To the Graduate Council:

I am submitting herewith a thesis written by Scott D. Roberton entitled "Characterization and Distribution of the Human Renal Cell Carcinoma Glycoprotein Recognized by Monoclonal Antibody 469 - 5A." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Life Science.

Stephen J. Kennel, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

Characterization and Distribution of the Human Renal Cell Carcinoma Glycoprotein Recognized by Monoclonal Antibody 469 - 5A

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Scott D. Robertson

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DEDICATION

TO MY WIFE

Mrs. Brenda G. Robertson

AND

MY PARENTS

Charles R. and Norma D. Robertson

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Finally, I am most grateful to God, who sent His son that I might have everlasting life. During the roughest times when I thought I could go no further, Jesus carried me through.

ABSTRACT

Renal cell carcinoma is responsible for 2% of all malignant tumors in adults and is one of the most challenging tumors to treat by the clinical oncologist. Unless early primary lesions (stage I or stage II) are identified and completely removed, cures are uncommon. Patients presenting with late stage or metastatic disease have a relentless progression of disease and a short survival. The exact etiology of renal cell carcinoma is poorly understood and effective measures of prevention have not been determined. Current therapeutic methodologies used in the treatment of late stage and metastatic renal cell carcinoma have been generally ineffective, failing to realize a prolonged control of tumor spread.

A monoclonal antibody, 469-5A, has been generated using human renal cell carcinoma lysate as an immunogen in BALB/c mice. The monoclonal antibody recognizes a glycoprotein, designated gp 45/55, which is overexpressed in 18/18 renal cell carcinomas observed. Evidence from Western blot, including staining pattern, molecular weight range, and increased detectability after neuraminidase treatment demonstrated that monoclonal antibody 5A recognized a similar glycoprotein in both normal kidney and tumor tissues. However, Western blot data indicates that gp 45/55 expression is 2 to 6-fold greater in RCC than normal kidney tissues. Furthermore, treatment with various combinations of other glycosidases caused shifts in the detected molecular weight

in Western blot, indicating that the 5A epitope was probably protein in nature. Immunohistochemistry supported Western blot data, demonstrating an increase in monoclonal antibody 5A staining in tumor tissue compared to normal kidney tissue. Monoclonal antibody 5A staining was restricted to proximal tubule epithelium in normal kidney but was detected on most of the tumor epithelial cells.

Expression of 5A antigen was also detected by Western blot and/or immunohistochemistry in other normal human tissues as well as in lymphoma tissues. Based on the same identification criteria, the 5A monoclonal antibody detected a similar molecule, gp 45/55, in Western blot of other normal tissues as had been detected in normal kidney and renal cell carcinoma. However, distribution data comparing results from immunohistochemistry and from Western blot of various normal and tumor tissues demonstrated that gp 45/55 was not detected in IHC of small intestine and spleen but was detected in Western blot.

The results presented demonstrate the overexpression of detectable gp 45/55 in renal carcinoma cells. It is also shown that the probable epitope for the 5A monoclonal antibody is protein in nature. Western blot data indicates that monoclonal antibody 5A recognizes the same antigen, gp 45/55, in normal kidney and tumor cells. Similarly, gp 45/55 is expressed in cells of other normal tissues as well as in lymphoma tissues. The potential of gp 45/55 as a renal carcinoma marker and of the immunodiagnostic and immunotherapeutic use of monoclonal antibody 5A is discussed.

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CHAPTER I

INTRODUCTION

RCC Epidemiology

Renal cell carcinoma (RCC) is an unpredictable neoplasm (Reese, 1992) with a poor prognosis and is responsible for 80% of all malignant renal tumors in man (Papadopoulos et al., 1994). RCC comprises approximately 2% of all malignant tumors in human adults (Papadopoulos et al., 1994) and 20% in children (Dayal and Kinman, 1983). Each year, 25,000 individuals develop RCC and more than 10,000 deaths are attributed to this type of cancer (Gnarra et al, 1993). Overall, the incidence of the disease is approximately 7.5 cases per 100,000 population, and it is twice as common in males as in females (Garnick and Richie, 1991; Stadler and Vogelzang, 1993). Although RCC occurs in individuals of any age, most patients are between the ages of 50 and 70 years (Linehan et al, 1989). The extent of primary tumor spread at the time of diagnosis has been correlated with patient survival (Delahunt et al., 1991). In approximately 50% of RCC patients, the primary tumor has metastasized before an initial diagnosis is made (Papadopoulos et al., 1994).

Epidemiological studies have revealed that RCC is most prevalent in Iceland, Denmark, and Sweden, moderately prevalent in the U.S. and western Europe, and

least prevalent in Africa, Asia, Canada, and the United Kingdom (Wynder et al., 1974; Walter et al., 1989). RCC has been divided histologically into different subtypes: papillary, nonpapillary, clear cell, granular, glomerular, and spindle cell (Javadpour, 1984). The most significant prognostic indicator of survival seems to be the anatomical extent of the tumor at the time of diagnosis and/or treatment (Thrasher and Paulson, 1993) rather than subtype. Treatment modalities for RCC have expanded over the last decade into areas including chemotherapy, radiation therapy, cytokine-mediated therapy, vaccine therapy, and monoclonal antibody therapy (Chiou, 1991; Reese, 1992; Uemura et al., 1994). In spite of this expanded knowledge regarding alternative forms of therapy for RCC, surgical extirpation remains the most effective method of treating the disease (Thrasher and Paulson, 1993).

RCC Etiology

Knowledge of renal cell tumors has existed for centuries. Specific attention was not drawn to them until Grawitz, in an 1883 publication, suggested that the yellowish tumors, previously thought to be lipomas, were in fact derived from ectopic adrenal tissue and should therefore be termed hypernephromas (Garnik and Richie, 1991; Walter et al., 1989). This nomenclature, although still widely used today, is incorrect since the cellular origin of most RCCs has been determined to be

the proximal tubule epithelial cell located in the cortex of the kidney (Ericsson et al., 1966), as judged by the presence of the proximal tubular brush border antigens and the absence of the distal tubular Tamm-Horsfall glycoprotein in tumor samples (Holthöfer, et al., 1983). However, even today the exact etiology of RCC is still debated (Gnarra et al., 1993), and it is likely that most RCCs are derived from proximal tubule epithelium, while other normal kidney cells may give rise to carcinomas as well.

There is little evidence to implicate any specific factor in the etiology of RCC (Belldegrun et al., 1991). However, various environmental (reviewed in Linehan et al., 1989), hormonal (reviewed in Jaske and Müller-Holzner, 1988), cellular (reviewed in Droz et al., 1994), and genetic (reviewed in Meloni et al., 1992) factors have been studied as possible causal factors. Tobacco use has been implicated as a definite risk factor for the development of kidney cancer (Linehan et al., 1989; La Vecchia et al., 1990). In a study by Yu et al. (1986), it was estimated that 30% of RCCs in men and 24% in women may be directly due to smoking. Men who smoke and who are also at risk for exposure to cadmium (either by inhalation or ingestion) are thought to have a higher than normal risk for RCC (DeKernion and Smith, 1984), although this conclusion has been disputed (Dayal and Kinman, 1983). Overall, the evidence that suggests cigarette smoking as a possible

causative agent in RCC is very weak and is based upon conflicting statistics in a small number of case-control studies (La Vecchia et al., 1990).

Well studied cellular factors that contribute to the RCC phenotype are found in both familial and sporadic renal cell carcinomas. Cellular aberrations, which serve as molecular markers associated with the RCC phenotype, include the varied expression of growth factors, growth factor receptors, extracellular matrix components, and cytokines. Studies of the role of transforming growth factor-alpha (TGF-α) indicate that it may have an important role in the transformation and/or proliferation of renal neoplasms (Walker et al., 1991; Atlas et al., 1992), especially in the regulation of glycolipid sulfotransferase (GSTase) activities at the cellular level in RCC via the epidermal growth factor (EGF) receptor (Kobayashi et al., 1993). The mechanism of cellular regulation used by TGF-α has not been determined exactly, but is thought to be one of three distinct endocrine-type pathways - autocrine (influence of a cells own activities by a factor released by that cell), paracrine (influence of cells within the immediate microenvironment of the factor-secreting cell) or juxtacrine (influence of cells located near the factor-secreting cell).

Synthesis of abnormal glycolipids found in neoplastic cells is due at least in part to the aberrant expression of the responsible glycosyl transferases. In

particular, the activity levels of GSTases which catalyze the transfer of sulfate from 3'-phosphoadenosine-5'-phosphosulfate to galactose on cerobroside-type glycolipids (Cumar et al., 1968) are markedly elevated in RCC tissues (Sakakibara et al., 1989). Another growth factor, basic fibroblast growth factor (bFGF/FGF-1), has also been implicated in the neoplastic growth of RCC. It is well established that bFGF acts to promote the vascularization of RCC tumors as an angiogenic agent (Eguchi et al., 1992; Singh et al., 1994), but there is also some evidence that this growth factor may function in an autocrine manner to stimulate the growth of the transformed proximal tubular epithelia in RCC tissues. Studies by Mydlo et al. (1993) and Emoto et al. (1994) indicate that bFGF works via an autocrine mechanism *in vitro* in two separate carcinoma cell lines, RC29 and VMRC-RCW respectively, to maintain unrestricted proliferation.

As previously indicated, the EGF receptor (EGFR), a transmembrane glycoprotein with tyrosine kinase activity, has also been implicated in RCC tumorigenesis. In normal, non-pathological kidney tissue expression of the EGFR is low, while expression in most RCCs is high (Ishikawa et al., 1990; Weidner et al., 1990). Interestingly, expression of the HER2/*neu* gene product, a glycoprotein receptor with a cytoplasmic tyrosine kinase domain, demonstrates an inverse relationship in expression to that of the EGFR, namely, the HER2/*neu* is

high in normal tissues and low in RCC tissues (Weidner et al., 1990). It appears therefore, in humans, that the increased expression of the EGFR associated with a transformed phenotype relates to the RCC cells' ability to respond to TGF-α in an autocrine manner (Walker et al., 1991). The biological role of the HER2/*neu* receptor is still unknown, although its expression has been correlated with survival time in human breast cancer (Slamon et al., 1987). Its overall sequence homology (approximately 50%) with the EGFR (Yamamoto et al., 1986) suggests that they may be functionally related.

The role of altered expression of integrin subunits and other cell adhesion molecules, such as neural cell adhesion molecule (NCAM), have also been implicated in the proliferation and malignant transformation of RCC (Dedhar and Saulnier, 1990; Zocchi et al., 1994). Since the interaction of cells with the extracellular matrix (ECM) is an important determinant of tissue morphogenesis and of cell differentiation, any alterations in this interaction may explain aspects of malignant cell behavior (Adams and Watt, 1993; Stetler-Stevenson et al. 1993). For example, Korhonen et al. (1992, 1994) has recently shown that increased expression of the integrin α_v and alterations in the α_6 subunit may change integrin-mediated, cellular-basement membrane interaction and therefore may be associated with more malignant, histologically anaplastic RCC

forms. Zocchi et al. (1994) has demonstrated that the homolog of N-CAM, or CD56, is involved in the adhesion of RCC tumor cells to endothelial cells through interaction with heparan sulfate (a component of ECM). This binding can modulate transduction of an activation signal across the tumor cell membrane that leads to proliferation.

Directly related to the expression of receptor molecules on the surface of renal tumor cells is the composition of the tumor ECM. Recent studies have indicated that a truncated form of fibronectin may be excreted by some RCCs and may act as an autologous growth factor (Kochevar et al., 1991). Thrombin, along with plasminogen activating factor and other matrix factors, is active in the conversion of fibrinogen into fibrin in the RCC tumor ECM. Fibrin is an important constituent of the tumor matrix that may contribute to the structural integrity of the tumor and support the vascularization of the tumor mass (Wojtukiewicz et al., 1990).

Finally, the roles of various cytokines in the RCC phenotype are beginning to be reported in the literature. The cytokine, tumor necrosis factor (TNF), has been reported to possibly stimulate RCC tumor growth. This is especially true in advanced stages of RCC, according to *in situ* hybridization and immunohistochemistry data from patients studied with different stages of disease (Waase et al., 1992).

However, TNF's role in the progression of RCC has not yet been fully established. Overexpression of the cytokine interleukin 6 (IL-6) and its receptor has also been detected in some RCC tumors *in vivo*, indicating that IL-6 may have a role in the transformation and/or proliferation of RCC, again via an autocrine pathway (Takenawa et al., 1991; Blay et al., 1992). As the functions of growth factors, growth factor receptors, integrins, ECM components, and cytokines involved in the transformation and proliferation of RCC become more clearly defined, their roles in the etiology of the RCC phenotype will also be better understood.

Genetic markers that are associated with the RCC phenotype in both familial and sporadic renal cell carcinomas have also been well documented. Today, after extensive studies (For review see Zbar, 1989; Meloni et al., 1992) there is little doubt that chromosome 3, and in particular, the short arm of chromosome 3 (3p), is the most commonly involved region of the human genome in both familial (also called von Hippel-Lindau-associated RCC) (Walter et al., 1989; van der Hout et al., 1991; Meloni et al., 1992) and sporadic forms of papillary-type RCC tumors (Kovacs and Kung, 1991; Gnarra et al, 1993). However, the genes or gene products that are involved in the actual initiation and progression of both forms of RCC which bear this marker have yet to be identified (Gnarra et al., 1993). It has been suggested that the development of RCC is due to the loss of a

recessive tumor suppressor gene on the short arm of chromosome 3 (3p) (van der Hout et al., 1991). It should be noted, however, that nonpapillary tumors are rare forms of RCC that do not demonstrate any significant 3p involvement (Presti et al., 1991).

Mutation of the p53 gene, found on the short arm of chromosome 17, may also be involved in the progression of RCC (Reiter et al., 1993). The loss of p53 expression has also been correlated with malignancy in several systems (Merlo et al., 1994; Symonds et al., 1994). The p53 gene product normally functions by controlling cell proliferation by directing cells to an apoptotic pathway (Symonds et al., 1994).

RCC Clinical Presentation and Diagnosis

RCC tumors are generally found in the kidney cortex as solitary masses that are most often spherical and well defined (O'Toole et al., 1993). The tumor is also highly vascular in 85% of cases (Stadler and Vogelzang, 1993). However, RCC may remain clinically occult for most of its course (Linehan et al., 1989) and is most often discovered incidentally during the course of various diagnostic studies, such as ultrasound or computed tomography (Parks and Kellett, 1994). Unfortunately, without early detection RCC ultimately progresses to the point that up to 50% of patients present with metastatic disease (Papadopoulos et al., 1994), most often in the lung (Belldegrun et al., 1991).

Other metastatic sites include soft tissues, bone, liver, cutaneous sites, and the central nervous system (Linehan et al., 1989; Brantley et al., 1993).

Several methods for the detection and diagnosis of RCC have been used, including abdominal X-ray, intravenous urography with nephrotomography, ultrasonography, and percutaneous cyst puncture (Garnick and Richie, 1991). The mainstay for both diagnosis and staging of RCC has been selective renal arteriography using a radiopaque dye (Watson et al., 1968). Computed tomography (CT) has recently become the method of choice to diagnose RCC because of its reproducibility and non-invasive nature (Cronan et al., 1982; Miles et al., 1990). This high resolution technique allows the visualization of cross-sectional anatomy, and therefore, the relationship of mass lesions in the kidney to surrounding structures. The ability to detect and determine the amount of tumor involvement efficiently is critical in the staging of RCC. The development of magnetic resonance imaging (MRI), and its use as a tool in evaluating renal masses, was at first thought to be an important advance (Hricak et al., 1988; Amendola et al., 1989). However, numerous recent studies have demonstrated that MRI offers little advantage over conventional CT scans (Reese, 1992). Efficient diagnostic evaluation is critical to the determination of patient tumor involvement and to indicate the best therapeutic approach (Couillard and deVere White, 1993).

RCC Grading and Staging

Once diagnosis of RCC has been established, attention must be turned to the delineation of the extent of involvement at the primary site and any distant, metastatic sites (Strohmeyer and Ackermann, 1991). Although the total extent of tumor anatomical involvement seems to be the most important factor in prognostic evaluation, histologic features of the primary RCC do have some bearing on the prognosis (Delahunt and Nacey, 1987; Parks and Kellett, 1994). It has been demonstrated that "clear cell" variants of RCC have the highest 5-year survival rate at 58% (Meloni et al., 1992), closely followed by the granular cell variants. Spindle cell carcinoma is the most aggressive form of RCC (Ro et al., 1987) and has the poorest 5-year survival rate of 23% (Meloni et al., 1992). Recently, interest has focused on DNA content (ploidy) of the tumor cell, as indicated by a comparison of nephrectomy specimens from normal and tumor tissues, as a possible prognostic indicator in RCC (Banner et al., 1990; Papadopoulos et al., 1994). Results from their studies indicate that DNA content of RCCs correlates closely with tumor differentiation grade and tumor stage. Evidence demonstrates that diploid RCC tumors tend to be solitary, localized masses, while aneuploid tumors tend to metastasize. Similarly, tumor specimens have been assessed for nuclear shape abnormalities (Murphy et al.,

1990) and for the presence of increased numbers of nucleolar organizer regions (NORs) (Delahunt et al., 1991), associated with nuclear synthesis activity, as prognostic tools in staging RCC. Quantitative increases in the morphometric shapes associated with tumor cell nuclei and in the number of intranuclear NORs observed have indicated poorer prognoses in RCC patients. Although these different grading systems have achieved some degree of success as prognostic indicators, they have received only limited acceptance in clinical practice.

Clearly, the single most important determinant of prognosis with RCC is the pathologic stage of the tumor when it is initially diagnosed and treated (Strohmeyer and Ackermann, 1991). A pathologic staging system for RCC modified by Robson et al. (1969) is as follows:

Stage I	Tumor confined within the kidney
Stage II	Perirenal fat involvement but confined within Gerota's fascia
Stage IIIA	Gross renal vein or inferior vena cava involvement
Stage IIIB	Regional lymph node involvement
Stage IIIC	Venous plus lymph node involvement
Stage IVA	Involvement of adjacent organs other than adrenal
Stage IVB	Distant metastases

Figure 1 represents a graphic depiction of this staging system.

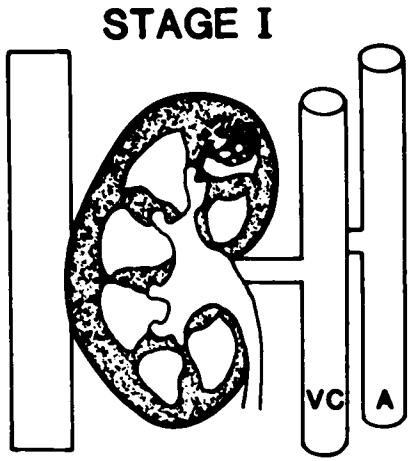
The most important feature of the histologic grading and the staging of RCC tumors, as it relates to this thesis, concerns the differential expression of those proteins involved in the transformation, differentiation, proliferation, and metastasis of RCC disease. These oncogenic-related events are associated with marked changes of tumor cell surface antigens, especially changes in the carbohydrate structures associated with glycolipids, glycoproteins, proteoglycans, and mucins (Hakomori, 1989; Hoon et al., 1991; Shimizu and Shaw, 1993). Early diagnosis and/or predicting the progression of cancer can be facilitated by the discovery of tumor-associated antigens that can be used advantageously either for diagnostic and/or for therapeutic treatments of the disease.

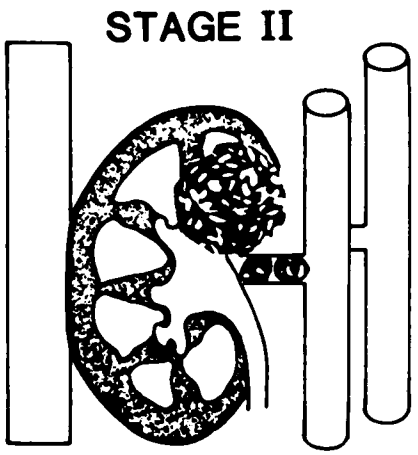
Treatment of Primary RCC

a. Surgical therapy

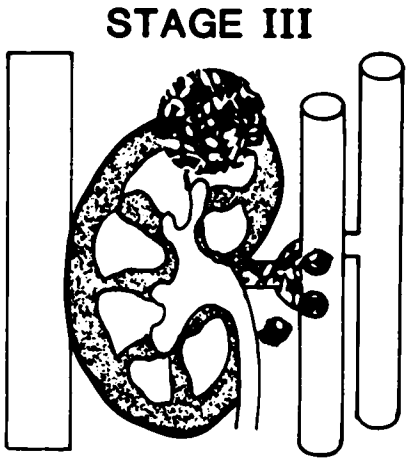
Surgery is the only known effective therapy for localized RCC (Linehan et al., 1989; Couillard and deVere White, 1993). Radical nephrectomy continues to be the recommended surgical treatment, although recent evidence suggest that this may not be mandatory in all patients with localized tumors (Belldegrun et al., 1991; Licht et al., 1994). Nephron sparing surgery is a

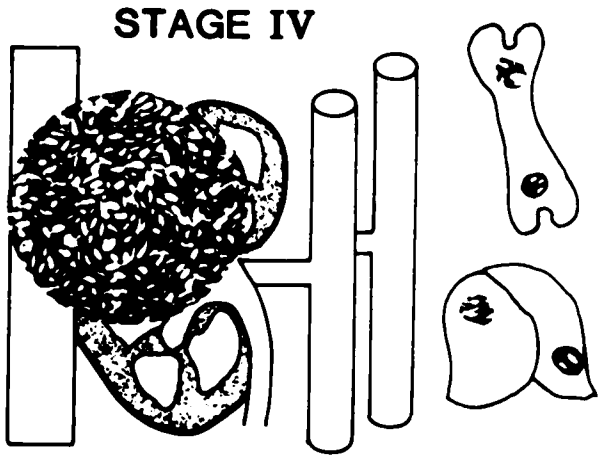
Figure 1. Graphic representation of the pathologic staging system for renal cell carcinoma developed by Robson (1969). Stage I represents tumor confined within the kidney. Stage II represents the involvement of peritoneal fat but the confinement of tumor mass within Gerota's fascia. Stage III is divided into three substages: A - gross renal vein or inferior vena cava involvement, B - regional lymph node involvement, and C - venous and lymph node involvement. Stage IV is divided into two substages: A - involvement of adjacent organs other than the adrenals, and B - formation of distant organ metastases.





(Brodsky and Garnick, 1989)





successful form of treatment for patients with localized (unilateral), stage I RCC tumors less than 4 cm in size when there is a need to preserve the functioning renal parenchyma and there is evidence of a normal contralateral kidney (Licht et al., 1994). When nephron sparing surgery is not feasible, radical nephrectomy is performed. For specific, well described and illustrated techniques of the surgical approaches to radical nephrectomy, refer to the text of Glenn (1991).

b. Chemotherapy and Radiation therapy

Chemotherapy, the use of cytotoxic drugs to treat cancer, remains the cornerstone of therapy for advanced solid tumors. However, despite the advances made in the treatment of some tumors, RCC remains one of the tumors most refractory to standard chemotherapy (Belldegrun et al., 1991). The use of cytotoxic chemotherapy has demonstrated little or no antitumor activity against advanced RCC and, therefore, has played no positive therapeutic role adjuvantly or neoadjuvantly (Yagoda and Bander, 1989; Parks and Kellett, 1994). Recent pilot studies using new therapeutic drugs, including suramin, methyl-gag and melphalan, cimetidine and coumarin, navelbine (Reese, 1992), and carboplatin (Trump and Elson, 1990), have likewise demonstrated minimal responses in primary tumors as well as metastatic RCC. One basis for this multidrug resistance appears to be related to a transmembrane glycoprotein (P-glycoprotein,

P170) encoded by the Multiple Drug Resistance (MDR1) gene that functions as an energy-dependent drug efflux pump (Fojo et al., 1987). It should be noted that studies where chemotherapy and immunotherapy were combined have also offered no therapeutic advantage in the treatment of advanced RCC (Neidhart et al., 1991; Merimsky and Chaitchik, 1990).

The role of radiation therapy for RCC has also been investigated, with conflicting reports on its efficacy. Preoperative radiation therapy does appear to have some impact upon improving the potential for local control of the disease even though it may not have an effect on long-term survival (van der Werf-Messing, 1973). However, in some cases, radiation damage to the contralateral kidney or adjacent liver has been reported, making preoperative radiation therapy a less favorable alternative (Gonick, 1983). Postoperative radiation therapy is often considered for those patients who have evidence of metastatic disease, namely an invasion into the capsule, a transection of the tumor at surgery, a spillage of tumor at surgery, an invasion into the perinephric fat or adjacent renal structures, or an invasion into the regional lymph nodes draining the kidney or into the inferior vena cava (Linehan et al., 1989). The precise role of postoperative radiation therapy is not clear, but the data do suggest that it does not have a significant impact upon reducing the potential for local and regional failure or for

increasing the potential for long-term survival (Couillard and deVere White, 1993).

c. Immunotherapy

Recent advances in tumor immunology offer new prospects for the treatment of advanced or metastatic RCC with immunotherapy. Patients with RCC are considered strong candidates for immunotherapy for several reasons: (1) primary RCC tumors have been shown to be immunogenic tumors (Haas, et al., 1993; Uemura et al., 1994); (2) RCC in most cases is a highly vascular tumor (Fischer et al., 1992); (3) the documented spontaneous regression of RCC metastases from some distant sites implicates an organ-specific immunological basis for regression (Kirk, 1987; Palmer et al., 1993); and (4) other forms of adjuvant therapy have been ineffective (Yagoda and Bander, 1989; Haas et al., 1993). Several strategies for immunotherapeutic intervention are currently being studied. Immunotherapy with autologous tumor cells, tumor cell vaccines, and immune RNA has demonstrated little or no beneficial effect (Vugrin, 1987). Immunotherapy with biological response modifiers has shown some limited promise in the treatment of advanced and metastatic RCC. Cytokines, such as interferons, IL-2, and TNF as well as various combinations of these cytokines, have been tested in the treatment of advanced RCC (Baisch et al., 1990; Figlin et al., 1991). However, the rates of objective

remission (partial and complete) in patients with good performance status and low tumor burden were disappointing, rarely exceeding 20% (Wirth, 1993).

Rosenberg et al. (1987) used RCC patient's lymphokine-activated killer (LAK) cells and high-dose IL-2 as a form of adoptive immunotherapy in the management of metastatic RCC to achieve 12 objective and seven minor responses in 36 patients (53%) with RCC. Further phase II trials with IL-2 and LAK could not confirm the results of the preliminary findings (Palmer et al., 1992). Two further large scale studies conducted by the National Cancer Institute and by a European multicenter consortium reported remission rates of 9% and 21%, respectively (Stahl et al., 1992).

Treatment with tumor-infiltrating lymphocytes (TILs) is a second form of adoptive immunotherapy. For this approach, TILs are isolated from resected renal tumors or biopsies, then expanded *in vitro* in the presence of IL-2 before being reinfused over several days (Kradin et al., 1987). Despite their specific cytotoxic activities against autologous tumor cells *in vitro*, clinical results with TILs in metastatic RCC were not superior to any other form of immunotherapy (Kradin et al., 1989). Although the response rate to various modalities of immunotherapy are small in RCC, preclinical and clinical studies of immunotherapy have contributed to a better understanding of the complex interactions between elements of the immune system

involved in antitumor responses (Haas et al., 1993).

The Role of Monoclonal Antibodies in RCC Detection and Treatment

The generation of monoclonal antibodies (MAb) that can be used in tumor imaging or in tumor therapy has been the focus of a great deal of research. Because MAbs are derived from single antibody-producing clones, they are relatively homogeneous, demonstrate specific binding for their antigens, and are easier to purify than antibodies obtained from conventional antisera (Kennel et. al, 1983). MAbs have provided a variety of new probes to investigate molecular changes at the cell surface during development, differentiation, and malignant transformation. These probes can be used as tools of basic biology to learn about cellular function(s), as markers for diagnosis of disease, or as therapeutic tools. Although true tumor-specific antigens have not been found, altered expression of normal protein antigens have been studied as 1.) markers of malignancy and tumor heterogeneity, and 2.) targets for antibody-mediated tumor imaging and/or therapy. With the basic ineffectiveness of chemotherapy, radiation therapy, and established immunotherapy against advanced, metastatic RCC, interest in such RCC-associated antigens is strong.

a. Diagnosis

Malignant transformation of proximal tubule cells may lead to expression of cell surface proteins that are not expressed or expressed in much lower quantities on the non-transformed cells (Oosterwijk et al., 1986A). Such antigens may or may not be restricted to one tumor type and in addition may be present on unrelated normal adult or fetal tissues (Old, 1981). Table 1 lists MAbs that recognize RCC-associated antigens. Studies by Finstad et al. (1985) and Oosterwijk et al. (1986B) have not been able to detect consistent differences in antigen expression in primary versus metastatic RCC tumors. Both of these groups as well as others (Chiou et al., 1985; Kaiser et al., 1988; Dijk et al., 1989) acknowledge that their respective panels of MAbs also react with normal tissues (including normal kidney in some cases) to various degrees.

Three potential diagnostic applications of RCC-reactive MAbs are 1) the localization of occult metastatic disease, 2) the development of serum tests for tumor-related antigens to monitor disease, and 3) the imaging and identification of questionable renal masses (Yagoda and Bander, 1989). Several studies have been reported that indicate the significance of using MAbs in the detection of RCC tumors. Tumor-bearing human kidneys have been used in an *ex vivo* perfusion model to screen MAbs that recognize RCC-associated antigens for diagnostic potential *in vivo* (van Dijk et al., 1988).

Table 1

MAbs that Identify RCC-expressed Glycoproteins

MAb	Antigen Characteristic	Refs.
URO-2	160 kD gp	Ueda et al., 1981 Ebert et al., 1990
URO-3	140 kD gp; amino peptidase N	ibid.
URO-4	120 kD gp; adenosine deaminase binding protein	Ueda et al., 1981 Andy et al., 1984
URO-7	115 kD gp	Ueda et al., 1981 Bander, 1984
URO-10	85 kD gp	Fradet et al., 1986
S23	120 kD gp (reducing SDS-PAGE)	Ueda et al, 1981 Bander, 1984
S6	120 kD gp (non-reducing SDS-PAGE)	ibid, ibid.
AJ2	Multiple Mr forms of gp; 28, 140, 170 kD	Bander, 1984
R23	130 kD gp	ibid
C26	40 kD	ibid
S25	Undetermined Mr; heat labile gp	Ueda et al., 1981
G250	Undetermined Mr gp	Oosterwijk et al., 1986
RC4	170 kD gp	Oosterwijk et al., 1988
K45	Recognizes 150 and 177 kD gps	Luner et al., 1986
K20	Unknown Mr with gp characteristics	ibid
T5A7	Lactoseries type II antigen (LSII): lactosylceramide	Fukushi et al., 1988
1B2	LSII: lactoneotetraosylceramide (paragloboside)	ibid
FH2	LSII: Le^x	ibid

Table 1 (cont.)

MAb	Antigen Characteristic	Refs.
FH4	LSII: difucosyl Le^x	ibid
FH6	LSII: sialosyl-difucosyl Le^x	ibid
KRC-1	135 kD gp	Tokuyama and Tokuyama, 1988
MCA	350 kD secretory mucin	Ammon et al., 1994
BW 495/36-P	200+ kD gp	ibid
EGP-2	38 kD gp	Leij et al., 1994 Kroesen et al., 1994

The kidneys were perfused with ^{99m}Tc-labeled G250 (Oosterwijk et al., 1986A) and RC38 (Oosterwijk et al., 1986B) MAbs in order to visualize tumors via gamma scintillation images. Using this model the investigators were able to demonstrate increased MAb uptake in RCC tissues, with maximal tumor-to-kidney tissue ratios of 8.6 and 2.7, respectively. In a similar study, human kidneys were perfused with a ^{99m}Tc-labeled MAb to γ-glutamyltransferase (GGT) (Fischer et al., 1992). Their study indicated that GGT uptake by RCC tumors was up to 25-fold greater than by the surrounding normal kidney tissue. The reason for this dramatic increase in tumor uptake of labeled MAb can be explained, not by GGTs tumor-specific expression, but rather by its cellular site of expression. The polarized luminal localization of GGT in the proximal tubule cells of normal kidney greatly diminishes contact with the blood flow or perfusion fluid. In contrast, in tumors GGT is located on the outer, contraluminal side of the tumor cells (Fischer et al., 1991), where it is more accessible to circulating MAb.

b. Therapy

Monoclonal antibodies have also been used in several studies to determine their possible therapeutic advantage over other more conventional therapies. The cytotoxic potential of MAbs alone, or in combination

with cytotoxic agents or in combination with cytokines, when targeted to RCC tumor cells has been investigated in both *in vitro* and *in vivo* systems. In an early *in vitro* study (van Dijk et al., 1989), bi-specific MAbs were developed by somatic hybridization of two mouse hybridomas, one producing MAb against the G250 RCC-associated antigen (Oosterwijk et al., 1986A) and the other against the T-cell receptor antigen CD3 (Meuer et al., 1983). These dual specificity hybrid MAbs were then used to test the ability of the complex to recognize the RCC-related antigen G250 in various RCC cell lines and subsequently bind to cytotoxic T cells (CTLs) via the CD3 antigen. The results demonstrated the cytolysis of RCC cell lines via the activity of the CTLs. In a similar study, Kerr et al. (1990) chemically produced bi-specific MAbs (anti-CD3 x anti-tumor MAb) to direct human lymphocyte effector cells (TILs) to RCC cells *in vitro*. Results with RCC antigen-associated MAbs URO 3 (Ebert et al., 1990) or K20 (Luner et al., 1986) linked to anti-CD3 MAbs indicated that bispecific MAbs may be useful as immunotherapeutic reagents for directing the killing of RCC tumor cells by autologous TILs. Studies such as these indicate the potential for targeting RCC tumor cells *in vivo* using MAbs to RCC-associated antigens.

The use of MAbs to localize in human RCC tumors growing in nude mice has also been studied. Singh et al. (1991) investigated the localization of MAb DAL K29

(Luner et al., 1986) conjugated to methotrexate (MTX)-containing liposomes in a nude mouse ascites tumor model using the human RCC line Caki-1. Their results indicated that the MAb-conjugated liposomes containing MTX prolonged the survival of Caki-1 xenografted mice at a dose level of MTX at which the MTX alone had no effect. The lack of therapeutic efficacy of non-MAb-conjugated MTX liposomes demonstrated the importance of carrier antibody localization in the RCC tumor for the antitumor effect of the conjugate. Studies using solid tumor models in nude mice have also demonstrated promising results for the use of RCC-localizing MAbs. For example, Van Dijk et al (1994) have suggested that combination therapy, consisting of interferon-α, TNF-α, and MAb G250, may also have therapeutic value in the treatment of RCC.

These preclinical results from *in vitro* and *in vivo* experiments have provided the impetus for testing the therapeutic efficacy of MAbs in man. The use of MAbs alone or combined with cytotoxic agents and other biological response modifiers for directed RCC tumor cell imaging and/or destruction is now moving into the clinical trial stage. A recent study by Oosterwijk et al (1993) defined the imaging and biodistribution characteristics of ^{131}I-labeled MAb G250 in patients diagnosed with RCC. None of the patients had received prior chemotherapy, radiation therapy, or immunotherapy. Results of intravenous (i.v.) administration of the

labeled MAb gave clear tumor images in 12 of 13 patients with G250-positive tumors and in 1 of 3 patients with G250-negative tumors (possibly caused by the presence of a few G250 positive tumor cells or the nonspecific accumulation of labeled MAb). Another phase I trial investigated the toxicity and immunomodulatory effects of combined treatment with i.v. bispecific MAb BIS-1 and subcutaneous (s.c.) IL-2 in RCC patients that had not responded to single agent s.c. IL-2 alone (Kroesn et al., 1994). The bispecific MAb, BIS-1, is reactive to both the CD3 complex on all T lymphocytes and a pancarcinoma-associated antigen called epithelial glycoprotein (EGP-2) (De Leij et al., 1994). Only one partial remission response was observed in 14 evaluative patients. Side effects, including transient leukopenia and preferential depletion of CD8+ T lymphocytes from the blood, were observed, although the treatment did endow T lymphocytes with a specific anti-EGP-2-directed cytotoxic potential.

The demonstrated potential use of MAbs as diagnostic or therapeutic agents warrants continued study to fully exploit the use of this type of immunotherapy. A better understanding of antigens that typify the phenotype of RCC cells will also play an important role in the diagnosis and/or therapy of RCC. The plausibility of bringing MAbs from laboratory studies into clinical trial programs has been established. The diagnostic and therapeutic

implications for the use of MAbs is an important step in improving the life expectancies of RCC patients (Yagoda and Bander, 1989).

The Significance of Glycoproteins in the Cancer Phenotype

Aberrant glycosylation in human cancer was implicated in several immunological studies performed more than 50 years ago (cited in Hakomori, 1988). Aberrant glycosylation of glycoproteins was subsequently detected by Buck et al. (1970) by observing changes in the gel filtration pattern of glycopeptides from the cell surface. However, the biochemical basis of this aberrant glycosylation phenomenon was not known until 1976, when Ogata et al. discovered that multiantennary carbohydrate structures attached to cell surface proteins were more prevalent on transformed than on normal cells. These original observations have since been extended to human tumor cell systems.

Since the introduction of the hybridoma technique by Köhler and Milstein (1975), MAbs have been used successfully to define the chemical nature of a large variety of tumor-associated antigens. Many tumor-associated antigens defined by MAbs were found to be altered forms of carbohydrate-containing molecules such as glycolipids, proteoglycans, and glycoproteins. The altered glycosylation of some cell surface glycoproteins is probably responsible for many antigenic differences

between carcinomas and adjacent, normal epithelial tissue (Gendler et al., 1991; Devine and McKenzie, 1992). In addition, recent advances in the amino acid sequence analysis of glycoproteins has shown that heterogeneity is also due to differences in the protein backbone to which carbohydrates are attached, possibly the result of mRNA splice variants produced as part of new the cancer cell phenotype (Yonezawa et al., 1991; Verma, 1994).

Elucidation of the structure and function of tumor-associated glycoproteins has obvious importance in cancer research, diagnosis, and therapy. For example, mucinous glycoproteins associated with cancer cells may actively promote tumor cell attachment to normal cells, create an extended functional domain to shield the tumor cell from self aggregation (therefore promoting metastasis), or shield the tumor cell from immune system responses (Devine and McKenzie, 1992). Other glycoproteins that are the result of aberrant modifications in protein backbone or carbohydrate structure may be potential vaccine candidates (Strous and Dekker, 1992). Another useful application of cancer-associated glycoproteins would be in the production of MAbs that recognize tumor-associated or tumor-specific epitopes, based on their unique carbohydrate and/or protein backbone structures.

Glycoproteins and Monoclonal Antibodies in RCC

As indicated by Table 1, there are a large number of MAbs that identify glycoprotein antigens expressed in RCC tissues. However, none of the glycoproteins associated with RCC discovered thus far are uniquely expressed on tumor cells. For example, the URO series of MAbs (refer to Table 1 for specific refs.), developed and characterized by Old's group, demonstrates varying degrees of specificity for RCC cells, normal kidney cells, other normal and fetal cells, and other tumor cell types. The URO MAbs are mainly used to identify different normal kidney cell types (ie., glomeruli, proximal tubules, collecting ducts, and portions of Henle's loop) and subsets of RCC tumor types (ie., clear cell, granular cell, and spindle cell). Other RCC-associated MAbs (and their respective glycoproteins) that have been reported in the literature also appear to recognize similar antigens on various normal fetal and normal adult cells as well as cancer cells. Although MAbs that recognize RCC-associated glycoproteins also bind to normal cells, they still may have diagnostic and therapeutic applications.

For example, a study done by Nanus et al. (1990) showed that the presence of a 160 kilodalton (kD) glycoprotein on some RCC cell subsets may predict the sensitivity of these tumor cells to the antitumor effects of interferon-α. Their study of several RCC cell lines indicated that those cell populations which

expressed the 160 kD glycoprotein resisted IFN-α mediated cell death. Also, *in vivo* studies using tumor xenografts in nude mice formed from glycoprotein 160-positive cell lines demonstrated resistance to IFN-α therapy. Another study from the same laboratory identified a tyrosine phosphorylated glycoprotein that is preferentially expressed in RCC cell lines but is not expressed in normal proximal tubule cells *in vitro* (Hoffman et al., 1994). The future exploitation of MAbs to RCC-associated glycoproteins, such as the two mentioned above, shows signs of promise as diagnostic and/or therapeutic tools.

However, there is still a great deal to be learned about RCC tumor glycoprotein biology. Important areas of research include 1) the mechanisms of regulating carbohydrate expression at the transcriptional and post-transcriptional levels, 2) the functional concept of carbohydrate expression and the phenotypic changes associated with oncogenesis, and 3) the mechanism of immune recognition of carbohydrate epitopes.

Preliminary characterization of MAb 469-5A has indicated that it recognizes a previously undescribed glycoprotein expressed on most RCC tumor cells, on some normal kidney proximal convoluted tubule epithelial cells, and on a variety of other normal and cancer tissues. This work describes the molecular characterization and distribution of expression of the MAb 469-5A target antigen.

Research Goals

The goals of this research were to produce MAbs that identify proteins overexpressed on RCC cells. These goals were accomplished by meeting the following objectives:

1. By producing a MAb, 469-5A, to an overexpressed RCC protein.
2. By examining the range of reaction of MAb 469-5A.
3. By examining the structure of the antigen recognized by MAb 469-5A.

CHAPTER II

METHODS AND MATERIALS

Mice

BALB/c/Bd mice (Oak Ridge colony) were bred and maintained in a specific-pathogen-free facility. Animals were fed a commercial diet (Purina Rodent Chow, gamma sterilized), given water ad libitum, and maintained on a 12 hour light/dark cycle in a temperature/humidity controlled environment (21 $\pm$ 1^{o}C; 46%). Female mice were used between 8 to 12 weeks of age.

Cell Lines

A human renal cell carcinoma cell line was obtained from Dr. Neil Bander of The New York Hospital-Cornell Medical Center Urology Department. This cell line, designated, SK-RC-7, was established from a primary RCC tumor that displayed main renal vein involvement but no metastatic disease at the time the tumor was removed from the patient (Ebert et al., 1990).

The cell line was maintained in Ham's Modified MCDB 107 medium (McKeehan and McKeehan, 1980) containing 10% fetal calf serum (FCS, Bio-Whittaker), 30mM HEPES (J.T. Baker, Inc., Phillipsburg, NJ), traces of inorganic salts and trace elements, 1000U/ml heparin (Grade 1-A, from porcine

intestinal mucosa, Sigma, St. Louis, MO), 2 mM L-glutamine, 100 units per ml penicillin and 100 µg per ml streptomycin. Cells were cultured in 100 mm plastic culture dishes (Falcon, Oxnard, CA) and passaged weekly at a ratio of 1:10 (approximate plating density of 1.5×10^6 cells per plate).

A mouse mammary tumor cell line, EMT6, from the laboratory of Dr. Steve Kennel (Oak Ridge National Laboratory Biology Division, Oak Ridge, TN) was used as a negative control in some experiments. The cell line was grown in Dulbecco-Vogt Modified Eagles Minimal Essential Medium containing 10% FCS.

ELISA Assay

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described (Engvall and Perlmann, 1971). Briefly, 96-well Immulon 4 flat bottom plates (Dynatech Laboratories, Inc., Chantilly, VA) were used to bind whole cell lysate as a solid state antigen source. Antigen was bound to plates by first adding 50 µl per well of a 1/500 dilution of a 50 mg/ml poly-L-lysine (PLL) stock solution in 0.01 M sodium phosphate plus 0.15 M NaCl (PBS). The PLL was allowed to bind to the wells for 2 hrs at 4°C on a rotator plate angled at 60° from normal. The PLL solution was then aspirated, and 50 µl of 1% glutaraldehyde in PBS was added to each well for 5 mins at room temperature to provide crosslinking between the antigen source and the PLL. The 1%

glutaraldehyde solution was then aspirated, and 50 µl of the antigen source (approximately 1 - 2 µg total protein) was added per well. The protein antigen was allowed to bind for 8 - 12 hrs at 4°C on a rotator plate angled at 60° from normal. Following this incubation period, 100 µl of 25 mg/ml BSA was added to each well for 1 hr and further incubated at 4°C for 1 hr.

Antigen-bound plates were then aspirated and washed 3X with PBS. Primary antibody (MAb 5A) was then added to each well, either in growth medium (for hybridoma screening) or diluted in 5 mg/ml BSA in PBS (for ascites or purified MAb screening), in a total volume of 50 µl per well. Primary antibody was incubated at 4°C on a rotator plate angled at 60° from normal for 2 hrs. Media was then aspirated from the wells, the wells washed 3X with PBS, and 50 µl per well of a 1/1500 dilution of Goat-Anti-Mouse, horse radish peroxidase conjugated (GXM HRPO) antibody (BioRad, Richmond, CA) diluted in 5 mg/ml BSA in PBS was added. The secondary GXM HRPO antibody was incubated at 4°C on the rotator. The solution was then aspirated, wells were washed 3X with PBS, and an ABTS Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) was used to determine MAb binding. The intensity of color formation was then visually scored or measured using an EIA Reader (Model 2550, BioRad).

Production of Monoclonal Antibody 469-5A

BALB/c/Bd mice were immunized with a human renal cell tumor tissue homogenate, containing approximately 5-10 µg total protein, in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) ip. The patient from which the tumor sample was take was a 69 year old male. The tumor was classified as a clear cell carcinoma. The tumor tissue was stored at -70°C until used to prepare a tumor whole cell lysate as the immunogen source. Briefly, whole cell lysates were prepared using one gram of tumor tissue combined with a cocktail containing 10 µg/ml phenylmethylsulfonylfluoride (PMSF) (Sigma), 10 mg/ml leupeptin (Sigma), and 0.5% Nonidet-40 (Particle Data Laboratories LTD, Elmhurst, IL) in 10 mls cold PBS. The mice were subsequently boosted ip at two weeks with the original immunogen in incomplete Freund's adjuvant (Difco). Prior to the production of hybridomas (Köhler and Milstein, 1975), immunized mice were bled and the serum titer was determined against the original immunogen using an ELISA. Hybridomas were derived from the fusion of immune mouse spleen cells with mouse myeloma SP2/O cells (Shulman et al, 1978) according to the procedure of de St Groth and Scheidegger (1980). Fusion products were selected based upon their ability to grow in hypoxanthine-aminopterin-thymidine medium (Littlefield, 1964). To increase the plating efficiency of hybridomas, normal peritoneal exudate cells were used as a feeder layer as previously described (de

St. Groth and Scheidegger, 1980), except Dulbecco-Vogt Modified Eagles Minimal Essential Medium containing 20% FCS was used throughout. Hybridoma supernatant fluids were preliminarily screened by ELISA for antibody to human RCC cell antigens using the immunogen whole cell lysate as the antigen source. Secondary screening was carried out in a similar manner on all positive hybridomas from the preliminary ELISA screen, using the same antigen source as well as using normal human serum as a negative control. Positive cultures from the secondary screening were cloned by limiting dilution before expansion for large-scale antibody production. MAb 469-5A containing ascites fluid was prepared in BALB/c/Bd mice that had been primed with pristane (2,6,10,14-tetramethylpentadecane, Aldrich Chemical Company, Inc., Milwaukee, WI) and inoculated i.p. with 5×10^6 cloned hybridoma cells in PBS. Immunoglobulin was isolated from ascites fluid by precipitation with saturated ammonium sulfate, followed by DEAE-celluose chromatography (DE52; Whatman Chemical Separation, Maidstone, Kent) using a 0.0 to 0.5 M NaCl gradient in 10mM phosphate buffer, pH 8.0 (Kennel et al., 1981). MAb 469-5A was then purified by high performance liquid chromatography (HPLC) using a Waters HPLC Protein Pak Column DEAE 5PW (Millipore Corporation, Milford, MA) and NaCl gradient elution (Kennel et al., 1990).

Isotyping of MAb 469-5A

The isotype of MAb 469-5A was determined using Mouse Monoclonal Antibody Isotyping Kit (Sigma). The method used

was an antigen-mediated ELISA, employing the original immunogen as the solid phase antigen source. The subclass was determined to be IgG_1.

Immunohistochemistry of Human Tissues

Human renal carcinoma and normal tissues, from surgical or autopsy specimens obtained through The Jewish Hospital of Cincinnati, OH, were fixed in 10% buffered formalin before embedding in paraffin. Sections used for immunohistochemistry (IHC) were cut at a thickness of 4-5 μm and placed on ProbeOn (Fisher Scientific, Fair Lawn, NJ) glass slides. Slides from individual patients' tissues were stained with hematoxylin and eosin by use of standard protocols. IHC was also done using Multitissue Normalgrid and Tumorgrid slides (Biomedia Corp., Foster City, CA) containing multiple samples of various normal tissue types or various tumor tissue types fixed overnight in standard 10% neutral buffered formalin.

IHC staining was done on xylene deparaffinized sections that were rehydrated through graded ethyl alcohol. Sections were then blocked, to decrease background staining caused by non-specific binding of primary and secondary antibodies, by incubating for 20 min in 2% normal goat serum (NGS) in PBS. The sections were then placed in a humidified box and treated for 2 hours with purified MAb 469-5A antibody used at a concentration of 10 μg antibody per ml 2% NGS. Slides were then washed in PBS (2X; 5 min each) and then incubated in 1% H_2O_2 in 100% MeOH for 15 min to destroy endogenous peroxidase

activity. Slides were washed once again with PBS (1X; 5 min), blocked again in 2% NGS for 5 min, and incubated for two hours in a humidified box with GXM HRPO antibody (BioRad) diluted 1/1500 in 2% NGS. Slides were again washed in PBS (2X; 5 min each) and then treated with 3,3'-diaminobenzidine (DAB; Sigma) substrate (5 mls of 1 mg/ml DAB in 0.1 M Tris-HCl, pH 7.2 added to 7.5 mls dH_2O containing 5 µl 30% H_2O_2) for 5 min. To stop the reaction, slides were washed in distilled water (dH_2O) for 5 min prior to graded alcohol and xylene dehydration. Sections were coverslipped using Permount (Fischer Scientific).

^{125}I-Labeling of MAb 469-5A

MAb 469-5A was radioiodinated as previously described (Kennel et al., 1981) using limiting amounts of chloramine T (Sigma). Briefly, 50 µg of antibody was incubated with 500 µCi of $^{125}I^-$ (Amersham Corp., Arlington Heights, IL) with 5 µl of the oxidizing agent chloramine T (1 mg/ml dH_2O). The coupling reaction was stopped by adding 5 µl of sodium bisulfite (2 mg/ml dH_2O) (J.T. Baker), and the labeled MAb was separated from the free $^{125}I^-$ and protein aggregates by size exclusion chromatography on Ultrogel AcA-34 (Pharmacia LKB Biotechnology Inc., Rockville, MD). Fractions containing labeled MAb were pooled, the MAb fraction recovered was estimated based on total radioisotope in the pooled fraction,

and the concentration of labeled MAb was estimated based on the total volume of the pooled fraction (Kennel et al., 1983).

Western Blot Analysis

Tissue samples were obtained from surgical or autopsy specimens obtained through The Jewish Hospital of Cincinnati, OH, the University of Tennessee Memorial Hospital in Knoxville, or Saint Mary's Hospital in Knoxville and stored frozen at -80°C. The use of human tissue samples was performed under the permission of and the guidelines set forth by the Human Use Committee at ORNL and the University of Tennessee. The protein concentration of tissue and cell line lysates (prepared as described above) were determined using the Lowry assay (Lowry et al., 1951). Lysate proteins were combined with 1% SDS (sodium dodecyl sulfate)/8M urea, separated by SDS-PAGE (polyacrylamide gel electrophoresis), and Western blotted onto membranes as previously described (Towbin et al., 1979.). SDS-PAGE separated proteins were transferred to a 0.45 μm Immobilon-P Transfer Membrane (Millipore Corporation, Bedford, MA) in a Hoefer TE Series Transphor apparatus (Hoefer Scientific Instruments, San Francisco, CA) at 200 mA at approximately 20°C for 1 hour.

Blots were developed using two different detection methods. Immunoperoxidase detection was accomplished by first washing the transfer membrane overnight in 0.1% Tween-20 in PBS. The membrane was then blocked, to decrease

background detection, by incubating with Blotto-Plus (5% powdered milk, 0.05% Tween 20 in PBS, 10% FCS) for 1 hour at room temperature. After blocking, the membrane was incubated at room temperature with 10 μg/ml purified MAb diluted in Blotto-Plus for 2 hours, followed by washing with 0.1% Tween-20 in PBS (4X; 5 min each). The membrane was then incubated at room temperature for 2 hours with a 1/1500 dilution of GXM HRPO antibody (BioRad) diluted in Blotto-Plus, followed by a repeat of the washing steps as previously described. The membrane was then stained using a DAB substrate (5 mls of 1 mg/ml DAB in 0.1 M Tris-HCl, pH 7.1 added to 5 mls H_2O containing 5 μl 30% H_2O_2) for approximately 5 min at room temperature. The reaction was stopped by washing the membrane in dH_2O. Molecular weight markers (BioRad) were separated and transferred in parallel lanes and stained on the Immobilon-P filters using Coomassie Blue R-250 (Eastman Kodak Co., Rochester, NY) in 50% methanol plus 10% glacial acetic acid.

A second method of detection was employed utilizing ^{125}I-labeled MAb. The washed membrane was blocked with Blotto-Plus as previously described. The membrane was then incubated for 3 hours at room temperature in Blotto-Plus containing 500 ng/ml of ^{125}I-MAb (approx. 8,500 to 10,000 cpm/ng MAb). The membrane was then washed in 0.1% Tween-20 in PBS (4X; 5 min each), dried at room temperature, wrapped in plastic wrap, and placed on X-Omat™ RP XRP-5 Diagnostic

film (Eastman Kodak Co.) for autoradiography using double intensifier X-ray Exposure Holders (Eastman Kodak Co.).

Enzymatic Digestion of Carbohydrate Residues

Whole cell and whole tissue lysates were treated with neuraminidase (Sigma) to cleave terminal sialic acid residues from the 469-5A antigen as determined by the effect of treatment on the electrophoretic mobility of the glycoprotein. Five microliters (approx. 15 µg total protein) of each whole cell lysate was treated with 1 µl neuraminidase (1 unit/mg; 10 mg/ml) in the presence of 5 µl of 0.15 M sodium acetate (NaAc), pH 5.5 and 4 µl of Milli-Q water (total reaction volume of 15 µl) in a 37°C water bath. The optimal incubation time was experimentally determined. The digestion reaction was stopped by addition of an equal volume of 1% SDS/8M urea. The sample was then heated to 90°C for 10 minutes, cooled, and electrophoresed as previously described.

To further examine the effect on electrophoretic mobility caused by enzymatic removal of serine/threonine and/or asparagine-linked carbohydrates, SK-RC-7 RCC cell line whole cell lysate and IAC purified gp 45/55 were treated with N-glycanase (Genzyme Corp., Cambridge, MA) alone, neuraminidase and O-glycanase (Genzyme Corp.), or N-glycanase, neuraminidase, and O-glycanase. Details of the digestion conditions are presented in the legend for Figures 9 and 10.

Determination of Binding Affinity Constant ± Neuraminidase

The binding affinity constant (K_a) for MAb 469-5A was determined before and after treatment of a RCC whole cell lysate with neuraminidase. RCC lysates were digested with neuraminidase as described above. Samples without neuraminidase were given 1 µl Milli-Q water instead of enzyme. The methods used to determine binding of MAb to protein antigen bound to solid supports has been described previously (Kennel, 1982). Briefly, proteins from RCC whole cell lysate (approximately 1 - 2 µg per well) were bound using a PLL-glutaraldehyde bridge to Immulon 4 Removawell Strips (Dynotech Laboratories). Wells were washed 3X with PBS. The microwells were then blocked for two hours with 25 mg/ml BSA prior to addition of labeled MAb. Two-fold dilutions of ^{125}I-labeled MAb 5A in 5 mg/ml BSA were used, starting at 200 ng down to 6.25 ng, in duplicate microwells to assess the binding to gp 45/55. Labeled MAb was incubated with the antigen source for three hours at room temperature on a rocker platform. Microwells were then carefully washed 3X with PBS prior to individual wells being counted for 1 minute in a Beckman Gamma 5500B counter (Beckman Instruments, Inc., Fullerton, CA).

The amount of bound ^{125}I-labeled MAb 5A was determined by averaging the counts per minute (cpm) in duplicate wells and dividing by the number of cpm per ng of labeled MAb 5A in

1 µl of stock labeled MAb 5A. The amounts of bound MAb 5A versus the amounts added of MAb were plotted on a double reciprocal plot. The K_a of MAb for gp 45/55 before and after neuraminidase digestion was determined graphically as described previously (Kennel, 1982).

469-5A Antigen Purification Procedure

Protein used for amino acid analysis was purified from the SK-RC-7 RCC cell line. Cells were grown to confluence in 100 mm culture dishes as previously described, usually in sets of 180 to 200 dishes. The cells were scraped from plates using a plastic policeman after the addition of 1 ml of lysis buffer (10 mls cold PBS, 10 µg/ml PMSF, 10 µg/ml leupeptin, 0.5% NP-40) per 5 plates. The lysate was stored on ice until it was briefly sonicated to disrupt membranes and centrifuged at 2000 rpm for 15 minutes to pellet non-soluble components. The sonication step was repeated to fragment DNA and was followed by a centrifugation step of 15,000 rpm for 10 minutes. The resultant supernatant was then incubated for 1 hour (at 4°C with gentle mixing) with BSA-bound (10mg/ml) Sepharose beads (prepared as previously described) to remove non-specific proteins and protein aggregates. The BSA beads were then gently pelleted (1000 rpm for 10 secs) and the supernatant was carefully removed. The whole cell lysate supernatant was then centrifuged for 10 mins. at 15,000 rpm to remove any remaining debris and aggregates.

Wheat germ agglutinin lectin (WGA) beads (Sigma) were added to the whole cell lysate supernatant in a ratio of 20 µl WGA beads per ml of whole cell lysate. This reaction mixture was incubated overnight at 4°C with gentle mixing. The beads were then gently pelleted by centrifugation (1000 rpm for 10 secs), the supernatant was carefully removed from the WGA beads, and the WGA beads were washed (as previously described) with decreasing concentrations of 0.5% NP-40 in PBS. WGA-bound glycoproteins were eluted (three to four times) at room temperature for 5 mins using a 1X volume of 1% SDS/8M urea per elution. The eluates were pooled and dialyzed against 10 mM phosphate buffer, pH 7.0 for 2 hours at 4°C. Before dialysis, BSA was added to the pooled eluates to a final concentration of 10 µg per ml to compete for non-specific sticking of protein to the dialysis bag.

Sepharose bead-linked 5A MAb was added to the dialyzed WGA-bead eluates. KSCN pre-eluted 5A beads (as previously described) were used at a concentration of 10 µl 5A beads per 200 µl of WGA eluant. This reaction mixture was then incubated with gentle mixing at 4°C overnight. The 5A beads were then gently pelleted, supernatant carefully removed from the beads, and beads washed with decreasing concentrations of 0.5% NP-40 in PBS. The 469-5A antigen was eluted from the 5A beads with three equal volumes of 1% SDS/8M urea. This final eluant was also dialyzed against 10 mM phosphate buffer, pH

7.0 at 4°C for two hours and then lyophilized prior to amino acid analysis.

Amino Acid Analysis

Amino acid analyses of the purified 469-5A protein were carried out at the University of Tennessee Memorial Research Hospital in the laboratory of Dr. Alan Solomon. To determine N-terminal sequence of the 469-5A antigen, the concentrated and purified sample previously described was used. One fifth of the total lyophilized sample amount was dissolved in 10% acetic acid, loaded onto polybrene-treated glass fiber disks, and air dried. The disks were placed in an Automated Biosystems Incorporated (ABI) model 477A gas-phase sequenator and the resulting phenylthiohydantoin (PTH) amino acids were identified by using an on-line ABI model 120A PTH amino acid analyzer (Solomon et al., 1994).

To determine total amino acid composition, the remaining portion of the 469-5A protein sample was sealed in a sample container and then placed into a larger vessel containing 6 N HCl solution. This device allows only the acid vapor to come in contact with the sample, therefore excluding non-volatile contaminants. Vapor-phase hydrolysis was done under vacuum. The hydrolysate was then dissolved in 50 µl of a 1 mM EDTA-tripotassium phosphate solution and loaded onto the polybrene-treated glass fiber disk of an ABI model 420A amino acid derivatizer. The resulting phenylthiocarbamyl amino acids were separated by using an ABI model 130A HPLC apparatus (Solomon et al., 1994).

CHAPTER III

RESULTS

Characteristics of Monoclonal Antibody 469-5A

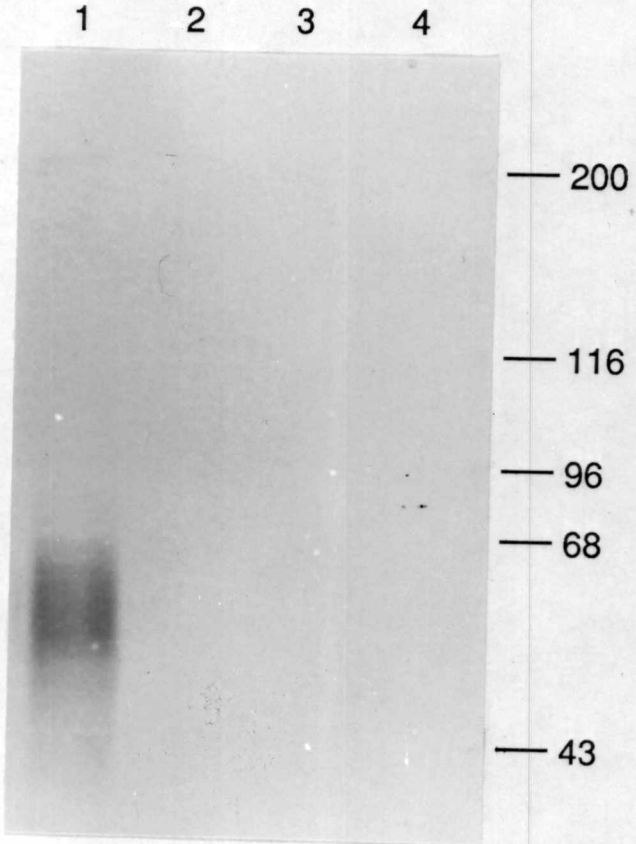
a. Binding to Reduced vs. Non-reduced Antigen

Protein samples known to contain the target antigen were separated by SDS-PAGE in the presence (reducing) or absence (non-reducing) of 2-mercaptoethanol. In Western blot analyses, monoclonal antibody 469-5A (MAb 5A) binds to a 45 to 55 kDa glycoprotein in the membrane protein fraction of human RCC tissue under non-reducing conditions (Figure 2, lane 1). When mercaptoethanol was added to reduce disulfide bonds in this protein fraction, no MAb 5A reactive protein was detected on the Western blot (Figure 2, lane 2). The binding of MAb 5A to its epitope in all normal and RCC kidney tissue samples as well as other normal and cancer tissues was lost when samples were reduced. This indicates the presence of disulfide bridge structures within the protein recognized by MAb 5A.

b. Increased epitope recognition after treatment of tissue samples with Neuraminidase

Known sources of gp 45/55 were separated by SDS-PAGE. However, prior to electrophoretic separation, the protein

Figure 2. The Binding of MAb 469-5A to non-reduced and reduced forms of gp 45/55. Equal amounts (10 μg) of whole cell lysate were separated on SDS-PAGE and transferred to Immobilon-P P membrane. After incubation with MAb 5A, detection of antigen was accomplished using enzyme immunoperoxidase and DAB. Lanes 1 and 3 contain non-reduced samples of RCC and EMT6 (BALB/c mammary tumor cell line) lysate respectively; lanes 2 and 4 contain reduced samples of RCC and EMT6 respectively.


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samples were incubated for two hours with or without the enzyme neuraminidase. Neuraminidase treatment of glycoproteins causes the cleavage of terminal sialic acid residues from carbohydrate chains. The ability of MAb 5A to bind to its target epitope in whole cell lysates from normal kidney tissue, RCC tissue, and SK-RC-7 RCC cell lines was increased after the enzymatic removal of sialic acid residues from carbohydrate side chains (Figure 3, compare lanes 1, 3, 5, and 7 with lanes 2, 4, 6, and 8 respectively), indicating that gp 45/55 antigen probably contains sialic acid. The removal of sialic acid residues may increase exposure of the protein backbone or an inner carbohydrate epitope of gp 45/55 antigen, allowing better MAb 5A recognition and/or binding to its epitope. Removal of sialic acid residues also effected the mobility of gp 45/55 in SDS-PAGE. Observable increases in the downward mobility of detectable gp 45/55 occurred after treatment with neuraminidase (Figure 3).

c. Binding Affinity Constant

The binding affinity constant for MAb 5A was determined before and after treatment of a RCC whole cell lysate with neuraminidase. Data from ^{125}I-labeled MAb binding to gp 45/55 with or without neuraminidase treatment was plotted on a double reciprocal plot (Figure 4). The k_a for gp 45/55 prior to digestion with neuraminidase was determined to be 0.375×10^8 M^{-1}. After digestion with neuraminidase, the value for k_a was determined to be 0.976×10^8 M^{-1}.

Figure 3. Normal kidney tissue, RCC tissue, normal prostate tissue, and RCC cell line SK-RC-7 whole cell lysates were incubated in the presence or absence of neuraminidase for two hours at 37°C. Ten micrograms of total lysate protein was loaded per lane. Glycoprotein 45/55 was detected with ^{125}I-MAb 5A after SDS-PAGE separation and Western blotting. The blot was exposed for 48 hrs at liquid nitrogen vapor phase temperature with double intensifier screens. Lanes contained: normal kidney plus (1) and minus (2) neuraminidase treatment, RCC plus (3) and minus (4), normal prostate plus (5) and minus (6), and SK-RC-7 cell line plus (7) and minus (8).

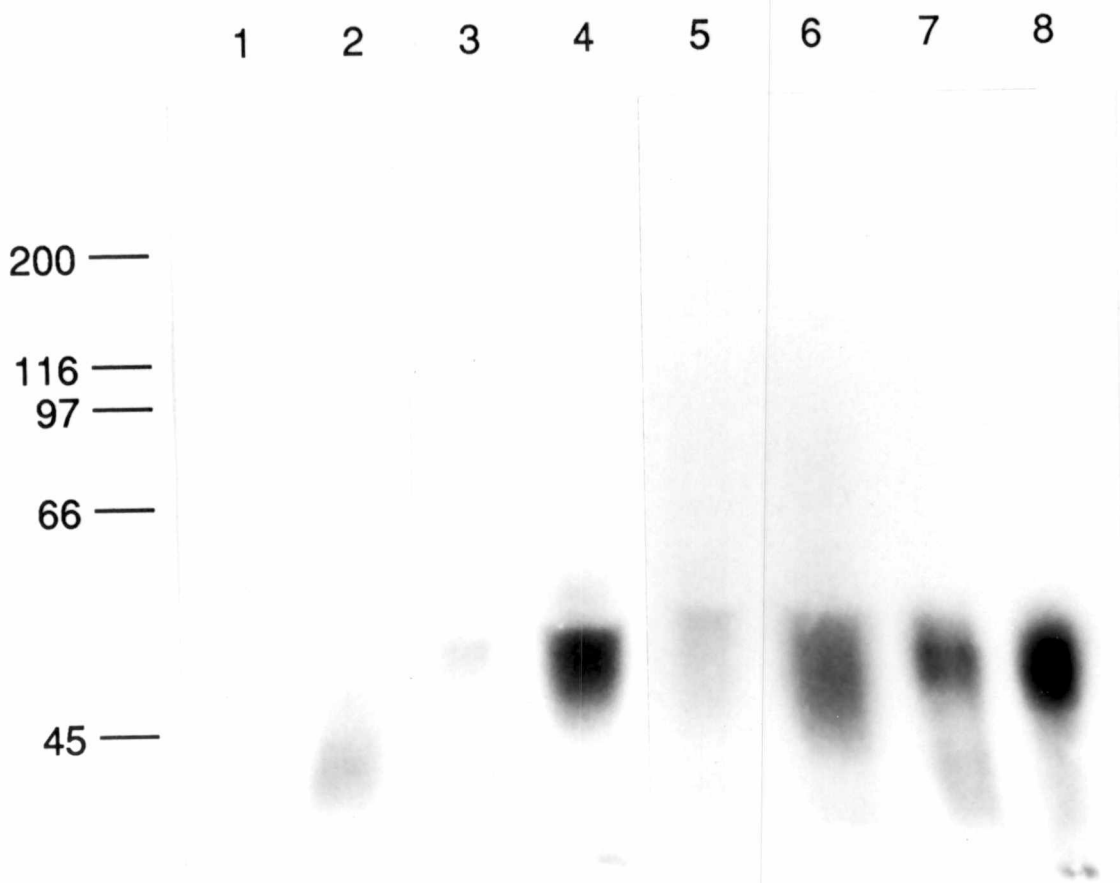
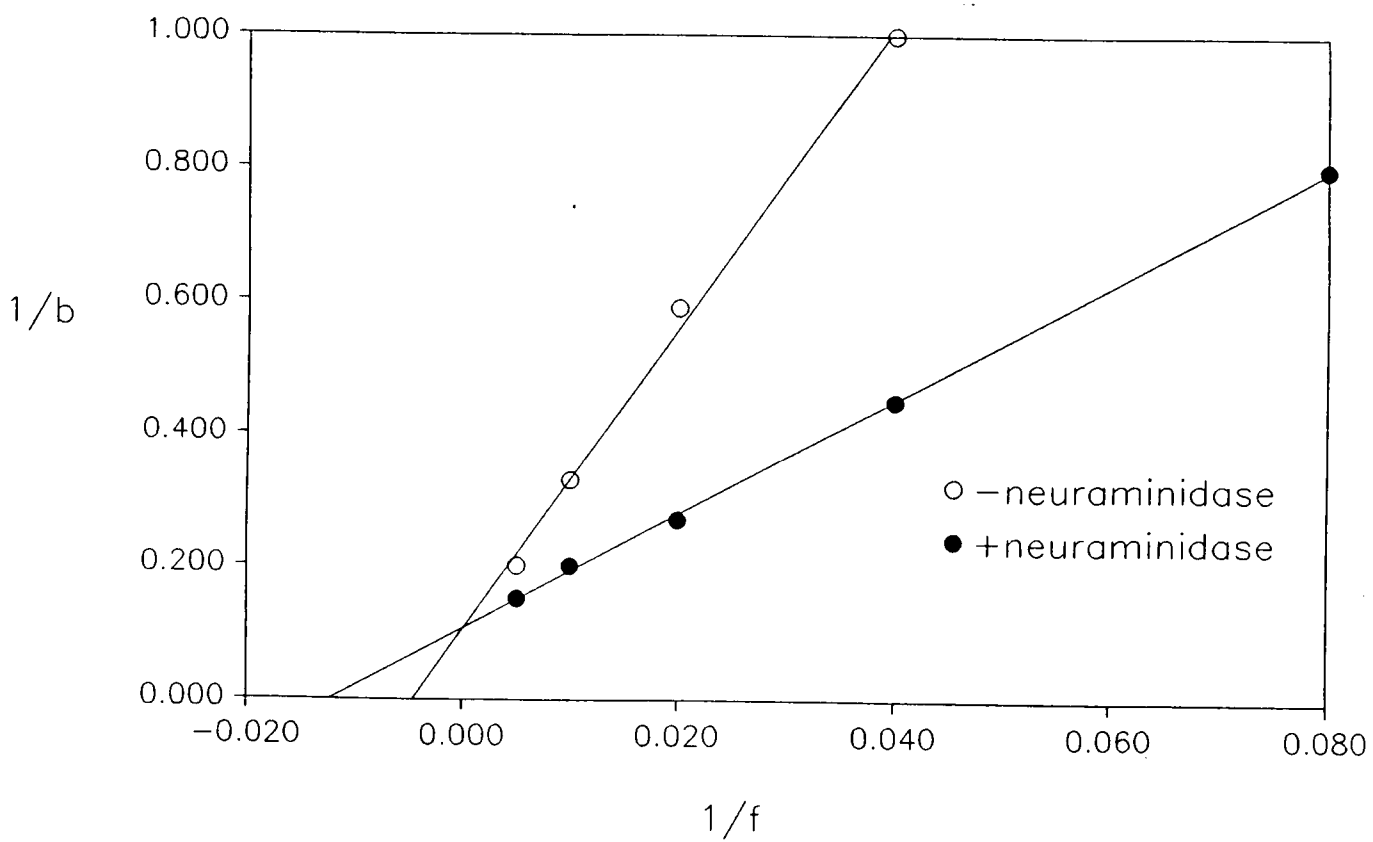

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Figure 4. Double reciprocal plot of ^{125}I-labeled MAb 5A binding to RCC whole cell lysate before (○) and after (●) digestion with neuraminidase.


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Characterization of gp 45/55

a. Variations in Molecular Weight of the 5A Antigen

The molecular target of MAb 5A was identified after non-reducing SDS-PAGE separation of target proteins and Western blot transfer to Immobilon-P membranes. As shown in Figure 5, the SDS-PAGE mobility of the 5A antigen is a broad band ranging from 45 to 55 kilodaltons (kDa). The appearance of a banding pattern this broad implies the presence of several molecular species. Similar gel patterns are seen in cytosolic and membrane fractions of both normal and RCC kidney tissues. However, there is an apparent increased intensity in the staining pattern of the membrane fraction (Fig. 5, lanes 2 and 4) as compared to the cytosolic fraction (lanes 1 and 3) in both the normal and RCC tissues. Likewise, there is an increased intensity in the gp45/55 staining patterns of both the cytosolic and membrane protein fractions of the RCC tissue (lanes 1 and 2) as compared to those from the normal kidney tissue (lanes 3 and 4).

In subsequent experiments, proteins transferred to membranes were detected by direct binding with ^{125}I-MAb 5A followed by autoradiography. This detection method is very sensitive (detecting the presence of as little as 1 to 2 ng of protein) and has low background staining. Use of this antigen detection method demonstrated that the same broad banding pattern was found in all normal and RCC tissue samples examined (Figure 6). Notable variations were observed in the apparent molecular mass (Mr) banding patterns

Figure 5. MAb 5A detection of a broad Mr pattern in Western blot of normal kidney cytosolic and soluble membrane lysates and in RCC cytosolic and soluble membrane lysates. Ten micrograms total lysate protein were loaded per lane and separated by SDS-PAGE. After incubation with MAb 5A, detection of antigen was accomplished using immunoperoxidase with DAB as substrate. Lanes contained: (1) RCC cytosol, (2) RCC solubilized membrane, (3) normal kidney cytosol, (4) normal kidney solubilized membrane, (5) EMT6 solubilized membrane.

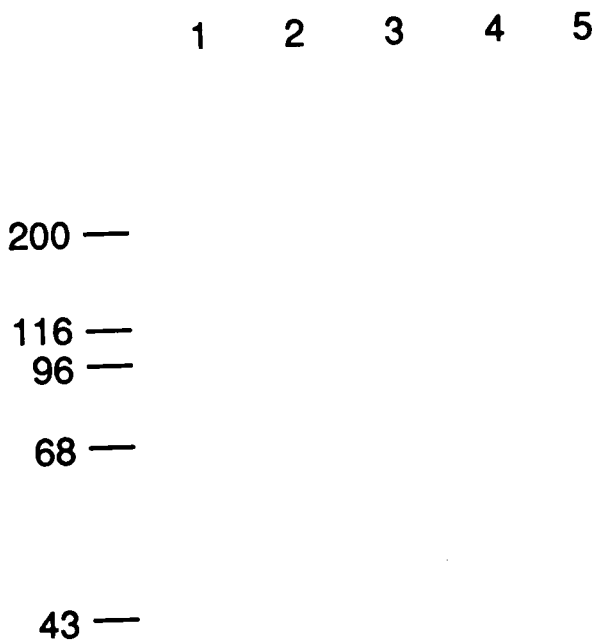
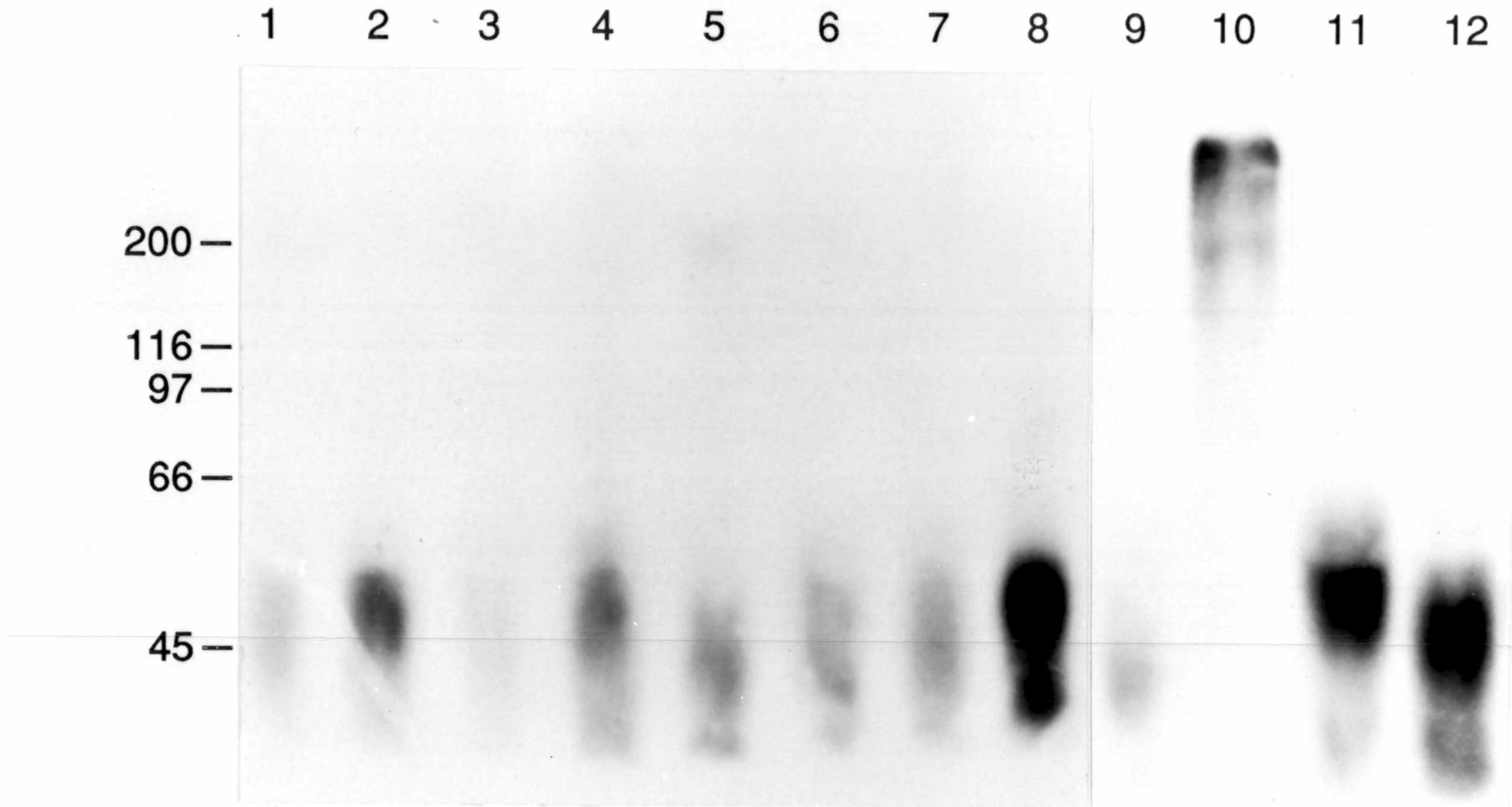

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Figure 6. Banding pattern detected by ^{125}I-labeled MAb 5A in Western blot after two hour neuraminidase digestion of normal kidney, RCC tissues, and SK-RC-7 RCC cell line whole cell lysates. Ten micrograms of total lysate protein were loaded per lane and separated by SDS-PAGE. The blots were exposed for 48 hrs at liquid nitrogen vapor phase temperature with double intensifier screens. Lane 1: 12/11 (date of sample acquisition) normal kidney (NK), lane 2: 12/11 RCC, lane 3: 7/5 NK, lane 4: 7/5 RCC, lane 5: 7/89 NK, lane 6: 1/4 NK, lane 7: 7/23 NK, lane 8: SK-RC-7 RCC cell line, lane 9: 5/14 NK, lane 10: 9/4 RCC, lane 11: 11/20 RCC, lane 12: 6/16 RCC.


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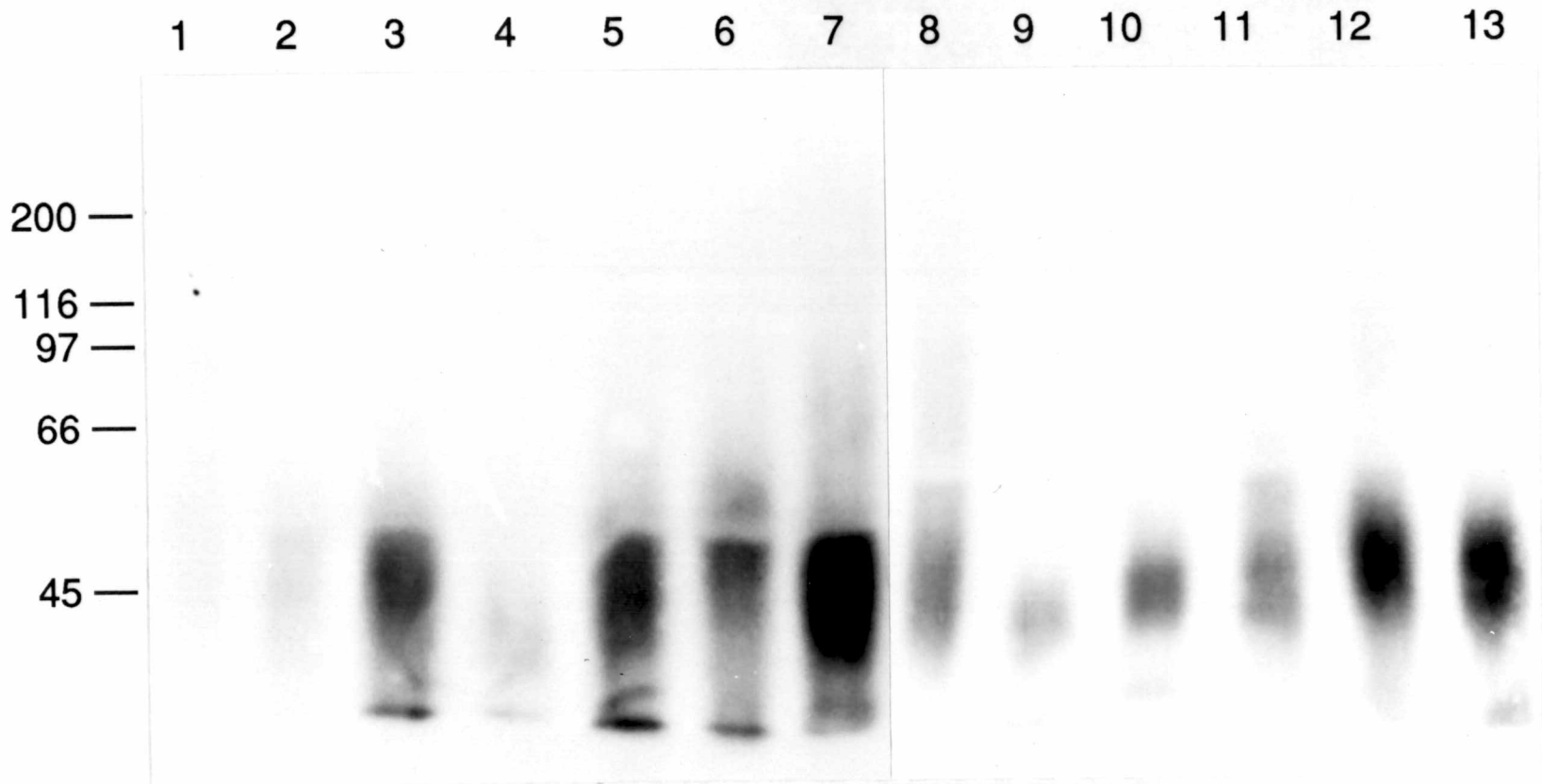
of gp 45/55 in some of these samples. Although the samples from normal and RCC kidney tissue of two patients (Fig. 6, lanes 1 - 4) demonstrate little difference in the gel mobility of gp 45/55, comparisons among other normal and RCC kidney tissues and the SK-RC-7 RCC cell line (Fig. 6, lanes 5 - 12) demonstrated measurable variations in the gel mobility of glycoproteins detected with MAb 5A. The most striking shift from the common gp45/55 pattern was seen in samples from a large (approximately 800 gm kidney), highly necrotic RCC mass (Fig. 6, lane 10). In this sample MAb 5A detected three discernible bands greater than 200 kDa, although there was still some detection of a band at 45 to 55 kDa as well.

To further investigate the variations in gel mobility of gp 45/55, other human tissue samples were examined. The characteristic gp45/55 banding pattern, observed in most normal and RCC kidney samples, was seen in samples from other normal tissue sources (Fig. 7), although variations were observed in the apparent Mr banding patterns of gp 45/55 in a few samples. Comparison of band patterns from different tissue sources in the same patient (for example, lanes 3 and 9) or from the same tissue source in different patients (for example, lanes 3 and 4 or 9 and 10) demonstrated these variations. These observed variations (as much as 5 to 10 kDa) in Mr may be caused by organ-specific and/or by cell-specific glycosylation differences in gp 45/55.

b. Carbohydrate Analyses

The detection signal of gp45/55 in Western blot of

Figure 7. Banding patterns detected by ^{125}I-labeled MAb 5A in Western blot after two hour neuraminidase digestion of normal human tissue whole cell lysates. Ten micrograms of total lysate protein were loaded per lane and separated by SDS-PAGE. The blots were exposed for 72 hrs at liquid nitrogen vapor phase temperature with double intensifier screens. Samples are identified by the date on which they are acquired. Lane 1: 1364 bronchi, lane 2: 6/6 lung, lane 3: 12/8 lung, lane 4: 7/5 lung, lane 5: 10/3 lung (upper lobe), lane 6: 10/3 lung (lower lobe), lane 7: 1364 prostate, lane 8: 1437 colon, lane 9: 12/8 liver, lane 10: 7/5 liver, lane 11: 1420 small intestine, lane 12: 1420 spleen, lane 13: 7/5 spleen.


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normal kidney, RCC, and SK-RC-7 RCC whole cell lysate proteins increased after the antigen source had been treated with neuraminidase. As shown previously in Figure 3, intensity of bands detected with radiolabeled MAb from neuraminidase treated samples was approximately 5- to 10-fold (depending on the tissue sampled) greater than in untreated samples. Table 2 presents data from image analysis evaluations (Molecular Dynamics Model 300A Computing Densitometer and ImageQuant software, Sunnyvale, CA) of these band intensities. Direct densitometry comparisons of gp 45/55 detected in whole cell lysates with or without neuraminidase treatment from a normal kidney, a RCC, a normal lung, and the SK-RC-7 cell line are also included in Table 2. Other tissue sources of gp45/55, including various normal human tissues were also tested (for examples see Fig. 8A). Each sample that contained detectable amounts of gp45/55 demonstrated an increase in the amount of detectable gp after neuraminidase treatment of the 5A antigen source.

Increases in the detection of gp 45/55 varied between different tissue sources as well as between similar tissue sources from different patients. To determine if longer neuraminidase treatment further enhanced detection with MAb 5A, whole cell lysates of SK-RC-7 RCC cells were incubated with neuraminidase for two or 24 hours (Fig. 8B). Increased time of digestion had no effect on the detectability of the epitope by MAb 5A. However, the Western blot indicates a downward shift in the banding pattern of gp 45/55, from approximately 51 kDa to approximately 39 kDa, after a 24 hour

Table 2

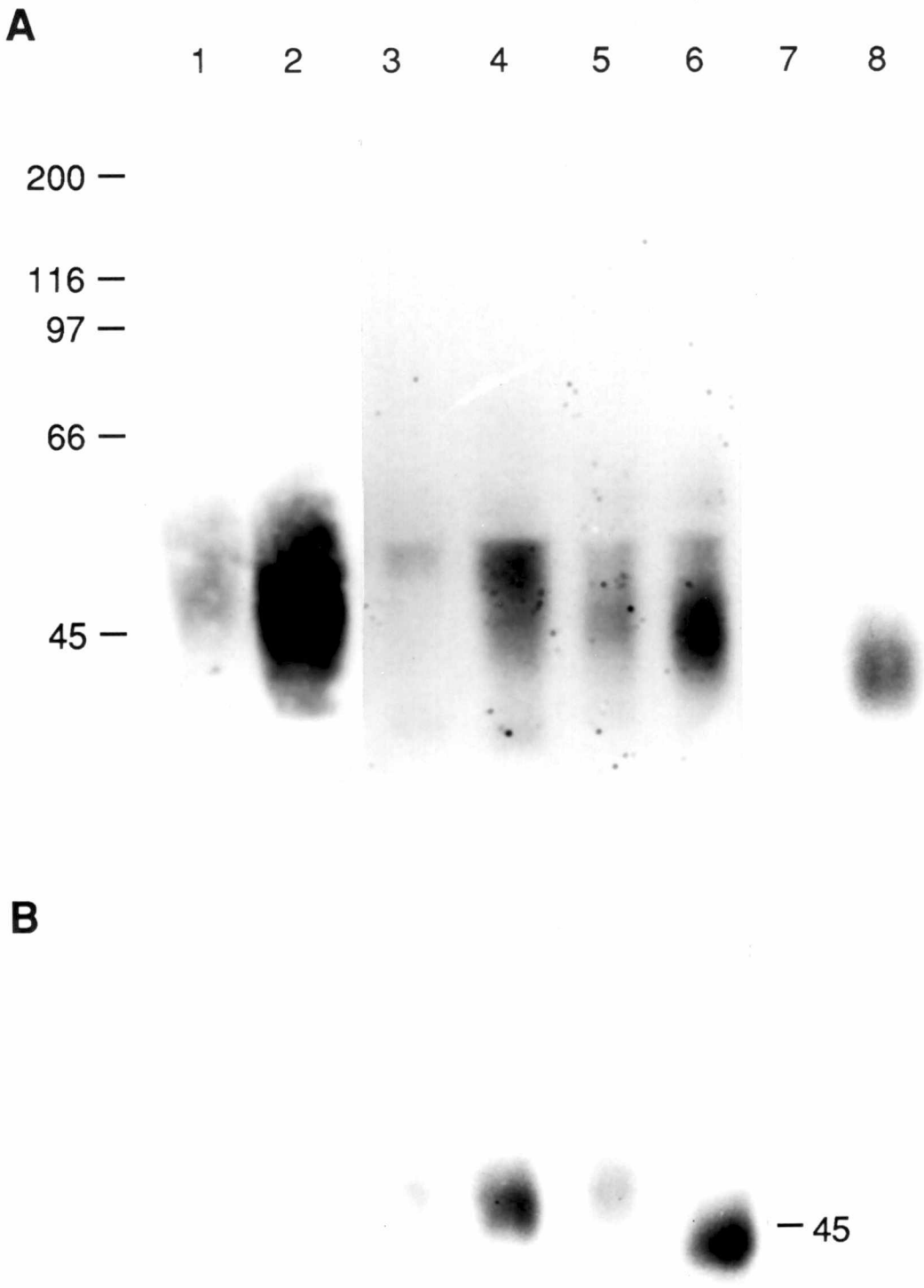
Dosimetry Measurements of MAb 5A Detection of gp 45/55 in Normal Kidney, RCC, and Other Normal Tissues ± Neuraminidase

Tissue	Neuraminidase Treatment	
	control[a]	treated
12/11 Kidney	ND	15.5
12/11 RCC	ND	23.5
7/5 Kidney	ND	3.3
7/5 RCC	ND	25.0
7/89 Kidney	ND	21.8
1/4 Kidney	ND	14.0
7/23 Kidney	ND	21.9
5/14 Kidney	ND	30.6
9/4 RCC	ND	58.9
4/23 RCC	ND	3.3
9/24 RCC	ND	7.5
11/20 RCC	ND	86.7
6/16 RCC	ND	86.9
12/88 Lung	6.9	51.5
7/89 Kidney	5.6	21.0
7/5 RCC	30.0	68.8
SK-RC-7 RCC cell line	93.5	112.5

[a] Based upon area integration within equally sized rectangles placed around detectable gp 45/55. Arbitrary density units.

ND = not determined

Figure 8. (A) Normal human tissue whole cell lysates were incubated in the presence or absence (± N) of neuraminidase for two hours at 37°C. Ten micrograms of total lysate protein were loaded per lane. gp 45/55 was detected with ^{125}I-MAb 5A after SDS-PAGE separation and Western blot transfer. The blots were exposed for 48 hrs at liquid nitrogen vapor phase temperature. Lane 1: 12/8 lung -N, lane 2: same +N, lane 3: 1364 prostate -N, lane 4: same +N, lane 5: 1420 small intestine -N, lane 6: same +N, lane 7: 7/5 spleen -N, lane 8: same +N. (B) RCC whole cell lysate incubated in the presence or absence (±) of neuraminidase for 2 or 24 hours. Lane 1: 2 hr incubation -N, lane 2: 2 hr incubation +N, lane 3: 24 hr incubation -N, lane 4: 24 hr incubation +N.

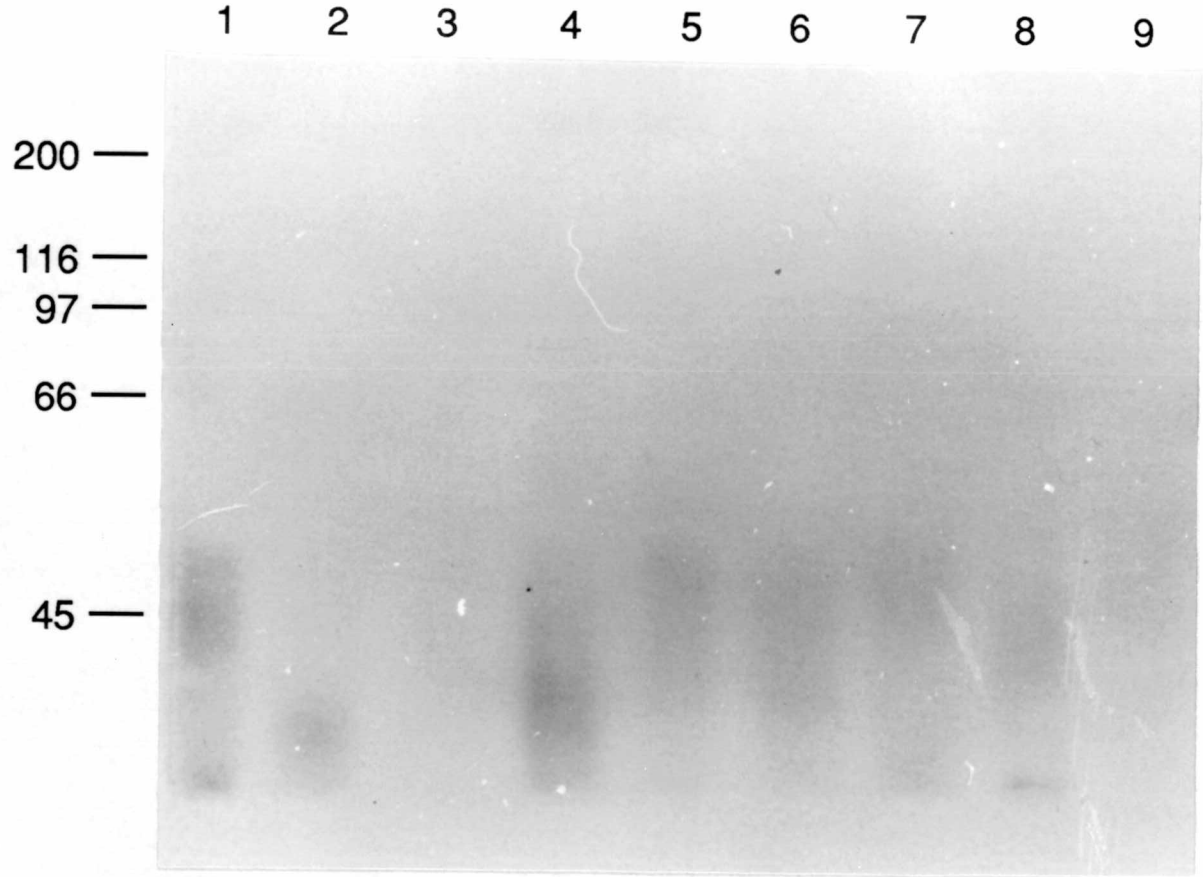

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digestion. The increase in mobility was probably related to the loss of sialic acid residues. These data indicate that the epitope recognized by MAb 5A is partially masked from antibody detectability by the presence of sialic acid and that sialic acid itself is unlikely to be part of the epitope.

Digestions with N-glycanase, O-glycanase, and neuraminidase were done to determine the effect of other glycosidase treatments on gp45/55 migration and detection in Western blot. RCC whole cell lysates and MAb 5A immunoaffinity chromatography purified samples of gp45/55 were treated with four different enzyme mixtures consisting of: 1) N-glycanase, neuraminidase, and O-glycanase; 2) neuraminidase and O-glycanase; 3) N-glycanase alone; or 4) neuraminidase alone. The reaction conditions are presented in the legend of Figures 9 and 10.

RCC whole cell lysate treated with all three glycosidases (Fig. 9, lane 2) demonstrated the greatest increase in 5A antigen mobility, with an observed change in apparent Mr from approximately 46 kDa to approximately 35 kDa. Treatment with neuraminidase and O-glycanase (Fig. 9, lane 4) also demonstrated an increase in gp 45/55 mobility, with an observed Mr shift from approximately 45 kDa to 38 kDa. Treatment with N-glycanase alone (Fig. 9, lane 6) demonstrated a smaller shift in mobility, from approximately 46 kDa to approximately 41 kDa. Finally, digestion for 6 hours with neuraminidase alone (Fig. 9, lane 8) demonstrated a very small shift in Mr compared to the lysate control

Figure 9. RCC whole cell lysate was digested with N-glycanase, O-glycanase, and/or neuraminidase. Lane 1: Undigested 5 µg RCC whole cell lysate (WCL). Lane 2: Rxn I digest of 15 µg RCC WCL. Rxn I digest consisted of 15 µg (2 µl) RCC WCL added to 12.5 µl Milli-Q water, 0.9 µl Na_2PO_4 buffer, and 1.25 µl 10% SDS in a 1.5 ml microfuge tube. The sample was boiled at 95°C for 7 mins, cooled, and microfuged. The supernatant was transferred to a new microfuge tube and 7.5 µl of 5% NP-40 was added, followed by the addition of 0.5 µl N-glycanase. The digest was incubated at 37°C for 20 hrs. Next, 0.5 µl neuraminidase was added for 6 hrs at 37°C, followed by the addition of 0.5 µl O-glycanase and incubation at 37°C for another 20 hrs. The enzymatic reactions were stopped with 25 µl loading buffer (1% SDS/ 8 M urea). Lane 3: Negative control for Rxn I (Add Milli-Q water in place of each enzyme as described above). Lane 4: Rxn II digest of 15 µg RCC WCL. Rxn II digest consisted of 15 µg (2 µl) RCC WCL added to 6.4 µl Milli-Q water, 0.6 µl Na_2PO_4 buffer, and 0.5 µl neuraminidase in a 1.5 ml microfuge tube. The sample was incubated at 37°C for 6 hrs prior to the addition of 0.5 µl O-glycanase and further incubation at 37°C for 20 hrs. The enzymatic reactions were stopped with 20 µl loading buffer. Lane 5: Negative control for Rxn II (See Rxn I negative control). Lane 6: Rxn III digest of 15 µg RCC WCL. Rxn III digest consisted of 15 µg (2 µl) RCC WCL added to 10.9 µl Milli-Q water, 0.6 µl Na_2PO_4 buffer, and 1 µl 10% SDS in a 1.5 ml microfuge tube. The sample was boiled at 95°C for 7 mins, cooled, and microfuged. The supernatant was transferred to a microfuge tube, and 5 µl of 5% NP-40 was added, followed by 0.5 µl N-glycanase. The digest was incubated at 37°C for 20 hrs. The enzymatic reactions were stopped with 20 µl loading buffer. Lane 7: Negative control for Rxn III (See Rxn I negative control). Lane 8: Rxn IV digest of 15 µg RCC WCL. Rxn IV digest consisted of 15 µg (2 µl) RCC WCL added to 16.9 µl Milli-Q water, 0.6 µl Na_2PO_4 buffer, and 0.5 µl neuraminidase. The sample was incubated at 37°C for 6 hrs. The enzymatic reaction was stopped with 20 µl loading buffer. Lane 9: Negative control for Rxn IV (See Rxn I negative control).


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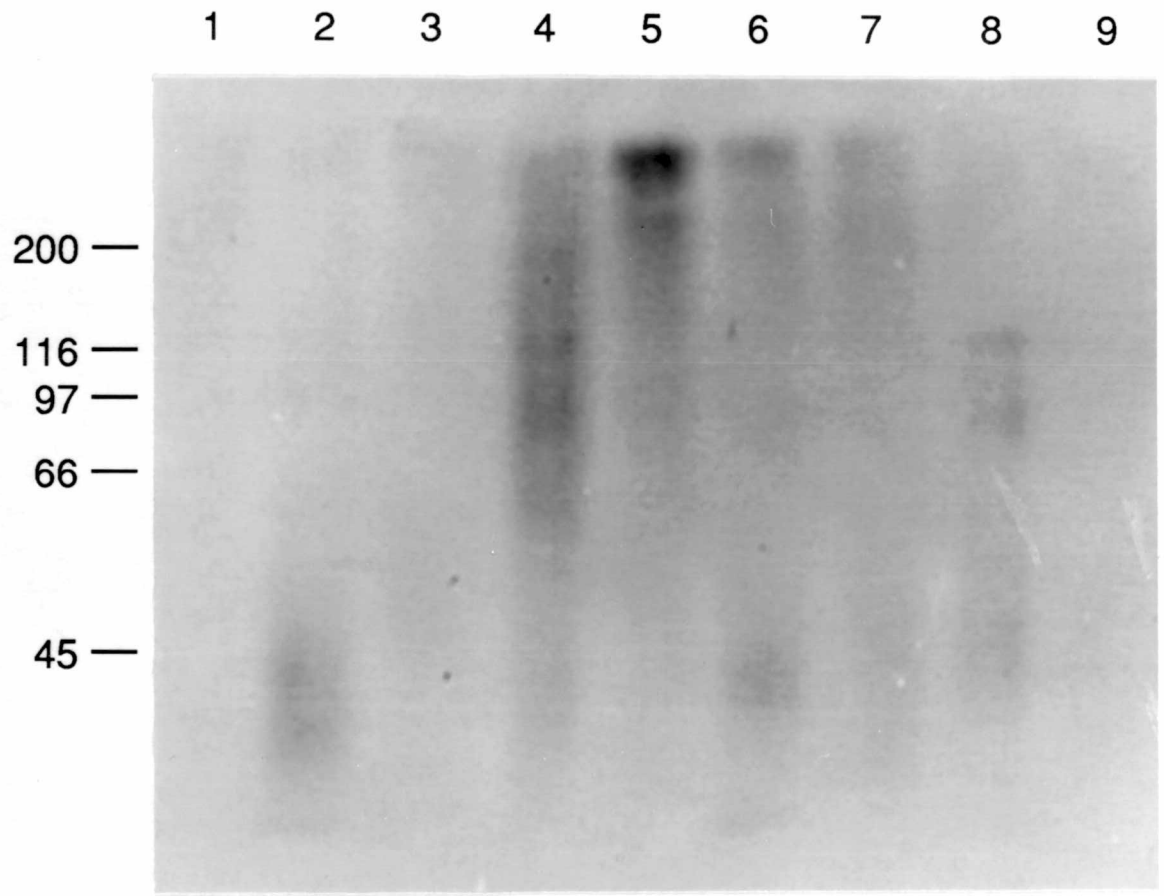
lane 1). However, it was evident from comparison with the negative control (lane 9) that neuraminidase digestion increased detection of gp 45/55 by MAb 5A, as noted earlier.

In IAC purified gp 45/55 (Fig. 10), treatment with all three glycosidases (Fig. 10, lane 2) increased the mobility of the gp45/55 the most, with an observed change in apparent Mr from approximately 46 to 40 kDa. Samples treated with neuraminidase and O-glycanase (Fig. 10, lane 4) demonstrated 5A antigen bands ranging from approximately 41 to 190 kDa. Buffer controls also showed large shifts in 5A antigen mobility indicating aggregation was occurring in the buffer. Treatment with N-glycanase alone (Fig 10, lane 6) resulted in an observable shift of the apparent Mr to approximately 43 kDa, compared to the negative control at approximately 46 kDa. Finally, treatment for 6 hours with neuraminidase alone (Fig. 10, lane 8) gave the expected slight shift in gel mobility as well as an appearance of two more discernible bands, at Mrs of approximately 88 kDa and 120 kDa, that were not observed in the buffer control or previously in neuraminidase treated whole cell lysate samples.

c. Purification procedure

Preliminary attempts were made to purify gp 45/55 directly from whole cell lysates using MAb 5A coupled to Sepharose beads. Results indicated that very little of the detectable gp 45/55 was bound by the 5A beads and that approximately 90% of gp 45/55 could be detected in the unbound fraction (data not shown). This result indicates

Figure 10. MAb 5A IAC-purified gp 45/55 was digested with N-glycanase, O-glycanase, and/or neuraminidase. Lane 1: Approximately 20 ng of undigested, IAC-purified gp 45/55. Lane 2: Rxn I digest of 20 ng gp 45/55 (Rxn I digest was similar to Fig. 9 description, except 12 µl volume of gp 45/55 and 2.85 µl Milli-Q water were used). Lane 3: Negative control for Rxn I (Milli-Q water was added in place of each enzyme as described above). Lane 4: Rxn II digest of 20 ng gp 45/55 (Rxn II digest was similar to Fig. 9 description, except 12 µl volume of gp 45/55 and 6.4 µl Milli-Q water was used). Lane 5: Negative control for Rxn II (Milli-Q water was added in place of each enzyme as described above). Lane 6: Rxn III digest of 20 ng gp 45/55 (Rxn III digest was similar to Fig. 9 description, except 12 µl volume of gp 45/55 and 0.9 µl Milli-Q water used). Lane 7: Negative control for Rxn III (Milli-Q water was added in place of enzyme as described above). Lane 8: Rxn IV digest of 20 ng gp 45/55 (Rxn IV digest was similar to Fig. 9 description, except 12 µl volume of gp 45/55 and 6.9 µl Milli-Q water used). Lane 9: Negative control for Rxn IV (Milli-Q water was added in place of enzyme as described above).


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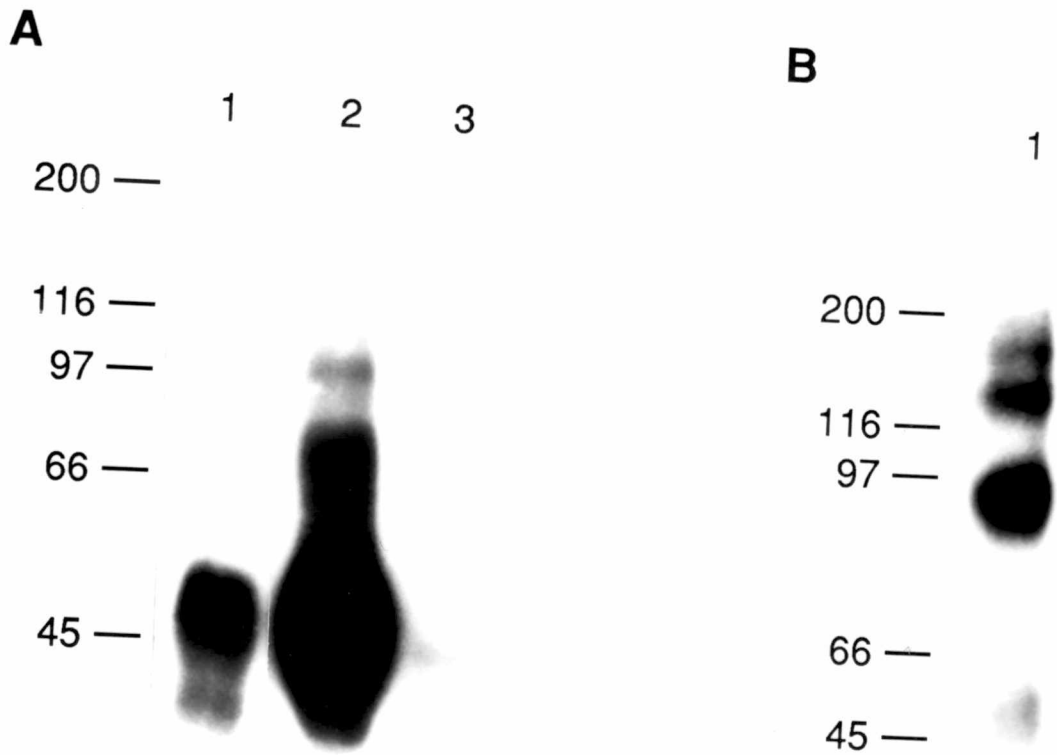
that MAb 5A was too low in affinity to bind significant fractions of gp 45/55 at the concentration available in the whole cell lysate. To overcome this problem, gp 45/55 was concentrated into a smaller volume in order to drive the biochemical binding reaction of MAb 5A and its target antigen.

Agarose bead-bound WGA was used to partially purify and concentrate gp 45/55. The gp 45/55 protein was concentrated approximately 16-fold in a single step. Although the majority of detectable antigen was still detected at the 45 to 55 kDa range in Western blot analyses, in addition there were bands detected at approximately 92 kDa, 145 kDa, and 195 kDa (Fig. 11A, lane 2). This would indicate that the 5A antigen aggregates when in concentrated solutions.

Glycoprotein 45/55 eluted from WGA beads in 1% SDS/8.0 M urea was then dialyzed against 10 mM sodium phosphate, pH 7.0 to remove the majority of SDS and urea. The removal of SDS and urea was required to facilitate binding of gp 45/55 with MAb 5A-linked Sepharose beads. To inhibit the loss of gp 45/55 caused by non-specific adherence of the glycoprotein to the dialysis bag during this procedure, BSA was added to a concentration of 10 µg per ml. This method resulted in increased detection of gp 45/55 (data not shown).

MAb 5A-linked Sepharose beads were used to specifically bind gp 45/55 from the partially purified WGA eluate. The ratio of MAb 5A beads to gp 45/55 was an important factor in efficient purification. An optimum volume of 10 µl of 5A

Figure 11. (A) Wheat germ agglutinin (WGA) concentration and purification of gp 45/55. Twenty µl of agarose bead-bound WGA was added to 1 ml of SK-RC-7 cell line whole cell lysate as described. Beads were eluted with 3X 20 µl of 1% SDS / 8 M urea, and eluates were pooled. Protein lysate, eluate, and bypass were separated by SDS-PAGE and Western blotted. After detection with ^{125}I-labeled MAb 5A, autoradiographs were exposed for 19 hrs at liquid nitrogen vapor phase temperature. Lane 1: 5 µl of SK-RC-7 whole cell lysate. Lane 2: 5 µl of WGA bead eluate. Lane 3: 5 µl of WGA bead bypass. (B) MAb 5A purification of gp 45/55. 10 µg of Sepharose bead-bound MAb 5A was added to 200 µl of dialyzed WGA bead eluate. 5A beads were eluted with 3X 10 µl of 1% SDS / 8 M urea, and eluates were pooled. Lane 1: 5 µl of 5A bead eluate was separated by SDS-PAGE and Western blotted. After detection with ^{125}I-labeled MAb 5A, autoradiographs were exposed for 15 hrs at liquid nitrogen vapor phase temperature.


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beads per 200 µl of WGA bead eluate were used. Glycoprotein 45/55 was eluted with SDS/urea. To determine the purity of the final product, a sample was analyzed by SDS-PAGE, and protein was detected by using a silver stain (Gelcode Color Silver Stain; Pierce, Rockford, IL). The banding pattern in the silver stain gel was then compared to a similar sample detected in Western blot (data not shown). Glycoprotein 45/55 was considered to be 95% pure. As shown in Figure 11B, the banding pattern of gp 45/55 after MAb 5A bead IAC purification was radically changed. ^{125}I-labeled MAb 5A detected a major broad band at approximately 95 kDa, although the presence of additional bands at 54 kDa, 165 kDa, and 210 kDa were observed.

d. Amino Acid Analysis

To prepare for amino acid analysis, the purified gp 45/55 in 1% SDS/ 8.0 M urea was dialyzed using 10 mM sodium phosphate, pH 7.0. The dialyzed sample was then lyophilized, and a portion of the final sample was subjected to N-terminal amino acid sequencing using a gas phase automated sequencer. Less than 10 pmole of product was detected, indicating that the N-terminus of the gp 45/55 antigen was blocked. In order to determine the gp 45/55 amino acid composition, an acid hydrolysis and amino acid quantification was performed on the remaining sample.

The preliminary amino acid composition (Table 3) of gp 45/55 showed that serine and threonine comprise 10.4% of the total detectable amino acid residues, and that the sum of

Table 3

Preliminary Amino Acid Composition of IAC Purified gp 45/55

Amino Acid[a]	% Total amino acids Recovered[b]
Glycine	24.0
GLX[c]	14.6
Lysine	10.5
Alanine	8.9
Leucine	8.0
Proline	7.1
Serine	6.2
Isoleucine	4.9
Threonine	4.2
ASX[c]	3.1
Tyrosine	3.0
Phenylalanine	2.4
Arginine	1.9
Histidine	1.3

[a] Determined as described in *Amino Acid Analysis* section.

b pmoles amino acid divided by total pmoles amino acid recovered x 100.

[c] The acid hydrolysis method converts Gln to Glu, therefore distinguishing between the two is not possible. The same occurs with conversion of Asn to Asp.

serine, threonine, glycine, proline, and alanine together constituted 50% of the total amino acid content. This amino acid composition data suggests that gp 45/55 may be a mucin-like glycoprotein (Lasky et al., 1992; Goswami et al., 1994). The relative percentage of the ASX residues (meaning that the residue was either aspartic acid or asparagine) was only 3.1% of the total amino acids recovered. Asparagine residues that are typically involved in the N-linked glycosylation of proteins were not differentiated from aspartic acid residues by the amino acid composition method used in the analysis of gp 45/55.

Distribution of gp 45/55

a. Immunohistochemistry of RCC and Normal Kidney Tissues

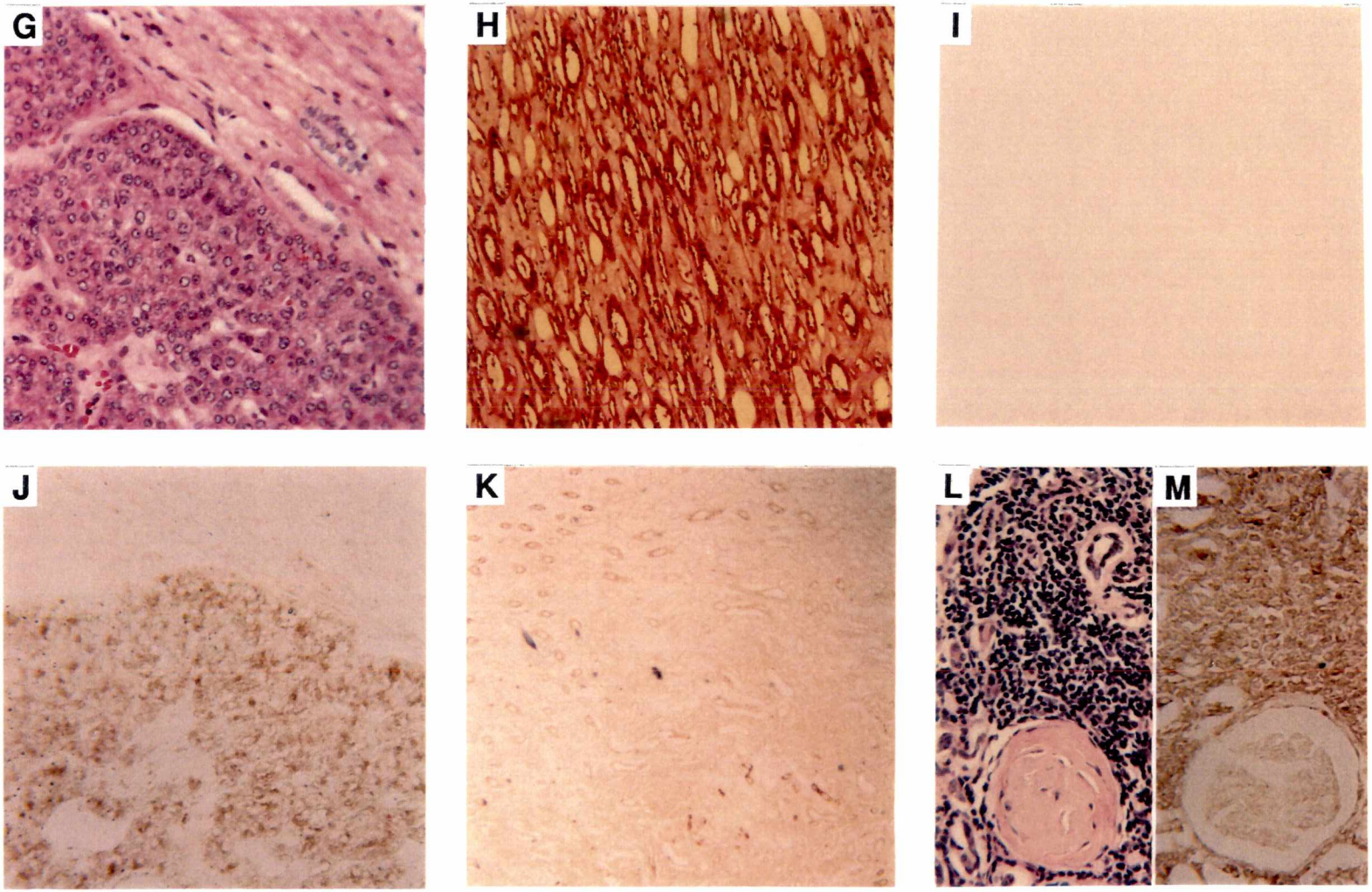
Immunohistochemistry (IHC) was used to determine the range of gp 45/55 expression in tissues. Frozen and paraffin sections of several different RCC and normal kidney tissues were examined using MAb 5A. Nineteen different RCCs of various histotypes were examined: 1 spindle cell, 4 clear cell, 7 granular cell, and 7 mixed (clear/granular) cell. In each of the 19 samples, MAb 5A staining was observed. The majority of 5A staining was confined to cell plasma membranes of tumor epithelial cells, although some staining was seen within the tumor cell cytoplasm as well. However, the staining of cells within the tumor was not homogeneous. The number of cells stained and the intensity of gp 45/55

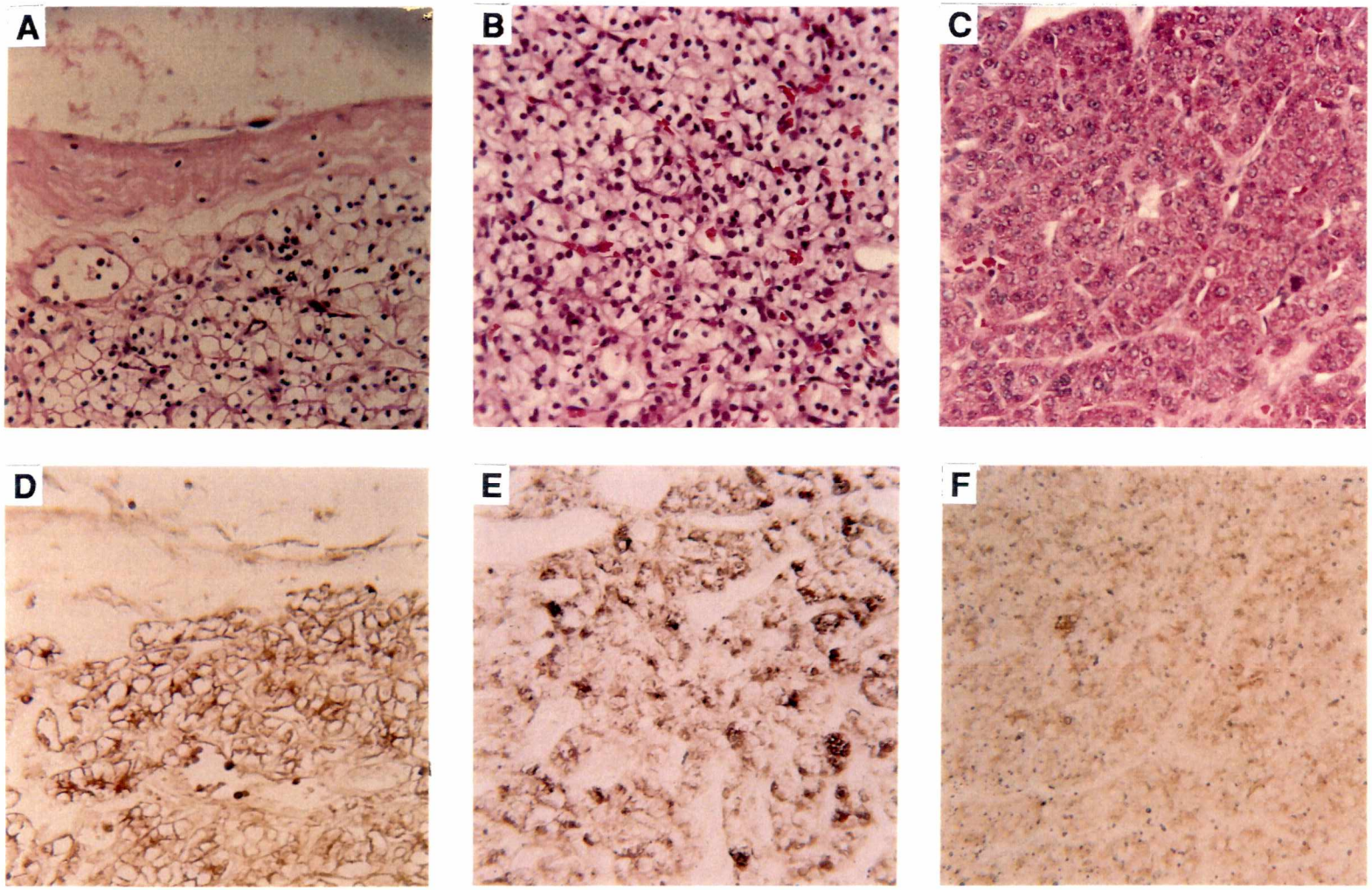
distribution varied among different RCC types (Figure 12D, 12E, 12F), but the pattern of expression remained fairly constant. Thus the expression of gp 45/55 is consistent in 19/19 RCC tumors including all the common histotypes.

Staining with MAb 5A in normal kidney tissue was restricted to the membranes of proximal tubule epithelial cells (Figure 12K), even though it could also be detected to a lesser extent in the cell cytoplasm. Although staining in normal kidney tissue was always restricted to tubules in different patient's samples, the intensity of staining was variable. A few (4/21) normal kidney tissues demonstrated a high staining intensity of tubule cells that was similar to that seen in cells from RCC tumors. However, compared to the intensity of staining seen in most samples of RCC tissues, the relative intensity of staining seen in most normal kidneys was lower. Figure 12J presents a typical staining pattern observed at the boundary between normal and tumor tissues. The intensity differences in staining agrees with western blot analysis of gp 45/55 expression that also indicated a higher expression of gp 45/55 in RCC tissues than in normal kidney tissues. The distribution pattern of gp 45/55 and its high expression may have functional significance and may serve as a marker of the RCC tumor phenotype.

Since it was demonstrated that sialic residues may mask the ability of MAb 5A to recognize its epitope in Western blot analyses, tissue sections were treated with neuraminidase before IHC was performed. Pretreatment of

Figure 12. Immunohistochemistry of normal kidney and RCC tissues with purified MAb 5A. Panels A and D: Haematoxylin-eosin stained and MAb 5A stained 4984 RCC clear cell carcinoma histotype, respectively. Panels B and E: Haematoxylin-eosin stained and MAb 5A stained 1922 RCC mixed (clear cell/granular cell) cell carcinoma histotype, respectively. Panels C and F: Haematoxylin-eosin stained and MAb 5A stained 1924 RCC granular cell carcinoma histotype, respectively. Panels G and J: Haematoxylin-eosin stained and MAb 5A stained 1924 RCC granular cell carcinoma histotype and adjacent normal kidney, respectively. Panel H: Haematoxylin-eosin stained 89-860 normal kidney tissue. Panel I: MAb P3 stained 4976 RCC granular cell carcinoma histotype and adjacent normal kidney (negative control). Panel K: MAb 5A stained 4968 RCC normal kidney tissue. Panels L and M: Haematoxylin-eosin stained and MAb 5A stained 4976 RCC granular cell carcinoma histotype, respectively.


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tissue specimens with neuraminidase did not increase gp 45/55 staining intensity perceptibly in RCC or normal kidney samples examined (data not shown). It is not known if neuraminidase treatment of these tissue samples was sufficient to digest sialic acid from the exposed gp 45/55 or if sialic acid was not masking *in situ*.

b. Immunohistochemistry of other human tissues

Table 4 lists the normal and tumor tissue types other than normal kidney and RCC that were examined by IHC with MAb 5A. The presence of staining was homogeneous in the general cell population of 3 out of 5 lymphoma samples tested. Monoclonal antibody 5A staining was also noted in the granular cells of one of two samples of ovarian adenocarcinoma. Of interest is the absence of gp MAb 5A staining in both samples of kidney adenocarcinomas, indicating that the gp may be expressed, at least in kidney tissue, in glandular, carcinoma-related epithelium as opposed to glandular, adenocarcinoma-related epithelium. This data is consistent with the staining of proximal tubule epithelium in normal kidney and of tumor epithelium in RCC.

Of the normal tissues assayed by IHC for gp 45/55 expression, the following demonstrated the presence of MAb 5A staining: acidophil and possibly basophil cells of the pituitary gland (3/3), cells of the cortex region in the adrenal glands, and zymogen granules of the pancreas (5/5). Although the intensity of staining again varied among the tissues sampled, the pattern of expression on cell plasma

Table 4

MAb 5A Staining in Human Normal and Tumor Tissues

Tissue	N	Immunodetection +	±	-
Normal Tissues				
Brain	5	0	4	1
Pituitary	3	3	0	0
Adrenal	2	2	0	0
Breast	3	0	0	3
Heart	2	0	0	2
Stomach	1	0	0	1
Small Intestine	1	0	0	1
Large Intestine	3	0	0	3
Fetal Liver	2	0	2	0
Adult Liver	3	0	3	0
Skin	3	0	1	2
Pancreas	5	5	0	0
Lung	5	0	0	5
Tonsil	3	0	0	3
Testis	2	0	0	2
Ovary	5	0	0	5
Uterus	3	0	0	3
Placenta	2	0	0	2
Prostate	5	0	2	3
Thyroid	5	0	0	5
Spleen	5	0	0	5
Tumor Tissues				
Lung Adenocarcinoma	2	0	0	2
Liver Adenocarcinoma	2	0	0	2
Kidney Adenocarcinoma	2	0	0	2
Colon Adenocarcinoma	2	0	0	2
Breast Adenocarcinoma	2	0	2	0
Thyroid Adenocarcinoma	2	0	1	1
Stomach Adenocarcinoma	2	0	1	1
Prostate Adenocarcinoma	2	0	0	0
Pancreas Adenocarcinoma	2	0	0	2
Ovary Adenocarcinoma	2	0	1	1
Lymphoma	5	3	2	0
Melanoma	5	0	0	5
Ewing's Sarcoma	1	0	0	1
Epithelioid Sarcoma	1	0	0	1
Malignant Fibrous Histiocytoma	2	0	2	0
Rhabdosarcoma Sarcoma	2	0	0	2
Carcinoid	4	0	1	3
Undifferentiated Carcinoma	3	0	0	3
Mesothelioma	3	0	0	3
Teratoma	2	0	0	2
Seminoma	2	0	0	2

N = number of different tissues of that type.
+ = positive staining of tissue.
± = possible light staining of tissue or undistinguishable background staining.
- = no staining.

membranes remained consistent. It should be noted that the staining of macrophages was observed in several different tissue types, especially in the spleen. This staining might reflect the unquenched endogenous peroxidase activity of this particular cell type.

c. Western blot analysis

The distribution of gp 45/55 in several normal tissues was also examined by Western blot analysis (Table 5). The majority of the normal tissue samples were taken from three patients and were stored at -70^{o}C prior to use. Western blot was performed on detergent solubilized tissue proteins separated by SDS-PAGE and detected by radiolabeled MAb 5A. As indicated in Table 5, the prevalence of gp 45/55 expression in normal tissues other than kidney was found in colon, liver, lung, prostate, small intestine, and spleen (also see Fig. 7).

The possibility was examined that normal tissues with no detectable expression of gp 45/55 (Table 5) in Western blot prior to neuraminidase treatment may subsequently demonstrate detectable gp 45/55 after neuraminidase treatment. Although neuraminidase treatment of samples prior to Western blot did increase the detectable amounts of gp 45/55, neuraminidase treatment did not expose new organ sites of gp 45/55 expression in the set of tissues originally found to be negative for target antigen expression (data not shown).

Table 5

Western blot Distribution of gp 45/55

in Normal Human Tissues

Tissue	N	Neuraminidase Treatment	
		Before	After
Breast	1	-	-
Bronchi	2	-/-[a]	-/+
Colon	1	+	++
Esophagus	1	-	-
Fat	1	-	-
Gall Bladder	1	-	-
Liver	2	-/-	+/+
Lung	5	±/+	+/++
Prostate	1	±	+
Small Intestine	1	±	+
Spleen	2	±/±	+/++

N = number of different tissues of that type tested.

± = weak detection of gp 45/55.

+ = positive detection of gp 45/55.

++ = strong detection of gp 45/55.

- = no detection of gp 45/55.

[a] indicates more than one tissue was available and detection varied.

CHAPTER IV

DISCUSSION

Introduction

Epithelial neoplasms are often distinguished by their excessive expression of various glycoproteins, which may be used as tumor markers in the monitoring of disease progress and recurrence (Ammon et al., 1994). Aberrations in cell surface carbohydrate structure are a common characteristic of malignant cells. Several recent studies suggest that changes in cell surface glycoconjugates have functional significance in malignant transformation and differentiation, although the molecular mechanisms involved are not clear yet (reviewed in Verma, 1994). Altered glycosylation is responsible for many of the antigenic differences between carcinomas and adjacent normal epithelial tissue. The altered activity of various glycotransferase pathways in tumor cells can cause changes in the carbohydrate structures expressed on tumor cell membrane core proteins and lipids (Singhal and Hakomori, 1990; Kobayashi et al., 1993). However, the differences in antigenicity between normal and tumor tissues may be due to actual differences in the protein backbone to which carbohydrates are attached as well (Devine and McKenzie, 1992). Three possibilities for target epitopes recognized by glycoprotein-recognizing MAbs have been described: (a) an

epitope composed of both peptide and carbohydrate; (b) an epitope that is solely peptide, but its correct conformation is dictated by carbohydrate(s); or (c) a peptide-epitope that is unmasked by the total absence or alteration of glycosylation (reviewed in Verma, 1994).

Chemical Nature of the gp 45/55 Epitope Structure

The antigen recognized by MAb 469-5A, gp 45/55, is a tumor-associated glycoprotein in RCC. Three pieces of evidence have been presented that give clues to the chemical structure of the epitope bound by MAb 5A. These data are 1) the increased binding of epitope after treatment with neuraminidase, 2) the recognition of epitope after treatment with N- and O-glycanases, and 3) the loss of epitope recognition after reduction of disulfide bonds. Based on this evidence, it is hypothesized that the epitope recognized by MAb 5A is part of the protein backbone of gp 45/55 and not a carbohydrate structure attached to the backbone.

The presence of sialic acid residues on gp 45/55 has been demonstrated in Western blot. Treatment of gp 45/55 positive tissues with neuraminidase for two hours increased detection with MAb 5A of the broad band at approximately 45 to 55 kD (Fig 3). Cleavage of sialic acid was evidenced by a slight shift to a lower Mr position. A longer (24 hour) incubation with neuraminidase generated a gp 45/55 which moved at an ever faster rate in SDS-PAGE, indicating that the two hour incubation had not removed all of the sialic acid residues although the detection by MAb 5A was not further

enhanced. The determination of MAb 5A's binding affinity (K_a) with or without neuraminidase also demonstrates how loss of sialic residues increased epitope binding. A 2.6 fold increase in K_a was found after antigen incubation with neuraminidase (Fig. 4).

These data indicate that sialic acid induces a poorer binding of MAb 5A to its target epitope. It is still possible that MAb 5A recognizes a carbohydrate epitope masked by the presence of the terminal sialic acid. On the other hand, the negatively charged sialic acid may block MAb 5A binding by electrostatic repulsion that is decreased by the removal of these residues. Likewise, the loss of surface charge after treatment with neuraminidase may induce a conformational shift in gp 45/55 that increases the exposure to MAb 5A of a peptide and/or carbohydrate epitope.

A second piece of information also supports the hypothesis that the 5A epitope is not a carbohydrate structure. Glycoprotein 45/55 was still detected in Western blot by MAb 5A after digestion with N-glycanase, O-glycanase, and neuraminidase and by combinations of these glycosidases (see legends and Figs. 9 and 10), although there were characteristic shifts in the Mr banding pattern with each reaction. The shifts in the broad banding pattern detected after deglycosylation indicated that N- and O-linked carbohydrate moieties were cleaved, but their loss had no effect on MAb 5A recognition of gp 45/55. However, it was not determined if these deglycosylation reactions were carried out to completion. Further studies need to be done

to assure that each deglycosylation reaction had gone to completion in order to demonstrate conclusively that carbohydrates are not part of the 5A epitope structure.

It has also been demonstrated that MAb 5A binds to its epitope under non-reducing conditions but not under reducing conditions (Fig. 2). The loss of epitope binding due to reduction with sulfhydryls indicates the presence of a disulfide bridge structure(s) that is important in MAb 5A recognition of gp 45/55. This also indicates that the peptide backbone structure is important in MAb 5A recognition of its epitope. Although these data suggest that the 5A epitope is composed of peptide, they are not conclusive. The disulfide bridge structure could be necessary to position peptide and carbohydrate moieties or to position two carbohydrate moieties that are recognized as the MAb 5A epitope.

Expression of gp 45/55 in Normal Kidney and RCC Tissues

Human cancer cells have been under intense study to define tumor-specific antigens (Old, 1981). Although antigens absolutely restricted to tumors have not been found, a variety of different antigens have been defined whose pattern(s) of expression in tumor tissues are being used to investigate the basis of tumor cell heterogeneity and as targets for antibody imaging and therapy (Fradet et al., 1986; Guadagni et al., 1994). The extent and heterogeneity of antigen expression are important considerations in

determining the efficacy of a MAb for immunodetection and/or immunotherapy. Data from Western blot and IHC indicate that gp 45/55 is expressed in both RCC and normal tissues, but is expressed in higher concentration in RCC tissues than normal kidney tissues. Based on this pattern of distribution, MAb 5A may prove to be an effective tool in the immunodiagnosis and/or immunotherapy of RCC.

a. Western blot Data

Three pieces of evidence demonstrated that MAb 5A recognized the same antigen in normal kidney tissue and in RCC tissue. First, the banding patterns on Western blot were similar. MAb 5A recognized a broad band between 45 and 55 kDa that, in some blots, had discernible, individual bands within the broad pattern. Secondly, the Mr range was similar in both normal and tumor tissues. Finally, as previously described, neuraminidase treatment of samples increased the detection of gp 45/55 in both normal and tumor tissues (Fig. 3).

Most glycoproteins are detected as broad bands in Western blot (Toyoshima et al., 1995). The banding pattern of the glycoprotein recognized by MAb 5A indicated unusual heterogeneity of protein and/or glycosylation patterns. Within this banding pattern, individual, focused bands are sometimes observed (Fig. 2, lane 1; Fig. 5, lanes 1-4). This banding pattern may represent breakdown products caused by partial proteolysis, or similarly, the partial cleavage and/or lack of carbohydrate components. Treatment with

various glycosidases (Figs. 9 and 10) did not consistently decrease the breadth of the banding pattern, but it did effect the detected Mr range. This indicated that carbohydrate heterogeneity alone does not account for the banding pattern.

In most normal and tumor tissues assayed by Western blot, MAb 5A recognized a glycoprotein with a similar banding pattern (Fig. 6, lanes 1-9, 11-14). One exception was found in a single RCC tumor (Fig. 6, lane 10). The majority of detectable signal was found at high Mr (greater than 200 kD). However, there was also a detectable band in the 45 to 55 kD range. The source of this RCC sample was a highly necrotic tumor specimen, containing few viable tumor cells. The reason for this Mr inconsistency is not known, but it is believed to be related to the extracellular environment of the necrotic tissue, since all other samples assayed came from viable tumor tissue samples. Differences observed in the mobility of gp antigens could also be attributed to genetic polymorphism or to aberrant glycosylation patterns (Beckett et al., 1991).

High Mr banding patterns were also detected in concentrated, purified samples of gp 45/55 (Fig. 11A, lane 2 and 11B, lane 1). This pattern of Mr banding was consistently observed in Western blot after WGA bead and MAb 5A bead purification of gp 45/55 from the SK-RC-7 RCC cell line. The detectable banding pattern of gp 45/55 from the RCC cell line was similar to those detected from both normal and RCC whole cell lysates, as previously described. The

presence of bands at approximately 92 kDa, 145 kDa, and 195 kDa indicated that the 5A antigen aggregated as it was concentrated and purified. This aggregation of the 5A antigen under these conditions may be a natural function of the glycoprotein or may be a function of the cohesiveness of exposed carbohydrate sidechains caused by the partial unfolding of the protein in SDS and urea.

The expression of gp 45/55 after neuraminidase treatment of normal kidney and RCC tissues from the same patient is presented in Figure 6 (lanes 1 - 4). These data indicate that the detectable expression of gp 45/55 in RCC tumor tissue is 2 to 8-fold greater than in adjacent normal kidney tissues (Table 2). Results observed from Western blot (Fig. 6 and Table 2) of independent tissue sources (normal and RCC tissues not from the same patient) demonstrate that overall the expression of gp 45/55 is 2- to 6-fold greater in 3/5 RCC tumors tested when compared to normal kidney tissues. However, the expression of gp 45/55 in <u>two</u> independent RCC tumors was greatly reduced compared to the other RCC tissues and to the other normal tissues. Samples of adjacent normal tissues were not available to determine the expression of gp 45/55 in normal versus tumor tissues from these two patients.

Based on the data presented, it could be hypothesized that gp 45/55 is a normal glycoprotein that is overexpressed in RCC. On the other hand, the actual amounts of gp 45/55 expressed and/or detected on the RCC cell surface may be the result of variances in the glycosylation of the core protein that exist between normal and tumor cells. However, based on

reports of similar glycoproteins found in the literature (Paradis et al., 1990; Fischer et al., 1991), it is more likely that gp 45/55 is a normal glycoprotein overexpressed on most, if not all RCCs. The absence of gp 45/55 on some RCCs would be understandable based on the various forms of RCC and the various biochemical pathways that lead to malignancy. From the data obtained in Western blot and IHC, it was concluded that the amount of gp 45/55 in RCC tissues detectable by MAb 5A was greater than that in adjacent normal kidney tissues.

b. IHC Data

The intensity of MAb 5A staining in IHC of normal kidney and RCC tissue sections indicated that gp 45/55 was expressed in RCC tissues in a higher concentration than in normal kidney tissues. Independently of their histological subtype and grade of differentiation, all RCCs tested showed at least some staining with MAb 5A, both in frozen and paraffin tissue sections (Table 4). In IHC of RCC tumor tissue, MAb 5A staining was detected mainly on the cell surface of tumor epithelium (Fig. 11, panel D), although in some tumors it was also distinctly found within the tumor cell cytoplasm as well (Fig. 11, panels E and M). The same was basically true of all normal tissues examined. However, the distribution of MAb 5A staining was limited to mostly proximal tubule cells (see Figure 11, panels K) and was sparsely detected on distal tubule and collecting duct epithelium (data not shown).

Although gp 45/55 is expressed in both normal and tumor

tissue cells, the relative amount of cellular staining and number of cells stained per given unit area by MAb 5A is increased in most RCC tissues. The intensity of staining also differs between proximal tubule epithelium and tumor cell epithelium (for example, compare panels D and K). The number and the staining intensity of MAb 5A positive cells was higher in neoplastic than in normal tissue (Figure 11, panels D, E, F, and M). These IHC data agreed with Western blot data, showing a distinct difference in staining intensity between normal kidney and RCC tumor tissues. In all cases (19/19) where tumor tissue and adjacent normal kidney tissue were stained by IHC, preferential staining was seen in the tumor tissue (for example, panel J). The difference in staining intensity may be related to the stage of progression of the tumor or the relative heterogeneity of the cells within a tumor (Oosterwijk et al., 1988). Both of these factors can influence the amount of glycoprotein and/or the degree of variations in glycosylation of the core protein structure. It can be hypothesized that the overexpression of gp 45/55 in most RCCs is related to the activity of either newly activated or over-activated glycotransferase pathways. These altered glycotransferase pathways may be linked to the degree of differentiation of various RCC cell populations.

Expression of gp 45/55 in Other Tissues

The distribution of glycoproteins expressed on tumor cells of various origins is rarely restricted to just the tumor and its organ/tissue of origin (Fradet et al., 1986;

Campbell et al., 1994; Shih et al., 1994). MAb 5A recognition of the gp 45/55 antigen is not limited to RCC tumors and normal kidney tissues. Western blot and IHC analysis revealed the presence of gp 45/55 in several normal as well as tumor tissues (Tables 5 and 6).

a. Western blot Data

Human epithelial cells from the mammary gland, salivary gland, digestive tract, respiratory tract, kidney, bladder prostate, uterus, and rete testis express glycoproteins with a broad range of Mrs on the apical cell membrane. Increased synthesis of the core proteins and alterations in the carbohydrates attached to glycoproteins are believed to play important roles in the function and proliferation of tumor cells originating from these tissues (Devine and McKenzie, 1992). To determine if the expression of gp 45/55 recognized by MAb 469-5A was restricted to normal kidney and RCC tissues, a panel of normal human tissues were tested for the presence of 5A antigen. The results of Western blot indicated that the glycoprotein antigen, gp 45/55, recognized by MAb 5A was detectable in several other normal tissues (Table 5).

Similar broad banding patterns and Mr mobilities were observed in gp 45/55 positive normal tissues, indicating the detection of the same protein in each tissue (Fig. 7). As with normal kidney and RCC tissues, however, there were observable variations in the Mr range between different tissue sources (Fig. 7, compare lanes 4 and 8) as well as

minor variations in the Mr range between the same tissue sources (ie. lung) from different patients (Fig. 7, compare lanes 3 and 4). As previously stated, the broad banding patterns observed could be caused by partial proteolysis or partial cleavage of carbohydrate moieties.

Of eleven normal human tissues (excluding kidney) assayed by Western blot, six demonstrated detectable gp 45/55, prior to neuraminidase treatment, in whole cell lysate preparations (for examples, see Fig. 8, lanes 1, 3, 5, and 7). After neuraminidase treatment, the amount of detectable gp 45/55 in these six gp 45/55 positive sources increased (Fig. 8, lanes 2, 4, 6, and 8). On the other hand, gp 45/55 only became minimally detectable in bronchus (Fig. 7, lane 1) and esophagus (data not shown) lysates after treatment with neuraminidase, indicating a much lower detectable expression of gp 45/55 in these tissues.

Each of the gp 45/55 positive normal tissue sources, including kidney, detected by Western blot contain glandular epithelial cells. Glycoprotein 45/55 may act as a receptor for some necessary nutrient or growth factor, as a component of cell-cell communication, or as an important factor in cell-extracellular matrix signaling processes. To elucidate the function(s) of gp 45/55 in tissues that expresses it, further work on the exact cellular distribution and structure of the glycoprotein must be done.

b. IHC Data

Immunoperoxidase-based IHC using MAb 5A was performed on

several human normal and tumor tissues (Table 3). Surprisingly, the IHC detection of gp 45/55 did not reproduce the results obtained by Western blot in all cases. However, the overall quality of both the normal and tumor tissues assayed by IHC using the Biomedia Corp. Multitissue slides was variable (personnel communication with Dr. Virginia Godfrey, UT Biomedical School, Oak Ridge, TN). Therefore, discussion of IHC results was limited to results that were observed in sections with good overall tissue morphology and cellular structure.

Of the 22 normal human tissues assayed by IHC for MAb 5A staining, only three (other than the normal kidney sections) gave detectable staining that was not attributable to background or unquenched peroxidase activity. The three were adrenal, pancreatic, and pituitary tissues. In the adrenal tissues, staining was light but fairly uniform within the cortex region. This region of the gland develops from epithelium. The major functions of the cortex are to control water and electrolyte balance via secretion of mineralcorticoid hormones and to maintain carbohydrate balance via secretion of glucocorticoid hormones. The pars distalis portion of the pituitary gland contains acidophils and basophils that were stained with MAb 5A. Both cell types are secretory cells, secreting various hormones. Zymogen granules located in the pancreas also stained with MAb 5A. Pancreatic enzymes are stored in these granules prior to exocytotic release from acinar cells. Each cell type that was stained by MAb 5A in IHC is involved in storage and

ultimately, secretory functions.

This secretory function contrasts with the function of the proximal tubule cell, which serves to reabsorb glucose, amino acids, and small peptides from the kidney filtrate. If each of the cell types mentioned above as well as the proximal tubule cells of the kidney share in common, by virtue of their IHC staining with MAb 5A, an expression of gp 45/55, it could be hypothesized that the glycoprotein functions in a secretory and/or binding role(s) that may not be mutually exclusive of one another. Either function would serve tumor cells equally well, in the binding of essential nutrients/growth factors or in the secretion of metabolic waste products or autocrine tumor cell growth factors. However, it is just as likely that expression of gp 45/55 is unrelated to any major cellular function.

Of 18 non-kidney tumor tissue sections assayed, only one, lymphoma tissue, was considered to stain with MAb 5A with any observable consistency. The uniformity of staining by MAb 5A within the lymphoma tissues, especially in cell membranes, indicated the probable importance of gp 45/55 to the lymphoma phenotype. It has been suggested that glycoproteins, especially mucin and mucin-like glycoproteins, may play an active role in the evasion of immune response by tumor cells (Hanna, 1985). Therefore it is plausible that gp 45/55 in tumor cells serves to mask the tumor cell from immune surveillance.

The presence of gp 45/55 in normal tissues other than kidney may be a drawback to MAb 5A's utilization as a

therapeutic or imagining tool. However, further quantitative investigation of the distribution gp 45/55 in normal human tissues must be done prior to ruling out MAb 5A's usefulness. Likewise, investigating and understanding the importance of gp 45/55's distribution in other tumor tissues may provide useful information about its importance in the overall cancer phenotype. Finally, data about the amino acid sequence and protein structure as well as the carbohydrate content should be completed in order to associate a possible functional role(s) to gp 45/55.

Conclusions

An important potential application of RCC-restricted MAbs is the identification of questionable renal masses through radioimmunoscintigraphy or in the localization of occult metastatic disease. As previously stated, however, the development of absolutely tumor-specific MAbs has not occurred. Alternative approaches to the identification and further characterization of RCC-associated antigens, nonetheless, is important in understanding more about the RCC phenotype. There are several MAbs already reported in the literature that have partial or restricted expression on RCC cells (Table 1). Currently, three MAbs that recognize different antigens on various forms of RCC have been used either diagnostically or therapeutically in clinical or pre-clinical trials. The three are MAb 138H11 to human γ-glutamyltransferase (Fischer et al., 1992), MAb G250 to a cell surface protein (Oosterwijk et al., 1993), and MAb EGP-2

to cell surface glycoprotein (Kroesen et al., 1994).

Of the three MAbs currently being used in clinical or pre-clinical trials, G250 has the most restricted expression, being found in most forms of RCC, in gastric mucosa, and in large bile duct epithelium but not in normal kidney cells. G250 has been used in phase I imaging and biodistribution studies and has demonstrated the highest reported accumulation of radiolabeled MAb in patients with solid tumors of any type (Oosterwijk et al., 1993). In a similar diagnostic pre-clinical trial, MAb 138H11 was used in a human kidney extracorporeal perfusion model to successfully demonstrate the effective uptake of radiolabeled MAb in RCC tumors (Fischer et al., 1992). In a phase I trial that was designed to test the therapeutic efficacy of combined MAb and IL-2 treatment, a bispecific antibody composed of EGP-2 and anti-CD3 was used (Kroesen et al., 1994). The bispecific MAb was used in conjunction with IL-2 therapy to assess tumor size reduction after treatment. The results from this approach were not encouraging, with only 1 partial response in 14 evaluable patients. Further clinical trials are expected to be performed with these MAbs to promote a better understanding of their diagnostic and/or therapeutic potential.

MAb 469-5A recognizes a previously undescribed glycoprotein, gp 45/55, that is overexpressed on most if not all RCCs. However, the further characterization of this MAb and its target antigen depends on the utility of either or both being effectively used to study, diagnose, and/or treat

RCC. One of the potential drawbacks to the use of 5A MAb is its low binding affinity for gp 45/55. Poor vascularization of most solid tumors effects MAb delivery to tumor cells (Jain, 1990) and high tumor interstitial pressure subsequently prevents MAb penetration into tumor tissues (Boucher et al., 1990). Therefore, low affinity for target antigen is disadvantageous for most therapeutic applications of tumor-targeted MAbs. Another potential drawback of MAb 5A as a diagnostic tool is the distribution of detectable gp 45/55 over a wide range of normal tissues. This may cause problems from high background binding and therefore poor RCC tumor accumulation of MAb.

On the other hand, there are several characteristics of MAb 5A and gp 45/55 that indicate its usefulness in the study, diagnosis, and possible treatment of RCC. Data that has been presented indicates that MAb 5A may be useful in the purification and further characterization of gp 45/55, in the clinical diagnosis of RCC tumor biopsy tissue via IHC assay and possibly, in the immunodiagnostic detection of RCC tumors *in vivo*. These conclusions are based on several significant results. First, MAb 5A can be used to efficiently purify gp 45/55 from RCC cell line whole cell lysates, which will allow for the further characterization and study of gp 45/55. These studies should lead to a better understanding of its function in RCC cells. The discovery of proteins important in the transformation, differentiation, and/or metastatic capability of tumor cells is important. MAb 5A purified gp 45/55 may also be used as an immunogen source to prepare

other MAbs with higher affinities to gp 45/55 that could be useful diagnostically and/or therapeutically.

As previously indicated, Western blot and IHC data demonstrated the overexpression of MAb 5A detectable gp 45/55 in most RCC tumors. MAb 5A also detects the presence of gp 45/55 on proximal tubule epithelium. These data indicate that MAb 5A could be useful in the clinical diagnosis of tumor biopsies from questionable RCC masses. The expression of detectable gp 45/55 on RCC tumor cells and its absence from renal adenocarcinoma cells could be used differentiate these two tumor types.

MAb 5A may also be useful in the *in vivo* detection of RCC masses using immunoscintigraphic techniques. The preliminary amino acid sequence of gp 45/55 indicates that it may belong to the mucin-like family of glycoproteins. Mucins are cell surface glycoproteins that have a very low rate of turnover. This would make mucin-like proteins, overexpressed on tumor cells, ideal targets for diagnostic imaging. Tumors as small as 0.5 cm, which are sometimes missed by other radiological methods, can be imaged with MAbs labeled with suitable radionuclides (Waldmann, 1991). MAb 5A's usefulness in the area of immunodiagnosis should be further explored.

The potential applicability of MAb 5A as an immunodiagnostic or immunotherapeutic tool warrants further investigation to fully exploit its full potential. Likewise, the results reported indicate the importance of gp 45/55 to the RCC phenotype and indicate the importance of further characterization of gp 45/55.

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