Differential Expression of the Enzyme That Esterifies Retinol, Lecithin:Retinol Acyltransferase, in Subtypes of Human Renal Cancer and Normal Kidney

Hui Chun Zhan, Lorraine J. Gudas, Dean Bok, Robert Rando, David M. Nanus, and Satish K. Tickoo

ABSTRACT

Purpose: Retinoids, a group of compounds, including vitamin A (retinol), and related metabolites, have been shown to regulate the growth and differentiation of many types of cells. IFN-α and either 13-cis-retinoic acid or liposomal all-trans retinoid acid have been shown to regulate the growth of patients with metastatic renal cell carcinoma. We knew that samples from renal cell carcinomas contained greatly reduced levels of retinol and retinyl esters relative to samples from normal human kidney. This prompted us to examine the levels of LRAT (lecithin:retinol acyltransferase) protein in various subtypes of human kidney cancers relative to normal human kidney by immunohistochemistry.

Experimental Design: We examined 31 partial or radical nephrectomy specimens diagnosed with kidney tumors between 1997 and 1998. Representative paraffin-embedded tissue blocks from each tumor, with each containing adjacent nonneoplastic renal parenchyma, were used for immunochemical analysis with affinity purified antibodies to human LRAT protein.

Results: LRAT protein was detected at high levels in the epithelial cells in the tubules and the lining of Bowman’s capsule in the glomeruli of normal, nonneoplastic kidney sections. Among the 31 tumors, there were 13 cases of conventional (clear cell) renal cell carcinoma (RCC; including 2 multilocular cystic RCCs), 7 papillary RCC, 6 chromophobe RCC, 1 RCC, unclassified, and 4 renal oncocytoma. All tumors showed diffuse immunoreactivity for LRAT. In each case, the staining was uniform throughout the tumor, with only minimal variation in the staining intensity between different areas. All 4 renal oncocytomas, 2 of 6 chromophobe RCCs, 1 conventional (clear cell) carcinoma, 1 RCC, unclassified, and 2 conventional RCCs, which were of the multilocular cystic-type stained strongly (3+) for LRAT. In contrast, the remaining conventional RCCs and the papillary RCC samples stained much less intensely for LRAT. Of the 10 tumors that stained 3+ for LRAT in the study, 9 were either benign tumors or tumors with low malignant potential.

Conclusions: These data show that LRAT expression is higher in renal tumors with an indolent biological behavior. Additional studies will ascertain if LRAT possesses any prognostic or therapeutic role in renal cancer.

INTRODUCTION

There are ~30,000 new cases of RCC diagnosed every year in the United States, with >10,000 deaths/year (1). The incidence of RCC has increased steadily over the past 20 years, by ~2–4%/year (1). There are few effective chemotherapeutic or biological treatment modalities for RCC. Once metastatic disease develops, the prognosis for long-term survival is poor. Approximately one-third of patients have metastatic disease at the time of diagnosis, and ~50% of the patients undergoing surgical resections for less advanced disease eventually relapse (2). Thus, the identification of new drugs for the treatment of RCC is a high priority. Two biological therapies, IFN-α and interleukin 2, have been used extensively for the treatment of advanced RCC. Both therapies produce responses in ~15–20% of patients (3).

Recent studies have revealed the potential of retinoids in combination with other agents in RCC therapy. Retinoids are prototypic differentiation agents that function as modulators of cell growth, differentiation, and apoptosis (4–9). Treatment of patients, experimental animal studies, and epidemiological analyses suggest that physiological levels of retinoids protect the organism against the development of premalignant and malignant lesions (10–14). In a Phase II trial of patients with metastatic RCC, the addition of CRA to IFN-α resulted in major responses in 30% of patients and related experiments with IFN-sensitive tumor cell lines suggested that CRA could augment the antiproliferative effect of IFN-α (15–17). A subsequent randomized Phase III trial confirmed that CRA could enhance...
the therapeutic response to IFN-α, leading to a higher response rate, a significantly longer duration of response, and greater progression-free survival rate (18). How retinoids and IFN-α interact to increase the antitumor effects in RCC has not been determined.

It is known that the local production of retinyl esters from retinol is an important mechanism for maintaining tissue retinoids in a form that is easily stored and readily mobilized for additional metabolism (19). LRAT is an enzyme that catalyzes retinol (vitamin A) esterification and plays an essential role in vitamin A metabolism. A greatly reduced intracellular metabolism of retinol to retinyl esters, which was correlated with a large reduction in LRAT protein level, was reported in renal cancers relative to normal human kidney (20). The low levels of retinol and retinyl esters, as well as the reduced LRAT level, may play a role in the aberrant differentiation of these neoplastic

Fig. 1 LRAT expression in formalin-fixed, paraffin-embedded sections of normal human kidney. A, normal human kidney section showing LRAT staining in the tubules (D-distal tubules, P-proximal tubules). In the glomeruli (G), positive staining is observed in the epithelial lining of Bowman's space. B, negative control nonneoplastic kidney; incubation with normal goat serum replaced the treatment with the primary LRAT antibody, otherwise negative controls were treated in identical manner as the test slides. Insets, higher magnification.
cells (20–23). We have now analyzed LRAT protein expression in tissue sections from various types of renal cell neoplasms.

MATERIALS AND METHODS

Tissue Specimens. Thirty-one partial or radical nephrectomy specimens diagnosed with kidney tumors in the Department of Pathology at New York Hospital in 1997 and 1998 were included in this study. Although 28 of these were consecutive renal epithelial tumors accessioned in the department between January 1997 and March 1998, 3 additional cases (1 each of chromophobe RCC, multiloculated cystic RCC, and renal oncocytoma) from later in 1998 were included to increase the sample size of the relatively less common tumors. H&E-stained slides were reviewed, and the tumors were reclassified, if necessary, according to the consensus Heidelberg criteria (24). A representative formalin-fixed, paraffin-embedded tissue block from each tumor, with each containing adjacent nonneoplastic renal parenchyma, was selected for immunohistochemical analysis (Table 1).

Immunohistochemistry. Tissue localization of LRAT protein was done by using an affinity-purified, polyclonal rabbit antihuman LRAT antibody (19). Five-μm tissue sections were cut from the blocks, and the sections were deparaffinized in Histo-Clear (National Diagnostics, Atlanta, GA) followed by rehydration in a graded series of ethanol. Antigen retrieval was performed by heat with Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) in a pressure cooker for 2 min. We had previously determined that these treatments resulted in antigen retrieval (23). A 3% solution of H2O2 was used to quench the endogenous peroxidase activity (15-min incubation). Slides were then incubated in primary rabbit anti-LRAT antibody diluted 1:250 in 1.5% goat serum for 1 h at room temperature and then treated with horseradish peroxidase polymer conjugate (Picture plus kit; Zymec) for 30 min. Color was developed with 3,3'-diaminobenzidine chromogen/substrate, followed by counterstaining with hematoxylin (Vector Laboratories). The negative control tumor sections were treated identically to all other sections, with the exception that 1.5% normal goat serum was used in place of the primary antibody. All samples were analyzed using this exact protocol and the same Picture plus kit reagents.

Grading of LRAT Immunoreactivity. The immunohistochemical expression of LRAT protein was scored in a semiquantitative fashion by a single pathologist (S. K. T.). Because each tumor showed diffuse immunoreactivity for LRAT, the number of positive-staining cells could not be used for grading the staining. Instead, we used the intensity of specific staining in each tumor compared with the staining intensity in the paired nonneoplastic renal tubules, and 3+ (staining equal to or more intense than that in nonneoplastic tubules). The immunohistochemistry procedure and sample evaluation were performed independently and blinded.

RESULTS

LRAT Protein Staining in Normal Human Kidney. The expression of LRAT protein in normal human kidney has not been examined previously. We found that the normal, non-

Fig. 2 LRAT protein expression in different subtypes of human kidney tumors. Samples of conventional (clear cell) RCC (13), papillary RCC (7), chromophobe RCC (6), RCC unclassified, of possible loop of Henle origin (1), and renal oncocytoma (4) were assayed. The grade of LRAT immunostaining is shown as 1+ (low), 2+ (moderate), 3+ (high). Two of the conventional RCC that stained 3+ were multilocular cystic RCC.

Table 1 Demographics of patients whose partial or radical nephrectomy specimens were included in the study

<table>
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<th>Age (yr)</th>
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<table>
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<tr>
<th>Pathology</th>
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<tr>
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<tr>
<td>RCC, papillary</td>
<td>7</td>
</tr>
<tr>
<td>RCC, chromophobe</td>
<td>6</td>
</tr>
<tr>
<td>Renal oncocytoma</td>
<td>4</td>
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<tr>
<td>RCC, unclassified (of possible loop of Henle origin)</td>
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* Of these, two tumors were multilocular cystic RCC.

Clinical Cancer Research 4899
Fig. 3 Immunohistochemistry of LRAT expression in various subtypes of human kidney tumors. Samples were incubated with the LRAT antibody, followed by horseradish peroxidase polymer conjugate. Hematoxylin was used as the counterstain. All samples included both tumor tissue (T) and nonneoplastic kidney tissue (N) for comparison. 3+ immunostaining with LRAT antibody in renal oncocytoma (A), RCC, unclassified “of possible loop of Henle origin” (B), and multilocular cystic RCC (C). Insets, higher magnification.
neoplastic human kidney exhibited diffuse cytoplasmic and membranous staining in the epithelial cells of the renal tubules. This LRAT staining was generally somewhat stronger in the distal and collecting tubules than in the proximal tubules (Fig. 1A). The epithelial lining of the glomerular Bowman’s space also stained strongly for LRAT (Fig. 1A). Other structures in the normal kidney, including the glomerular capillaries and mesangium, interstitium, and vessel walls, did not stain, although occasional endothelial cells lining the vessels did show focal and weak positivity. Because of the strong LRAT expression in the tubules of the normal kidney, we used this staining for the grading of the immunostaining in the tumors and as a positive internal control on each slide. None of the negative controls showed any staining either in the tumor or nontumorous adjacent renal parenchyma (Fig. 1B).

Subtypes of Renal Cell Neoplasms. Among the 31 tumors, there were 13 cases of conventional (clear cell) RCC, 7 of papillary RCC, 6 chromophobe RCCs, 1 RCC unclassified, and 4 renal oncocytomas. Two of the conventional RCCs were predominantly cystic (>95%) and qualified as multilocular cystic RCC. The 1 case of RCC, unclassified, showed features of the so-called “RCC of possible loop of Henle origin,” a recently described biphasic renal tumor (25–28). The tumors ranged in size from 1.2 to 18 cm (median, 3.8; mean, 4.9). Among the cases of carcinoma, 15 presented with pathological tumor stage 1 (pT1) disease, 7 with pT stage 2, 2 with 3b, and 1 with a pT4 disease.

LRAT Expression in Human Kidney Tumor Tissues. The case distribution in the LRAT immunostaining profiles of different tumor groups are shown in Fig. 2. All tumors showed diffuse immunoreactivity for LRAT. In each case, the staining was almost uniform throughout the tumor, with only minimal variation in the staining intensity among different areas. All 4 renal oncocytomas (e.g., Fig. 3A), 2 of 6 chromophobe RCCs, 3 of 13 conventional (clear cell) carcinomas, and 1 RCC, unclassified (Fig. 3B), showed strong and diffuse (3+) immunoreactivity. Two of the 3 conventional RCCs with 3+ positivity were the cases of multilocular cystic RCC (Fig. 3C). Four of the chromophobe RCCs stained a little less intensely than the surrounding nonneoplastic tubules (2+; Fig. 4), whereas none showed weak 1+ immunostaining. Among all of the cases of RCC, the grade of immunoreactivity was not related to tumor size, pathological tumor stage, or age of the patient at presentation, although the number of cases in each group was too small for any valid statistical analysis.

Tumors that are considered to be relatively more aggressive, i.e., conventional RCC (Fig. 5A) and papillary RCC (Fig. 5B) exhibited a lower degree of staining with LRAT antibodies. Thus, 5 tumors each of 11 conventional RCCs (excluding the 2 multilocular cystic RCC) showed only either 1+ or 2+ staining, whereas 5 of 7 papillary RCCs exhibited 2+ positivity and 2 were 1+ positive.

DISCUSSION

Retinyl esters are the predominant metabolites of retinol in most epithelial cells and tissues, and the esterification of retinol is believed to regulate important processes such as retinol uptake, storage, and function. Two enzymes have been reported to catalyze retinyl ester synthesis, acyl-CoA:retinol acyltransferase and LRAT. In human keratinocytes, LRAT activity could be induced by retinoic acid with a concomitant 50% reduction in retinol oxidation to retinoic acid (29). The authors speculated that this regulation worked as a sensor for retinoid status and
prevented retinol from being oxidized, allowing it to be sequestered in cells in the retinyl ester form. These retinyl esters could then serve as an internal source of retinol for the synthesis of retinoic acid during the differentiation process. Retinoic acid is a ligand for the nuclear retinoic acid receptors, which bind to retinoic acid response elements in target genes, thereby regulating gene transcription (30–33). If LRAT is required for the uptake and storage of retinol, it is likely that reduced expression of LRAT would result in reduced levels of one of the agonists for the retinoic acid receptors, all-trans-retinoic acid.

We and others (20–23, 34, 35) have shown that several types of human carcinomas, including breast, head and neck, skin, and prostate, exhibit a reduced ability to accumulate retinyl esters and major alterations in LRAT transcripts. We recently used immunohistochemistry to demonstrate that LRAT protein was observed in the basal cells of normal human prostate tissue but was not expressed in prostate carcinoma cells in formalin-fixed, paraffin-embedded tissues (23).

Evaluation of prognostic factors is an essential step in the assessment of RCC patients. Tumor-Node-Metastasis staging has been the primary source of prognostic information. An ideal prognostic marker, i.e., one that is specific, reliable, quantitative, easy to use, rapid, and inexpensive, for renal carcinomas has not yet been identified (36). On the basis of our prior research in which we showed that human kidney cancer samples contained extremely low levels of retinol and retinyl esters as compared with normal human kidney samples (20), we carried out additional studies to determine whether different subtypes of human RCC expressed different levels of LRAT protein.

In the results reported here, conventional (clear cell) RCC was the most common tumor (47%) in this cohort of 28 consecutive cases. Of all of the renal epithelial neoplasms in adults, conventional (clear cell) RCC is the most common form, constituting up to two-thirds of all of the cases. Clear cell RCC is also one of the most aggressive subtypes of RCC, with a metastatic rate of 27.4% at presentation (37). Compared with this, papillary RCC [the second most common RCC in this study and in the study by Amin et al. (37)] is a relatively less aggressive tumor that more often presents at a lower stage. Stage for stage, papillary RCC has better 5-year survival rates than the conventional RCC (37, 38). Chromophobe RCC has the best outcome among all RCCs, with up to 100% 5-year survivals and 90% 10-year survivals (37). Renal oncocytoma is a benign tumor, despite the occasional presence of apparently invasive features (39). Since 1991 there have been ~450 cases of histologically well-characterized renal oncocytomas, and no deaths have been directly ascribed to it (37).

With respect to LRAT expression, all of the benign tumors (4 of 4 renal oncocytomas) in this study show 3+ immunoreactivity for LRAT. Among the malignant tumors, 3+ positivity was observed in 2 of 6 chromophobe RCCs, 1 of 1 RCC of possible loop of Henle origin, 2 of 2 multilocular cystic RCC, and 1 of the other 11 conventional RCCs. Although multilocular cystic RCC is known to be a tumor of low malignant potential (40–43), the recently described RCC of possible loop of Henle origin is also a low-grade neoplasm (25–28). Only 1 of the >20 reported cases of this tumor in the literature had local lymph node metastasis. Thus, of the 10 tumors that stained 3+ for LRAT in this study, 9 were either benign or tumors with low malignant potential. From these initial observations, we suggest that high LRAT expression in renal tumors is a marker of a more benign phenotype or low malignant potential. In this regard, it is
interesting to note that using high-performance liquid chromatography analysis, we have also previously observed that the only tumor with relatively high retinyl palmitate levels, among 7 renal carcinoma specimens tested, was a chromophobe RCC (20). However, in the data reported here, high levels of LRAT protein were also seen in 1 conventional RCC. Reevaluation of the pathological features of this tumor showed it to be a classical RCC, with both clear cell and eosinophilic areas, with pathological stage 2. Thus, this specimen showed no significant differences from other conventional RCCs in this group.

The question of whether LRAT expression level has any prognostic value cannot be answered in a study of this size and will require evaluation of a much larger number of cases with a longer clinical follow up. However, Motzer et al. (15) reported
major clinical responses in up to 30% of patients with advanced RCC when they were treated with a combination of CRA and IFN-α. The presence of high LRAT expression by immunohistochemistry in 1 case of conventional RCC in our study and the clinical response to CRA in a proportion of cases (15, 18) raises an interesting possibility that the responders to the combination therapy may be the patients with tumors that express higher levels of LRAT. This hypothesis needs to be tested by the comparison of LRAT expression levels in patients who respond to the CRA plus IFN-α treatment versus those who do not respond.

In summary, in this preliminary immunohistochemical study, we have observed high LRAT expression primarily in renal tumors with indolent biological behavior. Whether high LRAT expression in rare tumors with otherwise expected aggressive behavior is related to their response to therapy with RA requires additional investigation and is currently being tested.

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