by other cell surface proteins, the vitronectin receptors, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (18). The penton base protein of the adenovirus interacts with the α_v subunit. Although adenovirus binds to cells that lack α_v, the presence of this subunit on the cell surface greatly increases the susceptibility to infection. Thus, for current adenoviral vectors in gene therapy to be efficacious, the presence of CAR and α_v on the cell surface of target tissues enhances the transduction of the virus.

This is the first study to examine the membrane status of CAR in paraffin-embedded tissues. Normal prostate had intense membrane staining in all epithelial cells in the region of cell-cell contact, which is consistent with that seen in human respiratory epithelia (19–22). In addition, there was equally strong luminal membrane staining of the prostatic secretory epithelium, which has not been observed in respiratory epithelia in studies using immunofluorescence techniques for CAR staining. We also observed this novel membrane staining pattern in colon epithelium and normal breast ductal epithelium (data not shown). There was some variability of CAR staining intensity in normal prostate tissue ranging from 2–3+ with the majority of staining in the 3+ range. This may result from minor variations in fixation or variation in the prostatic lobe of origin of the individual samples.

When compared with normal prostate, CAR expression decreased in both intensity and percentage of cells stained among the prostate specimens of all GSs. Well-differentiated, low-grade GS prostate cancers exhibited a similar membrane staining pattern as that seen in normal tissue, with most specimens exhibiting staining of 2+ intensity in the majority of cells. This was also the case for intermediate- and high-grade cancers. Interestingly, in those high-grade samples where luminal structures were preserved, CAR membrane staining patterns resembled those of lower grade tumors. In those samples with syncitial sheets of malignant cells, CAR membrane staining was greatly diminished. The variability of CAR membrane expression, especially in the androgen-independent group, suggests that the evaluation of CAR membrane status may be a useful criterion for predicting those patients who may best respond to adenoviral therapy. The expression of CAR in primary prostate cancer is similar to that seen in the expression of E-cadherin, a transmembrane glycoprotein important in cell-cell interaction and maintenance of the differentiated epithelial phenotype. The membrane expression of E-cadherin in prostate cancer has been well characterized (23–28). E-cadherin is strongly expressed in normal prostate epithelium and is localized to the lateral aspects of the cell where cell-cell contact occurs. E-cadherin expression becomes heterogeneous as GS increases and has been considered as a possible marker for prognosis attributable to its association with poor patient outcome (24, 27).

CAR exhibited robust membrane staining in the surgically derived metastases examined in this series. Bone, liver, and lymph node metastases all stained positive for CAR. The lung fine-needle aspirates had an increase in cytoplasmic staining, which made it difficult to distinguish membrane CAR staining. Again, CAR expression appears to mirror the expression of E-cadherin with a transient down-regulation of E-cadherin in the primary tumor (23, 27, 28) and a re-expression of the cell surface molecule in metastases (27, 28). Although the series of metastases stained in our study was small, staining these metastases with E-cadherin also exhibited a strong membrane staining pattern (data not shown). Unlike E-cadherin, the biological function of CAR in normal tissues is currently unknown. We have demonstrated that CAR expression in the normal prostate has robust luminal and lateral membrane staining. In the progression of prostate cancer, CAR expression becomes more heterogeneous, as indicated by a decreased intensity of membrane staining and a decreased percentage of cells that stain for CAR. CAR may function in a manner similar to E-cadherin in that it may help serve as a tumor inhibitor in its normal physiological state. *In vivo* studies have demonstrated that when CAR is transfected into the tumorigenic prostate cell line PC3, flank tumors produced from these cells in nude mice are growth inhibited as compared with controls (14). As proposed for E-cadherin, lack of cell adhesion molecule expression may promote the development of metastasis. Subsequently, metastatic tumor cells that can re-express cell adhesion molecules such as E-cadherin and CAR may possess a survival advantage, allowing the cells to gain a foothold in a foreign environment (27, 28). The strong correlation between high CAR expression and metastatic prostate carcinoma underscores the potential for adenoviral therapy in advanced disease.

The localization of CAR in prostate tissue and in human respiratory airway epithelium shows important differences. CAR has been well characterized in human respiratory epithelium (17, 21, 22, 29). Full-length CAR is limited to the basolateral aspect of respiratory epithelial cells with no apical expression. Multiple sorting regions within the cytoplasmic domain are responsible for the subcellular localization of

CAR on the membrane surface (22). When basolateral targeting domains are deleted or mutated, CAR is expressed not only in the basolateral region of the membrane but is translocated to the apical surface of the cell as well. Human CAR mRNA is expressed in a variety of tissues, including heart, pancreas, brain, testis, and prostate. Prostate tissue demonstrates the highest mRNA levels as determined by Northern blot analysis (30). Multiple species of RNA exist, with the 6–7-kb mRNA species being the most prevalent. Lung tissue has very little detectable CAR mRNA and appears to have only the 6–7-kb mRNA species. In contrast, prostate tissue exhibits mRNA species of approximately 6–7, 4.2, 3, and 1.5 kb. In a study by Fechner *et al.* (31), Northern blot analysis of human lung tissue also demonstrated the prominent 6–7-kb mRNA species, as well as two very weak, smaller mRNA species present. The biological role of these multiple species of CAR mRNA still remains unclear. The luminal expression of CAR exhibited in the prostate epithelium (as well as a similar membrane pattern seen in colon and normal breast epithelia) may be attributable to an alternate mRNA species found in prostate tissue that has altered basolateral sorting information in the CAR cytoplasmic domain, allowing it to localize to the luminal aspect of the cell. This hypothesis is supported in recent findings where alternate mRNA slice products for human CAR have been identified (32). An alternative hypothesis is that CAR undergoes posttranslational modification in prostatic epithelium such that it localizes to the luminal and lateral aspects of the cell.

In summary, we performed immunohistochemical evaluation of CAR on formalin-fixed, paraffin-embedded archival tissue sections to evaluate the membrane expression pattern of CAR in normal prostate and in prostate carcinoma. When compared with normal prostate, membrane CAR expression in tumors of increasing GS decreased in both intensity and percentage of cells stained. The expression of CAR in prostatic epithelium is substantially different from what has been described for respiratory epithelial cells. Because CAR is expressed on both the luminal border as well as the lateral cell membranes of prostate cells, secretory epithelial cells may be more likely to be targeted by replication-defective or replication-competent adenoviral therapy. In addition, metastatic lesions appear to have a high expression of membrane CAR after an apparent transient down-regulation of CAR seen in high Gleason grade tumors. Adenovirus therapy may provide an alternate modality in the treatment of prostate cancer, especially late-stage disease or metastatic disease, where there is currently no satisfactory treatment. Determination of CAR receptor status may thus prove valuable for predicting treatment outcomes of adenoviral therapy and therefore may aid in selecting those patients most likely to benefit from such treatment. Because integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ enhance viral infection, we are currently developing an immunohistochemical staining protocol to examine integrin α_v in formalin-fixed, paraffin-embedded tissues.

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REFERENCES

1. Logothetis, C., Hossan, E., Pettaway, C. A., Evans, R., Steiner, M. S., Wood, C. G., Troncoso, P., McDonnell, T. J., Fenstenmacher, M. J., and Pisters, L. L. AD-p53 intraprostatic gene therapy preceding radical prostatectomy (RP): an *in vivo* model for targeted therapy development. J. Urol., *161:* 297, 1999.
2. Rots, M. G., and Curiel, D. T. Towards the optimal vector for prostate cancer gene therapy: a CaPCURE meeting report. Cancer Gene Ther., *7:* 1507–1510, 2000.
3. Hassan, W., Sanford, M. A., Woo, S. L., Chen, S. H., and Hall, S. J. Prospects for herpes-simplex-virus thymidine-kinase and cytokine gene transduction as immunomodulatory gene therapy for prostate cancer. World J. Urol., *18:* 130–135, 2000.
4. Herman, J. R., Adler, H. L., Aguilar-Cordova, E., Rojas-Martinez, A., Woo, S., Timme, T. L., Wheeler, T. M., Thompson, T. C., and Scardino, P. T. *In situ* gene therapy for adenocarcinoma of the prostate: a Phase I clinical trial. Hum. Gene Ther., *10:* 1239–1249, 1999.
5. Shalev, M., Kadmon, D., Teh, B. S., Butler, E. B., Aguilar-Cordova, E., Thompson, T. C., Herman, J. R., Adler, H. L., Scardino, P. T., and Miles, B. J. Suicide gene therapy toxicity after multiple and repeat injections in patients with localized prostate cancer. J. Urol., *163:* 1747–1750, 2000.
6. Koeneman, K. S., Kao, C., Ko, S. C., Yang, L., Wada, Y., Kallmes, D. F., Gillenwater, J. Y., Zhau, H. E., Chung, L. W., and Gardner, T. A. Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. World J. Urol., *18:* 102–110, 2000.
7. Yu, D. C., Sakamoto, G. T., and Henderson, D. R. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. Cancer Res., *59:* 1498–1504, 1999.
8. Yu, D. C., Chen, Y., Seng, M., Dilley, J., and Henderson, D. R. The addition of adenovirus type 5 region E3 enables calydon virus 787 to eliminate distant prostate tumor xenografts [published erratum appears in Cancer Res., *60:* 1150, 2000]. Cancer Res., *59:* 4200–4203, 1999.
9. Hemmi, S., Geertsen, R., Mezzacasa, A., Peter, I., and Dummer, R. The presence of human coxsackievirus and adenovirus receptor is associated with efficient adenovirus-mediated transgene expression in human melanoma cell cultures. Hum. Gene Ther., *9:* 2363–2373, 1998.
10. Li, Y., Pong, R. C., Bergelson, J. M., Hall, M. C., Sagalowsky, A. I., Tseng, C. P., Wang, Z., and Hsieh, J. T. Loss of adenoviral receptor expression in human bladder cancer cells: a potential impact on the efficacy of gene therapy. Cancer Res., *59:* 325–330, 1999.
11. Miller, C. R., Buchsbaum, D. J., Reynolds, P. N., Douglas, J. T., Gillespie, G. Y., Mayo, M. S., Raben, D., and Curiel, D. T. Differential susceptibility of primary and established human glioma cells to adenovirus infection: targeting via the epidermal growth factor receptor achieves fiber receptor-independent gene transfer. Cancer Res., *58:* 5738–5748, 1998.
12. Pearson, A. S., Koch, P. E., Atkinson, N., Xiong, M., Finberg, R. W., Roth, J. A., and Fang, B. Factors limiting adenovirus-mediated gene transfer into human lung and pancreatic cancer cell lines. Clin. Cancer Res., *5:* 4208–4213, 1999.
13. Li, D., Duan, L., Freimuth, P., and O'Malley, B. W., Jr. Variability of adenovirus receptor density influences gene transfer efficiency and therapeutic response in head and neck cancer. Clin. Cancer Res., *5:* 4175–4181, 1999.
14. Okegawa, T., Li, Y., Pong, R. C., Bergelson, J. M., Zhou, J., and Hsieh, J. T. The dual impact of coxsackie and adenovirus receptor expression on human prostate cancer gene therapy. Cancer Res., *60:* 5031–5036, 2000.
15. Gleason, D. F. Classification of prostatic carcinomas. Cancer Chemother. Rep., *50:* 125–128, 1966.
16. Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., Horwitz, M. S., Crowell, R. L., and Finberg, R. W. Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. Science (Wash. DC), *275:* 1320–1323, 1997.
17. Pickles, R. J., Fahrner, J. A., Petrella, J. M., Boucher, R. C., and Bergelson, J. M. Retargeting the coxsackievirus and adenovirus receptor to the apical surface of polarized epithelial cells reveals the glycocalyx as a barrier to adenovirus-mediated gene transfer. J. Virol., *74:* 6050–6057, 2000.
18. Wickham, T. J., Mathias, P., Cheresh, D. A., and Nemerow, G. R. Integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ promote adenovirus internalization but not virus attachment. Cell, *73:* 309–319, 1993.
19. Zabner, J., Freimuth, P., Puga, A., Fabrega, A., and Welsh, M. J. Lack of high affinity fiber receptor activity explains the resistance of ciliated airway epithelia to adenovirus infection. J. Clin. Investig., *100:* 1144–1149, 1997.
20. Pickles, R. J., McCarty, D., Matsui, H., Hart, P. J., Randell, S. H., and Boucher, R. C. Limited entry of adenovirus vectors into well-differentiated airway epithelium is responsible for inefficient gene transfer. J. Virol., *72:* 6014–6023, 1998.
21. Walters, R. W., Grunst, T., Bergelson, J. M., Finberg, R. W., Welsh, M. J., and Zabner, J. Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia. J. Biol. Chem., *274:* 10219–10226, 1999.
22. Cohen, C. J., Gaetz, J., Ohman, T., and Bergelson, J. M. Multiple regions within the coxsackievirus and adenovirus receptor cytoplasmic domain are required for basolateral sorting. J. Biol. Chem., *276:* 25392–25398, 2001.
23. Umbas, R., Schalken, J. A., Aalders, T. W., Carter, B. S., Karthaus, H. F., Schaafsma, H. E., Debruyne, F. M., and Isaacs, W. B. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. Cancer Res., *52:* 5104–5109, 1992.
24. Umbas, R., Isaacs, W. B., Bringuier, P. P., Schaafsma, H. E., Karthaus, H. F., Oosterhof, G. O., Debruyne, F. M., and Schalken, J. A. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. Cancer Res., *54:* 3929–3933, 1994.
25. Cheng, L., Nagabhushan, M., Pretlow, T. P., Amini, S. B., and Pretlow, T. G. Expression of E-cadherin in primary and metastatic prostate cancer. Am. J. Pathol., *148:* 1375–1380, 1996.
26. Kuczyk, M., Serth, J., Machtens, S., Bokemeyer, C., Bathke, W., Stief, C., and Jonas, U. Expression of E-cadherin in primary prostate cancer: correlation with clinical features. Br. J. Urol., *81:* 406–412, 1998.

27. De Marzo, A. M., Knudsen, B., Chan-Tack, K., and Epstein, J. I. E-cadherin expression as a marker of tumor aggressiveness in routinely processed radical prostatectomy specimens. Urology, *53:* 707–713, 1999.
28. Rubin, M. A., Mucci, N. R., Figurski, J., Fecko, A., Pienta, K. J., and Day, M. L. E-cadherin expression in prostate cancer: a broad survey using high-density tissue microarray technology. Hum. Pathol., *32:* 690–697, 2001.
29. Walters, R. W., van't Hof, W., Yi, S. M., Schroth, M. K., Zabner, J., Crystal, R. G., and Welsh, M. J. Apical localization of the coxsackie-adenovirus receptor by glycosyl-phosphatidylinositol modification is sufficient for adenovirus-mediated gene transfer through the apical surface of human airway epithelia. J. Virol., *75:* 7703–7711, 2001.
30. Tomko, R. P., Xu, R., and Philipson, L. HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. Proc. Natl. Acad. Sci. USA, *94:* 3352–3356, 1997.
31. Fechner, H., Haack, A., Wang, H., Wang, X., Eizema, K., Pauschinger, M., Schoemaker, R., Veghel, R., Houtsmuller, A., Schultheiss, H. P., Lamers, J., and Poller, W. Expression of coxsackie adenovirus receptor and αv-integrin does not correlate with adenovector targeting *in vivo* indicating anatomical vector barriers. Gene Ther., *6:* 1520–1535, 1999.
32. Thoelen, I., Magnusson, C., Tagerud, S., Polacek, C., Lindberg, M., and Van Ranst, M. Identification of alternative splice products encoded by the human coxsackie-adenovirus receptor gene. Biochem. Biophys. Res. Commun., *287:* 216–222, 2001.

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