Expression of Pax-2 in Human Renal Cell Carcinoma and Growth Inhibition by Antisense Oligonucleotides

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ABSTRACT

Renal cell carcinoma (RCC) is the most common malignancy in the adult kidney. Because RCC is generally thought to arise from the epithelium of the proximal kidney tubules (1-3). Recently, the gene for VHL disease, a hereditary cancer syndrome predisposing affected patients to RCC, as well as other generally thought to arise from the epithelium of the proximal kidney tubules, the expression of Pax-2, a gene required for renal epithelium development, was examined in primary tumors and tumor-derived cell lines. Immunostaining of frozen sections from the primary tumors indicated Pax-2 expression in the malignant cells but not in the surrounding stroma. In a panel of human RCC-derived cell lines, 73% expressed Pax-2 protein and mRNA. Treatment of RCC cell lines with antisense oligodeoxynucleotides resulted in down-regulation of Pax-2 protein expression and growth inhibition after 3 days in culture. These data indicate that Pax-2 gene function is required for proliferation, as well as differentiation during embryonic development, and suggest a novel therapy for RCC.

INTRODUCTION

RCC occurs both sporadically and with a genetic predisposition. Based on ultrastructural and immunocytochemical analyses, RCC is generally thought to originate from the epithelium of the proximal kidney tubules (1-3). Recently, the gene for VHL disease, a hereditary cancer syndrome predisposing affected patients to RCC, as well as other tumors, was cloned (4), and VHL mutations were identified in nearly 60% of the sporadic, nonpapillary RCC tumors and derived cell lines examined (5, 6). In addition, the normally constitutive transcription of the VHL gene was silenced by hypermethylation in another 20% of RCC tumors (7). Thus, VHL plays an important role in the origins of RCC, although the nature of the VHL protein and its mechanism of action remains unclear.

Many other developmental control genes, the primary function of which is to regulate the rapid proliferation and differentiation of embryonic tissues, may actively participate in oncogenic transformation. During the organogenesis of the kidney, the epithelium of the glomerulus, proximal tubules, and distal convoluted tubules are believed to originate from the metanephric mesenchyme, a cell mass that proliferates and differentiates in response to inductive signals from the ureteric bud (8). One of the earliest genes activated after induction of the metanephric kidney is Pax-2, a member of a family of developmental transcription factors implicated in a variety of morphogenetic processes (9, 10). Pax-2 is expressed in the induced mesenchyme and the early epithelial derivatives thereof and is rapidly down-regulated as the tubular epithelium matures (11). Failure to repress Pax-2 in the proximal tubule epithelium, the expression of Pax-2 was examined in a panel of RCC cell lines and in primary tumors. A high proportion of RCC cell lines (73%) expressed Pax-2, as did the corresponding primary tumors tested. In the cell lines, Pax-2 protein synthesis could be inhibited by the addition of antisense ODNs directed against Pax-2 mRNA, resulting in significant growth inhibition of those cells. The data suggest a role for Pax-2 in the proliferation of tumor cells and to the reactivation of this developmentally important gene as a determinant for oncogenesis.

MATERIALS AND METHODS

Cell Lines and Tumor Samples. Established RCC cell lines were derived from primary or metastatic tumor isolates and characterized previously as described (17). Cells were cultured in DMEM with 10% FCS and antibiotics. Solid tumor tissues were frozen immediately after surgery and stored at -70°C until sectioning.

Western Blotting. The production and specificity of Pax-2 polyclonal antibodies were described previously (11). Soluble protein extracts from subconfluent cell line cultures were prepared using a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.2% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 μg/ml aprotinin, 1 mM sodium vanadate, and 25 mM sodium fluoride. Samples were electrophoresed in 8% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Proteins were stained with anti-Pax-2 IgG, 1 μg/ml, and visualized with an alkaline phosphatase-conjugated secondary antibody (Bio-Rad), according to the manufacturer’s protocol.

RNA Analysis. The S1 nuclease mapping procedure was essentially as described (17). The DNA probe was isolated from a plasmid containing a 600-bp BamHI/HindIII fragment of the Pax-2 cDNA by digesting with BstEII, dephosphorylating with calf intestinal phosphatase, and subsequently cutting the DNA with SnaI. The fragments were purified on low-melting-point agarose gels and end labeled with [γ-32P]ATP and polynucleotide kinase at the 5′ BstEII end only. The 490-bp probe spans nucleotides 887-1339 and generates a protected fragment of 327 bp for Pax-2b and 452 bp for Pax-2a. Approximately 10,000 cpm of probe was ethanol precipitated with 20 μg of total RNA from E18 kidneys, RCC cell line total RNA, or 20 μg of total yeast, control RNA. The hybridization conditions and S1 nuclease digestion was as described (18) with minor modifications. RNA and probes were resuspended in 25 μl of 80% formamide, 400 mM NaCl, 40 mM 1,4-piperazinediethanesulfonic acid (pH 6.5), and 2 mM EDTA. Samples were heated to 75°C for 15 min and then hybridized overnight at 55°C. Hybridization temperatures were titrated over a range (45–60°C) to maximize RNA/DNA hybrids relative to probe reannealing. Samples were placed on ice and digested with 150 units of S1 nuclease (Boehringer Mannheim) in 0.5 ml of 33 mM sodium acetate (pH 5.0), 200 mM NaCl, 30 mM ZnSO4, and 20 μg/ml denatured salmon sperm DNA. Digestions were done at 37°C for 60 min, followed by the addition of 0.1 ml stop buffer [0.5 M TRIS (pH 9.5)-0.1 M EDTA], extraction with phenol/chloroform, and...
ethanol precipitation. Samples were run on 6% denaturing acrylamide sequencing gels.

**Immunostaining.** Cryostat sections were cut at 8 μm, collected on gelatinized slides, and air dried for 30–60 min. RCC cell lines were grown on Lab-Tek chamber slides (Nunc, Inc.). Immunostaining was as described by Harlow and Lane (19). Frozen sections or cultured cells were fixed in 3% paraformaldehyde in PBS for 5 min, then washed in PBS, permeabilized in 0.05% Triton X-100 in PBS for 10 min, and rinsed again in PBS. A 10-μg/ml dilution of Protein A-purified anti-Pax2 IgG (II) was prepared in 2% goat serum in PBS, and 20 μl was applied to each sample. Slides were incubated at room temperature in a humid chamber and then washed twice in PBS containing 0.05% Tween 20. The TRITC-conjugated anti-rabbit second antibodies (Sigma Chemical Co.) were diluted 1:32 in 2% goat serum in PBS; the FITC-conjugated anti-rabbit second antibodies (Calbiochem) were diluted 1:250 in the same buffer. After a 30-min incubation, slides were washed twice in PBS containing 0.05% Tween 20 and covered with gelvatol. Photomicrographs were taken with a Zeiss Axiophot fluorescence microscope. Control sections were incubated with a preimmune rabbit IgG purified fraction and second antibodies.

**Antisense Inhibition Experiments.** For the initial dose response, approximately 5000 cells/well were seeded in 24-well plates, and increasing amounts of sense strand or antisense strand ODNs, resuspended in PBS, were added. The sequences of the phosphorothioate-substituted ODNs (Oligos, etc.) are as follows: AS18, 5'-ATGGGGAGGCAGAGGAGCG; S18, 5'-CGCTCCTCTCGCTCCCCAT; AS17, 5'-GGGAGGCCGTGCTGGGAAC; and S17, 5'-GTGCCTCACGGCCGCTCCC. The ODN pairs 18 and 17 correspond to nucleotides 527–544 and nucleotides 1039–1056 of the human cDNA sequence (20), respectively. For Western blotting, cells were cultured for 4 days and lysed directly in the well with SDS gel loading buffer. Because of differences in growth between sense- and antisense-treated cells, the protein lysates were adjusted for the number of cells in the well. A duplicate gel was run and stained with Coomassie blue to control for total protein levels. Growth curves were repeated three times by seeding 2000 or 5000 cells/well at day 0 and adding 20 μM sense ODNs, antisense ODNs, or an equivalent amount of PBS to the wells. At 24-h intervals, cells were trypsinized and counted with a hemocytometer. Medium was replaced after 3 days with fresh ODNs.

The MTT (Sigma) colorimetric assay was also used to measure proliferation in ODN-treated RCC cell lines. Cells were plated at 500 cells/well in microtiter plates in the presence of varying concentrations of ODNs, and the medium was replaced after 3 days with fresh ODNs. After six days of ODN treatment, 50 μl of MTT (5 mg/ml in PBS) was added to each well, and the plates were incubated at 37°C for 4 h. The medium was then removed, and the plates were air dried overnight. The blue formazan crystal precipitate was solubilized in 150 μl of mineral oil (overnight, 37°C), and the plates were read at a test wavelength of 570 nm on a scanning multiwell spectrophotometer (Titertek Multiscan model MCC/340 MKII; Flow laboratories, Inc.).

**RESULTS**

The expression of Pax-2 was examined in a panel of RCC cell lines and in the tumors from which the cell lines were originally isolated.

![Figure 1](image-url)
Protein extracts from RCC cell lines were analyzed by Western blotting using Pax-2-specific polyclonal antibodies. The expression of Pax-2 protein was variable among individual samples, with 14 of 19 cell lines tested showing detectable amounts of protein (Fig. 1A). The relative levels of Pax-2 protein ranged from high-expressing lines (UOK132, 115, and 117), with levels nearly equivalent to whole embryonic kidneys, to moderately expressing lines (UOK108, 109, 111, 112, 130, 151, and 134) and low-expressing lines (UOK121-LN, 122, 113, and 126). These results were confirmed by S1 nuclease mapping, which detected Pax-2 mRNA in cell lines that showed protein expression but not in several cell lines that were negative for Pax-2 protein (Fig. 1B). A 490-bp end-labeled probe, spanning an alternative splice site, was used in an S1 nuclease protection assay to detect the two alternatively spliced Pax-2 mRNAs (11). A 327-bp protected fragment is indicative of Pax-2b expression and can be clearly distinguished in cell lines UOK132, 115, 111, 117, and 125. As observed previously in embryonic kidneys (11), the positive-expressing cell lines also exhibited lower levels of the alternatively spliced Pax-2a mRNA, as indicated by the 452-bp protected band. Nonspecific degradation of the probe resulted in a band at approximately 430 bp that can be seen in all samples, including a negative control with no RNA added. Lines UOK123 and KN41 expressed neither Pax-2 mRNA nor protein (Fig. 1). Taken together, the protein and RNA data demonstrate persistent expression of Pax-2 in 73% of the RCC-derived cell lines examined.

Several RCC cell lines were further characterized by immunostaining with Pax-2-specific antibodies. This rabbit polyclonal serum has been used previously in embryonic tissues from mouse and fish.
and was shown to be highly specific (11, 21). RCC cell lines UOK111 and UOK117 show bright nuclear staining, whereas cell lines KN41 and UOK121 had no detectable staining with Pax-2 antibodies (Fig. 2). These data are entirely consistent with the Western blots and RNA analysis. During the growth and establishment of the RCC cell lines, Pax-2-expressing cells may have had a selective advantage and may only represent a fraction of the transformed cells in the primary neoplasm. Alternatively, Pax-2 expression could be the result of genetic changes occurring after the establishment of the cell line. Thus, frozen tumor sections from the original source of RCC lines UOK117 and UOK111 were stained with anti-Pax-2 and control antibodies (Fig. 3). Both tumors exhibited focal areas of Pax-2-expressing cells, as clearly evident by nuclear staining, and multiple necrotic areas with few remaining cells. Therefore, the expression of Pax-2 in the RCC-derived cell lines is not the result of positive selection nor due to additional genetic lesions occurring during the establishment of the cultures.

To determine if the persistent expression of Pax-2 contributed to the ability of the RCC cell lines to proliferate in culture, Pax-2 protein expression was down-regulated in high-expressing RCC lines using antisense ODNs directed against two disparate regions of the mRNA. Phosphorothioate substituted 19-mers were designed to span either the translation start site (AS18) or a splice site in the coding region of the mRNA (AS17). Similar ODNs inhibited Pax-2 expression in murine kidney organ cultures (13). Specific repression of Pax-2 protein levels could be detected by Western blot analysis over a range of antisense ODN concentrations from 5–20 µM (Fig. 4). The amount of Pax-2 protein detected decreased significantly as the concentration of AS18 increased. The effect of Pax-2 suppression on cell growth was examined in the RCC lines UOK111 and UOK117, as well as the Pax-2 negative cell line KN41 and COS-7. Typical growth curves for using ODN AS18 are shown in Fig. 5. These experiments were done in triplicate, and the average number of cells was plotted with time. Over the first 3 days of culture, antisense Pax-2 ODNs had no significant growth-inhibitory effects. However, by day 4, the antisense-treated
UOK 111 and UOK117 cells plateaued, and cell numbers actually decreased over the next 2 days. Note that cell lines not expressing Pax-2 showed no growth inhibition when cultured with antisense ODNs. Inhibition of growth by ODN AS17 was not as striking compared to AS18 (data not shown). Using UOK11 cells, total cell numbers after 6 days of culture with 20 μM AS17 were reduced to 22–47% of S17 and control media-treated cells. With UOK117 cells, cell numbers with AS17 were reduced to 15–25% of controls. These numbers reflect the range of growth inhibition of at least three independent experiments.

In independently run experiments, the effect of antisense ODN treatment on RCC cell line proliferation was examined by colorimetric, cell proliferation assay. Cells cultured in microtiter plates were treated with increasing amounts of antisense and control ODNs. After 6 days of treatment, the number of viable cells was determined by their ability to metabolize the indicator dye MTT. Absorbance was
measured and plotted against ODN concentration for two Pax-2-positive cell lines and one negative control line (Fig. 6). The absorbance levels of antisense-treated UOK117 cells were 5-fold less than sense controls at the highest concentration (30 μM). Note that AS18 was more inhibitory at lower concentrations of 3 and 10 μM. Using UOK117 cells, absorbance levels were reduced 3–4-fold with antisense ODNs. Again, AS18 was more inhibitory at lower concentrations. The kidney-derived cell line COS-7 did not show reduced absorbance when cultured with AS18 or AS17 over the range of concentrations used.

**DISCUSSION**

As the tissue of origin is thought to be the renal proximal tubule cells, the reactivation of developmental genes that regulate growth and differentiation of the renal epithelium may be a determining factor for the initiation and/or progression of RCC. The data presented in this report indicate a direct requirement for continued Pax-2 expression for the proliferation of at least some RCC cell lines in culture. Of the independently derived RCC cell lines examined, more than 70% tested positive for Pax-2 protein and mRNA. These cell lines have been described previously and differ in their growth rates in culture and in their ability to form solid tumors in nude mice (17). The individual cell lines also exhibit morphological heterogeneity. The levels of Pax-2 expression varied highly among positive cell lines, with no clear correlation to the growth rate or tumorigenicity of the different RCC cell lines. Nevertheless, the ability to inhibit cellular proliferation by reducing Pax-2 protein levels suggests that tumor growth can be dependent on Pax-2 reactivation, at least in some cases.

The Pax genes are directly implicated in a variety of morphogenic processes, including vertebral column segmentation, eye development, and neural crest migration (9, 10). At least one Pax gene, Pax-3, is the causative agent in some human rhabdomyosarcomas due to a chromosomal translocation that fuses the Paired homeodomain to a fork-head DNA-binding domain (22, 23) and generates a highly active transcription activator (24). However, such a mutational activation event for Pax-2 was not evident in the RCC examined. Southern blotting analysis of more than 100 RCC cell lines did not indicate any Pax-2 gene rearrangements.³ Pax genes are also able to promote contact-independent growth of NIH3T3 cells and tumorigenesis in nude mice (25).

The Pax-2 gene is activated immediately after kidney mesenchyme induction (11, 26) and is required for the conversion of the mesenchyme to renal epithelium (13). Concomitant with epithelial conversion is rapid proliferation of the induced mesenchyme cells and their early derivatives. During the course of normal development, Pax-2 is subject to negative regulation in the mesenchyme-derived epithelium that is mediated in part by the Wilms' tumor suppressor gene WT1 (27). Pax-2 expression is evident in embryonal-derived tumors, such as Wilms' tumor (11, 18), presumably because cellular transformation occurred prior to Pax-2 suppression. In affected individuals with only one functional copy of Pax-2, the kidneys are generally hypoplastic. Persistent Pax-2 expression alone results in renal abnormalities (12) but does not generate tumors in the kidneys of transgenic mice; and these kidney cells fail to form tumors when transplanted s.c. into nude mice.⁴ In addition, activation of Pax-2 is observed in regenerating proximal tubule epithelium after injury (29). These results indicate that Pax-2 expression correlates with proliferation and renal epithelial differentiation but alone is insufficient for transformation in vivo.

The successful use of antisense ODNs to inhibit gene expression has been reported in many in vitro systems (30). Because of the phosphorothioate substituted diester backbone, these ODNs have increased stability in tissue culture medium and a decreased melting temperature of the ODN/RNA hybrid (31). This decreased hybrid stability may reduce the propensity for nonspecific effects due to the formation of imperfect hybrids. Controls using the sense strand demonstrated that the substituted ODNs are not generally toxic. More significantly, cells not expressing Pax-2, including COS-7 and the RCC cell line KN41, were not sensitive to growth inhibition by antisense ODNs. These data demonstrate a specific effect due to inhibition of Pax-2 protein synthesis. However, cells appeared to undergo several divisions before the inhibitory effects of Pax-2 depletion was noticeable. This delay may reflect the rate of ODN uptake and/or the rate of turnover of the Pax-2 protein. Since Pax-2 is thought to potentiate transcription (32), the turnover of its target gene products must also be considered before any effects of depletion can be observed in vitro. The ability to modulate RCC growth in vitro by inhibition of Pax-2 protein synthesis indicates a novel role for Pax-2 in the development, and perhaps progression, of renal cell carcinoma. Although potential therapeutic applications of antisense technology are significant, oligonucleotide uptake mechanisms, retention and stability, and specificity of action must be considered (33) before modulation of gene expression in whole animals can be achieved.

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