ABSTRACT

Nine surgically resected Wilms' tumors (WIT) and nude mouse heterotransplants from one WIT were studied by histochemistry and immunohistochemistry. Histochemistry showed acid phosphatase in all cells, while alkaline phosphatase and γ-glutamyl transpeptidase were present in only some tubules. Using immunohistochemistry, antibodies to the intermediate filaments cytokeratin and vimentin distinguished tubular epithelium and mesenchyme, respectively. WIT tubules were also identified using antibody against a structural component (epithelial membrane antigen) and a secretory product (uromucoid) associated with distal convoluted tubules of normal kidney. Basement membrane surrounding the tubules of WIT was demonstrated using antibody to body to type IV collagen plus laminin. Different blastema subpopulations were negative or stained positively with antibodies to cytokeratin and vimentin. Production of basement membrane by blastema was also shown. Fetal antigen expression in WIT was examined using the monoclonal PI 153/3 and J5 antibodies. The blastema and tubules of WIT were strongly stained by PI 153/3, which did not label normal adult kidney, and weakly stained by J5, which strongly labeled glomeruli and proximal convoluted tubules of normal kidney. These studies show that WIT blastema is heterogeneous in intermediate filament subtypes, while WIT tubules more closely resemble distal than proximal convoluted tubules of adult kidneys but also retain expression of fetal antigens.

INTRODUCTION

WIT or nephroblastoma, a solid tumor of childhood, has a distinctive histological appearance, consisting of blastema, tubules, and mesenchyme (4, 24). In addition, WIT variants have been described which contain blastema and tubules, tubules alone, and blastema associated with differentiated structures such as skeletal muscle (4). The tubules of WIT have been shown to retain morphological and biochemical similarities to proximal and distal convoluted tubules, including production of BB antigen and uromucoid, respectively (39). Early attempts to prepare antibodies to the different cell types of WIT have met with some success (6, 33), but a more definitive study has not appeared. Thus, it is still unknown whether unique, stage-specific antigens are present in WIT, as reported in mouse and human teratocarcinomas (2).

MATERIALS AND METHODS

Samples of surgically resected WIT were taken for morphological (histology and ultrastructure), histochemical, immunohistochemical, and nude mouse heterotransplantation studies.

Morphology. Unfixed, 5-μm cryostat sections of 9 surgically resected WIT and nude mouse heterotransplants of one of these were examined for ALP, ACP, and γ-GT (29). Normal kidney was used as a control because these enzymes are located in different parts of the nephron.

Immunohistochemistry. Surgically resected and heterotransplanted WIT were stained by immunofluorescence and immunoperoxidase techniques using: (a) polyclonal antibodies to the intermediate filament cytokeratin (22), EMA (34), and uromucoid (Tamm-Horsfall secretory glycoprotein) (39) to label epithelial cells; (b) polyclonal antibody to the intermediate filament vimentin (8) to label mesenchymal cells; and (c) monoclonal antibodies, PI 153/3 and J5, in an attempt to label blastema cells. In addition, basement membrane material in WIT was labeled using polyclonal antibody to a mixture of type IV collagen plus laminin.

Mallory bodies were extracted from cirrhotic human liver, and a rabbit antibody to MBCK was produced (22) which reacted with various types of epithelial cells, including proximal and distal convoluted tubules and collecting ducts of normal human kidney. Goat antibody to EMA (Sera Laboratory, Crawley Down, Sussex, England), which reacted with the surface glycoproteins of secretory cells, was prepared against an organic solvent extract of milk fat globule membranes (34). Rabbit antibody to uromucoid, specific for the Tamm-Horsfall protein which is synthesized by epithelial cells of the loop of Henle and distal convoluted tubule, was purchased from Cappel Laboratories, Inc. (Malvern, PA). Rabbit antibody to basement membrane was prepared against a pepsin digest of placenta containing type IV collagen (Sigma Chemical Co., St. Louis, MO) (10). Immunoblotting showed that the anti-basement membrane antibody was directed against type IV collagen plus laminin (37). This antibody stained glomerular capillary and tubular basement membranes of normal kidney. A rabbit antibody to vimentin, which reacted with mesenchymal cells, was produced by immunization with vimentin extracted from a cultured line of human embryonic lung fibroblasts (8, 19). Mouse monoclonal antibody J5, which recognizes the CALLA on lymphocytes, was purchased from Coulter Diagnostics (Hialeah, FL), while PI 153/3, which recognizes an antigenic determinant on the human neuroblastoma cell line IMR-5, fetal brain cells, and normal and leukemic pre-B- and mature B-lymphocytes (21), was purchased from Bethesda Research Laboratories (Gaithersburg, MD). All antibodies prepared by us were produced by giving injections to New Zealand White rabbits s.c. or i.d. of 1 mg of antigen in Freund's complete adjuvant.

WIT can be successfully passaged as heterotransplants in nude mice (27, 31, 40), and histological examination has shown that the nude mouse tumors closely resemble the surgically resected specimens. In the present study, 9 surgically resected WIT and the nude mouse heterotransplants derived from one of them were studied by histochemistry and immunohistochemistry to identify the different tumor cell types present.
complete adjuvant. Booster injections were given at 2-week intervals, and sera were collected after 6 weeks.

**Immunofluorescence and Immunoperoxidase Labeling.** Two labeling methods were used, depending on the ability of the various antibodies to recognize antigenic determinants in formalin-fixed or frozen tissue sections. For rabbit anti-MBCK and goat anti-EMA antibodies, tissues were fixed in 10% buffered formalin and embedded in paraffin. Five-μm sections were deparaffinized, rehydrated, and briefly digested with pepsin to expose antigenic determinants (18). Immunolabeling was performed by the PAP method (20). Normal rabbit and goat sera were used as negative controls. In the case of rabbit antibodies to uromucoid, basement membrane (type IV collagen plus laminin), and vimentin and the mouse monoclonal antibodies, J5 and PI 153/3, tissues were snap-frozen in isopentane-liquid nitrogen, cut into 5-μm cryostat sections, air-dried, fixed in acetone for 15 min, air dried again, and immediately stained or stored at −20°C. For the rabbit antibodies, an indirect immunofluorescence method was applied, using fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin (B11, Miles-Yeda, Ltd., Rehovot, Israel) as the secondary reagent (19). For the mouse monoclonal antibodies, an indirect immunoperoxidase technique was applied, using peroxidase-conjugated F(ab')2 goat anti-mouse immunoglobulin as the secondary reagent. The method used was that described by Habeshaw et al. (11) for labeling of lymphocyte antigens. Positive controls for the monoclonal antibodies were normal kidney and a neuroblastoma. Negative controls were performed by substituting normal mouse serum for the monoclonal antibodies.

**Heterotransplantation of WIT.** The procedures for preparing tumor cells for injection of WIT into nude mice and assessment of tumor growth have been described in the preceding paper (40).

**RESULTS**

The results of histochemical studies of surgically resected WIT, nude mouse heterotransplants of WIT-1, and normal kidney are shown in Table 1.

ALP, ACP, and γ-GT were present in proximal and distal convoluted tubules of normal kidney (Figs. 1 to 3). ALP and γ-GT staining was stronger in proximal than in distal convoluted tubules, whereas ACP staining was stronger in distal tubules. Up to one-half of the tubules in some cases of WIT and nude mouse heterotransplants of WIT showed weak-to-moderate reactivity for ALP and γ-GT, but blastema was negative (Figs. 1 to 3). Overall, ALP and γ-GT reactivities were much stronger in normal kidney than in tumor. In addition, tubules and blastema showed staining for ACP in most cases. The results of immunohistochemical studies of surgically resected WIT, nude mouse heterotransplants of WIT-1, normal kidney, and renal cell carcinoma are shown in Tables 2 to 4.

A PAP technique was used to label the MBCK and EMA in tubular epithelium. In normal kidney, antibody to MBCK strongly stained the cytoplasm of collecting ducts, distal convoluted tubules, and portions of proximal convoluted tubules, whereas antibody to EMA labeled the apical portions of distal convoluted tubules (data not shown). In the case of WIT and nude mouse heterotransplants of WIT-1, both antibodies labeled the tubules in most cases. Staining was heaviest at the apices and lateral cell margins (probably corresponding to desmosomal contacts between cells) and weakest at the basal aspects (Figs. 4 and 5). Since antibody to MBCK and EMA labeled mainly distal convoluted tubules and collecting ducts of normal kidney, these results suggest that the tubules of WIT resembled these types of tubules in most cases. No labeling of mesenchyme was seen with these antibodies, whereas blastema was either negative or positive in discrete cytoplasmic areas, especially with the antibody to MBCK. Antibody to uromucoid produced diffuse cytoplasmic staining of the tubules of WIT and nude mouse heterotransplants of WIT-1, as detected by indirect immunofluorescence (Fig. 6).

In normal kidney, uromucoid is produced by epithelial cells of the ascending portion of the loop of Henle and distal convoluted tubules (15). Therefore, positive staining of the tubules of WIT suggested that they were similar to this portion of the nephron. The presence of a basement membrane around the tubules of WIT and nude mouse heterotransplants of WIT-1 was confirmed by indirect immunofluorescence using antibody to basement membrane components (type IV collagen plus laminin) (Fig. 7). In addition, deposits of basement membrane material were detected between blastema cells, suggesting that blastema was
Immunohistochemistry

Table 2

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<tr>
<th>Specimen</th>
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<th>Blastema</th>
<th>Tubules</th>
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</tr>
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Normal kidney

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† Staining intensity was graded negative (−), weak (+), moderate (++), and strong (+++).

Table 3

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</tr>
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<tr>
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Renal cell carcinoma

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Normal kidney

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<th>Stroma</th>
<th>PCT</th>
<th>DCT</th>
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<tbody>
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<td>+++</td>
<td>-</td>
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† Staining intensity was graded negative (−), weak (+), moderate (++), and strong (+++).

Table 4

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<tr>
<td>Anti-vimentin (1:20)</td>
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<tr>
<td>Anti-BM (1:40)</td>
<td>Mesenchyme</td>
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† Staining intensity was graded negative (−), weak (+), moderate (++), and strong (+++).

To label mesenchyme, antibody to vimentin was used by indirect immunofluorescence. This antibody stained endotheial and epithelial cells of glomeruli and stromal cells around the tubules of normal kidney. In WIT and nude mouse heterotransplants of WIT-1, antibody to vimentin also labeled the mesenchymal component. Tubules were negative, while some of the blastema cells were stained in a pattern resembling that seen with the antibody to basement membrane (Fig. 8).

In addition to anti-vimentin antibody, blastema was labeled capable of producing basement membrane.

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with 2 additional sets of antibodies. (a) Using antibody to MBCK in a PAP reaction, the blastema immediately peripheral to tubules or just beginning to form tubules stained positively (Fig. 4). In other areas of blastema were negative. Therefore, this antibody appeared to recognize those blastema cells which were committed to tubular epithelial differentiation. (b) Monoclonal antibodies, PI 153/3 and J5, were used in an indirect immunoperoxidase reaction. In normal kidney, PI 153/3 did not stain glomeruli or tubules, while both the blastema and tubules of WIT stained positively (Fig. 9). The adult tubular WIT also showed strong staining throughout the cytoplasm of tubular epithelium which was heaviest at cell apices. Therefore, the blastema and especially the tubular cells retained expression of fetal antigens during morphological and functional maturation. In normal kidney, however, J5 stained glomeruli and proximal but not distal convoluted tubules (Fig. 10). The blastema and tubules of WIT stained only weakly with this antibody or were negative. However, in the case of an adult tubular WIT, J5 strongly stained the epithelium. Thus, J5 recognized antigenic determinants on tubular epithelium of normal kidney and on an adult WIT, but not on childhood WIT, suggesting that these determinants were expressed later in development. In general, J5 or PI 153/3 did not stain stromal cells of WIT, but in a small number of cells, weak staining was observed. In a case of renal cell carcinoma, the tumor cells stained weakly with J5 and PI 153/3 (not shown). Since renal cell carcinoma is thought to arise from the proximal convoluted tubule (39), the J5 staining was not surprising, while reactivity with PI 153/3 indicated retention of fetal antigens and a histogenetic link to WIT.

DISCUSSION

The present study has utilized histochemical and immunohistochemical methods to shed light on the different cell components of WIT. The presence and degree of reactivity of ALP (38), ACP, and γ-GT, as detected by histochemical staining in tubules of WIT, suggest a similarity to the distal convoluted tubular portion of the nephron. Histochemical staining for ALP reactivity was demonstrated in a small portion of tubules of both primary WIT and a WIT heterotransplant. The degree of staining in the majority of WIT was considerably less than that found in the proximal convoluted tubules of normal kidney and more comparable to that seen in distal convoluted tubules. Waghe and Kumar (38) also found little ALP reactivity in WIT and showed a much simpler isoenzyme pattern for ALP and nonspecific esterase in WIT extracts. Thus, it would be interesting to know whether altered isoenzymes occur during the development of WIT and are linked in some way to specific genetic abnormalities associated with this tumor (25).

The results of our study and other reports (1, 9) have shown that the tubules of WIT contain cytokeratin andEMA, secrete uromucoid, and synthesize basement membrane material (3, 26, 36). Cytokeratin and EMA are produced mainly by collecting ducts and distal convoluted tubules of normal kidney, while uromucoid is synthesized only by the ascending portion of Henle’s loop and distal convoluted tubules. Therefore, these structural and secretory components are satisfactory for demonstrating the similarity of the tubules of WIT to the distal tubular system of normal kidney. Holthöfer et al. (13), using monoclonal antibodies to cytokeratins and a rabbit anti-vimentin antibody, described stage-dependent developmental patterns in the expression of intermediate filaments in fetal and adult human kidneys. Undifferentiated mesenchyme of fetal kidney expressed vimentin, while collecting ducts, which produce cytokeratin in adult kidneys, also showed a transient expression of vimentin. Developing proximal and distal tubular cells expressed cytokeratins, but certain cytokeratin staining patterns were lost during nephrogenesis. Thus, dynamic changes in intermediate filament composition and organization were observed. Differentiating WIT might be expected to show a similar complexity in intermediate filament expression.

Recently, Altmannsberger et al. (1) reported that the blastema of WIT stained for cytokeratin and vimentin, as detected by immunofluorescence. Such double expression of these 2 types of intermediate filaments by a single cell type occurs only rarely in vivo (28). In addition to staining tubules of WIT, the antibody to MBCK used in this study labeled blastema cells which were adjacent to and undergoing differentiation into tubules. This finding indicates that epithelial differentiation is accompanied by the turning on of genes which code for intermediate filament expression. This type of gene activation is also found when germ cells, which do not express intermediate filaments, develop into preimplantation embryos (17). Vimentin staining, albeit weak, was found in blastema cells in our study, suggesting that some of these cells retained mesenchymal characteristics. Moreover, vimentin expression appeared to be retained as that of cytokeratin was initiated (1). It is therefore clear from our studies and those of others (1) that the heterogeneity in intermediate filament expression in blastema reflects a multipotential capacity for differentiation by these cells. Moreover, it is likely that the majority of blastema cells are partially differentiated, since these cells have rudimentary intercellular junctions and produce basement membrane components.

Monoclonal antibody J5, which detects CALLA, has been reported to stain fetal tubules as well as proximal convoluted tubules and glomeruli of fully developed human kidney (30). In childhood WIT, there was only slight labeling of blastema and tubules by J5, but in a case of adult tubular WIT, there was strong staining of tubular epithelium. This suggests that tubules are more differentiated in adult than in childhood WIT. Therefore, the antigenic determinants recognized by J5 appeared to relate to the maturation of proximal convoluted tubules. In contrast, tubular epithelium of normal kidney was not labeled using PI 153/3, whereas blastema cells and tubules of WIT were strongly labeled. Since the determinants recognized by PI 153/3 (previously noted on neuroblastoma cells, B-lymphocytes, and fetal brain) are probably of fetal origin, both the blastema and tubules share fetal antigens. Fetal antigens are frequently expressed in a variety of tumor cell types and are much less common in normal tissues (16). For example, α-fetoprotein is often found in high concentrations on hepatomas and carcinoembryonic antigen on colon carcinoma. Thus, in the case of WIT, fetal antigenic expression by tubular epithelium appears to be retained while the cells undergo morphological and functional maturation.

No immunological marker is available to distinguish blastema cells of WIT from their differentiated derivatives. Platt et al. (30) reported that BA-1 monoclonal antibody, which identifies a surface antigen expressed by granulocytes and by normal and malignant B-lymphocytes at various stages of differentiation, also reacts with cells in the kidney at the earliest stages of
nephron development. Preliminary data suggest that BA-1 also labels the blastema cells of WIT. Another method for distinguishing blastema cells and differentiated derivatives of WIT might be to examine the surface glycoproteins of cells. This could be accomplished using lectins, which have been shown to stain different parts of the nephron of normal kidney (14). It has been claimed that benign and malignant tumors can be differentiated on the basis of lectin binding (23). Preliminary studies in other laboratories (12) are under way to determine whether lectins of known specificity will discriminate among the various types of WIT and selectively stain the blastema cells in this tumor.

It has been postulated that the blastema of WIT is generated from metanepric mesenchyme through a process of induction similar to that which occurs in the developing nephron (4, 7, 24, 32). However, in the case of WIT, further development of blastema is blocked, leading only to the formation of disorganized, nonfunctional tubules and abortive glomeruli. The factors that govern this limited differentiation of blastema are unknown. Some of the controlling events may be at the gene level, specifically in association with a recognized aberration in chromosome 11 (11p13) (25). By being able to identify the various cell types in WIT, it should now be possible to induce tumor cell differentiation using proven differentiating agents (5, 35). Such studies are in progress.

ACKNOWLEDGMENTS

The assistance of the Medical Publications Department, The Hospital for Sick Children, Toronto, Canada, is acknowledged.

REFERENCES

Figs. 1 to 3. Histochemistry of WIT and normal kidney. Fig. 1. ACP reaction, showing a strong staining of tubules and glomeruli (G) in normal kidney (a; x 200) and moderate staining of blastema (B) and strong staining of tubules (T) of WIT (b; x 400). Fig. 2. ALP reaction, showing a strong staining of proximal convoluted tubules, weaker staining of distal convoluted tubules, and no staining of glomeruli (G) of normal kidney (a; x 200) and moderate staining of some tubules (T) but no staining of others in WIT. Blastema (B) is also negative except in peritubular areas (middle bottom) (b; x 400). Fig. 3. γ-GT reaction, showing a strong staining of proximal convoluted tubules and weaker staining of distal convoluted tubules of normal kidney (a; x 200) and strong staining of the apices of some tubular cells (T) of WIT (b).
Figs. 4 and 5. Immunoperoxidase labeling of surgically resected WT and WT heterotransplant using anti-MBCK and anti-EMA antibodies. Fig. 4. Anti-MBCK antibody labeling, showing staining of the apices and plasma membranes of tubular cells (T) of surgically resected WT (a and inset, left top) and staining of tubules (T) but not blastema (B) of WT heterotransplant (b, left bottom). a, × 100; b, × 200. Fig. 5. Anti-EMA antibody labeling, showing staining of the apices of tubular cells (T) and the luminal contents of WT (a, right top) and staining of apices of tubular cells (T) of WT heterotransplant (b, right bottom). × 200.
Figs. 6 to 8. Immunofluorescent labeling of WIT using anti-basement membrane, anti-vimentin, and anti-uromucoid (Tamm-Horsfall glycoprotein) antibodies. Fig. 6. Anti-uromucoid antibody labeling showing staining at the apices of tubules (arrowheads) but not of the blastema of WIT (right bottom). × 400. Fig. 7. Anti-basement membrane antibody labeling, showing strong staining of the basal aspects of tubular cells and intercellular stroma of WIT-1, with no staining of blastema (a), and strong staining of the basement membrane (arrowheads) surrounding the tubular cells in WIT-10, a monomorphous tubular tumor (b). × 400. Fig. 8. Anti-vimentin antibody labeling, showing staining of blastema (arrowheads) and mesenchyme but not tubules of WIT. × 400.

Figs. 9 and 10. Immunoperoxidase labeling of normal kidney and WIT, using monoclonal anti-CALLA (J5) and anti-neuroblastoma (PI 153/3) antibodies. Fig. 9. Labeling with PI 153/3, showing no staining of normal kidney (a) and strong staining of the cytoplasm and plasma membranes of blastema (B) and tubules (T) of WIT (b). × 200. Fig. 10. Labeling with J5, showing strong staining of glomeruli and apices of proximal convoluted tubules of normal kidney (a) and weak staining of tubules of WIT (b). × 200.
Histochemical and Immunohistochemical Characterization of Surgically Resected and Heterotransplanted Wilms' Tumor

Herman Yeger, Reuben Baumal, Dennis Bailey, et al.

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