Attributes of Antiangiogenic Factor Plasminogen Kringle 5 in Glomerulonephritis

Jin-Shuen Chen, MD, PhD; Jyh-Chang Huang, MD; Li-Chien Chang, PhD; Chia-Chao Wu, MD, PhD; Yuh-Feng Lin, MD

Context.—Plasminogen kringle domain (K) 5 is known to inhibit endothelial cell growth, but limited data are available investigating the relationship between K5 and glomerulonephritis (GN).

Objective.—To understand the relationships among K5, GN, and glomerular endothelial cells in GN mice models and human subjects.

Design.—Two mice models of GN and 2 categories of human GN biopsy samples were collected to gain insight into the disease mechanism from the laboratory to bedside. In the mechanistic animal study, membranous nephropathy (MN) and focal segmental glomerulosclerosis mice models were used. Kringle domain 5 in the diseased kidney was located by immunofluorescence and quantified by Western blotting. In the kinetic animal study, different MN time points were stained with K5, immunoglobulin G, and C3 by immunofluorescence. CD31 and proliferating cell nuclear antigen were evaluated by immunohistochemical double staining for alterations in the glomerular endothelial cells. Biopsy samples from patients diagnosed with antibody (Ab)-mediated and non-Ab–mediated GN were collected for K5 analysis.

Results.—The expression level of K5 was found to be significant in MN, but not in focal segmental glomerulosclerosis, and was markedly elevated in the diseased glomeruli along the capillary walls. Kringle domain 5 levels increased steadily with the evolution of MN, appearing after the deposition of Abs. In altered glomerular endothelial cells, CD31 decreased with the evolution of MN. In human subjects, K5 occurred only in patients with Ab GN.

Conclusions.—Kringle domain 5 might be involved in the progression of Ab-mediated GN and associated with the alteration of MN glomerular endothelial cell growth.

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Received for publication February 26, 2010. From the Department of Internal Medicine, Division of Nephrology, Tri-Service General Hospital (Dr Chen and Wu) and the School of Pharmacy (Dr Chang), National Defense Medical Center, Taipei, Taiwan; the Department of Internal Medicine, Division of Nephrology, Chi Mei Medical Center, Tainan, Taiwan (Dr Hwang); and the Department of Internal Medicine, Division of Nephrology, Shuang Ho Hospital, Taipei Medical University, Taipei (Dr Lin). The authors have no relevant financial interest in the products or companies described in this article. Reprints: Jin-Shuen Chen, MD, PhD, Department of Internal Medicine, Division of Nephrology, Tri-Service General Hospital, 325 Cheng-Gung Rd, Section 2, Nei-Hu District, Taipei, Taiwan 114 (e-mail: dgschen@ndmctsgh.edu.tw).
domain contains 5 kringle domains, named K1 through K5. Plasminogen can be cleaved by plasminogen activators (eg, tissue-type plasminogen activators and urokinase-type plasminogen activators), matrix metalloproteinases, and elastase into specific fragments of K1 to K5, for example, K1, K2, K3, K4, K5, K1 to K3, K1 to K4, and K2 to K3. Since 1994, researchers have suggested that each of these fragments is involved in a number of biologic processes. The fragment containing the fifth domain, K5, exhibits greater ability for antiangiogenic cell growth than the other fragments. Hence, although no significant data regarding the functions of K5 in GN have been reported, to present, we speculated that the effects of K5 on glomerular endothelial cells (GECs) might also be deleterious.

We hypothesized that the antiangiogenic factor K5 plays a role in the pathogenesis of GN. To verify our hypothesis, 2 mice models of GN (MN and FSGS) were established, and 2 categories of human GN biopsy samples (Ab GN and non-Ab GN) were collected to gain insight into the disease mechanism from the laboratory to bedside.

MATERIALS AND METHODS

Experimental Design

To delineate the relevance between K5 and GN, we conducted animal and human studies. For the mechanistic animal study, the first experimental series used 2 mice models, mimicking human MN and FSGS, respectively, to study the connections of K5 with disease pathogenesis. For the kinetic animal study, in a second experimental mouse series, those relationships were further investigated by analyzing their development in the MN-diseased kidney at various time points. At the same time, CD31 and proliferating cell nuclear antigen (PCNA) were evaluated to understand the alteration of GECs. The aim of this series was to clarify the time course of the occurrence of K5 and CD31 in the diseased kidney. In clinical subjects, the third experimental series was conducted using biopsy samples. Several patients with GN were recruited to confirm the findings from the animal models.

Mouse Models

Animal studies were performed in accordance with institutional guidelines. Experiments were conducted on 8-week-old, female, BALB/c mice (20 g) that were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The animals were housed in laboratory cages and fed a standard diet. Experimental MN was induced by cationic bovine serum albumin as previously described. The animals were randomly divided into 2 groups (10 in each group): an experimental group and a normal control (NC) group. Disease onset was checked regularly using urine dipsticks (Bayer Corporation, Elkhart, Indiana). Once the dipstick showed a marked proteinuria (+++), it was termed full-blown MN (FBMN). In the first series of mechanistic studies, the animals were euthanized once severe proteinuria developed and, in the second kinetic study, at weekly intervals before or after onset of FBMN.

Experimental FSGS was induced by doxorubicin (Adriamycin) as previously described. The animals were divided into 2 groups (10 in each group). Animals in the experimental group received an intravenous injection of doxorubicin (Adriamycin; 10–11 mg/kg), and those in the NC group received saline. Animals were euthanized several days later, once severe proteinuria developed.

Blood and Urine Biochemistry

Blood and urine samples were collected as previously described. All blood and urine biochemical data were determined following previous methodologies.

Renal Histologic Examinations

Formalin-fixed, paraffin-embedded, and frozen sections of kidney tissue were cut and stained with periodic acid–Schiff stain, colloidal iron, immunofluorescence (IF) stain, and electron microscopy (EM) for general histologic examination as previously described.

Immunofluorescence for K5

Frozen kidney tissues were used in the IF analyses. Cryostat-cut tissue sections (4 μm in thickness) were incubated with goat anti-mouse K5 (R&D System Inc, Minneapolis, Minnesota), followed by a secondary, fluorescein isothiocyanate–labeled, rabbit anti-goat IgG (Santa Cruz Biotechnology Inc, Santa Cruz, California). Negative controls omitted the primary antibody. Slides were examined for development of fluorescence using an optical photomicroscope (Olympus Corporation, Tokyo, Japan), and for semiquantitative evaluation, at least 10 glomeruli per slide were examined for positively stained areas. For every glomerulus examined, a grade of 0 to 4 (0, no positive staining; 1+, minor staining; 2+, moderate staining; 3+, prominent staining with expanded positively stained areas; 4+, prominent staining of large areas) was given. A total fluorescence score for each specimen was calculated using the following equation: Total fluorescence score = (% glomeruli at grade 0) + (% glomeruli at grade 1+) + (% glomeruli at grade 2+) + (% glomeruli at grade 3+) + (% glomeruli at grade 4+). In the examination of the clinical biopsy samples, glomerular expression of K5 was semiquantified by the same method, but for each case, 3 or more glomeruli were investigated.

Western Blot Analysis

The expression of K5 in the renal cortices of mice was quantified by Western blotting. Renal cortices harvested from BALB/c mice were homogenized in ice-cold protein-extraction solution (Intron Biotechnology Company, Seongnam, Korea), followed by centrifugation at 19 980g for 20 minutes at 4 C. The protein concentration of the supernatants was determined, and samples (30 μg protein per lane) were run as previously described. Blots were incubated with goat anti-mouse K5 primary antibody and horseradish peroxidase-conjugated, anti-goat secondary antibody (Pierce Biotechnology Inc, Rockford, Illinois). Bands were detected using the Western Lighting Chemiluminescence Reagent Plus (PerkinElmer, Boston, Massachusetts) and Kodak film (Eastman Kodak, Rochester, New York). Western blot signal intensity was normalized to the actin band density.

Immunohistochemical Double Staining

Kruskall–domain 5 is known for its antiangiogenic cell growth ability. CD31 is an intrinsic endothelial cell antigen expressed early in development and persisting through adult life. Antibodies directed against CD31 were shown to detect angiogenesis. Proliferating cell nuclear antigen has been identified as an antigen that is expressed in the nuclei of cells and as a marker of cell proliferation. Therefore, the injury to glomerular endothelial cells was evaluated by staining with CD31 and PCNA. Immunohistochemical staining was performed on formaldehyde-fixed, paraffin-embedded sections. Paraffin was removed from the sections, followed by dehydration. The sections were incubated with a 1:100 dilution of the first primary rat polyclonal anti-CD31 antibody (BD Biosciences, San Jose, California) in phosphate-buffered saline at 4 C overnight. After incubation with 1:50 dilution of the biotinylated secondary antibody (Vector Laboratories, Burlingame, California) for 40 minutes, the sections were treated with VECTASTAIN ABC (Vector Laboratories) working solution for 30 minutes. The reaction was visualized by use of an AEC chromogen. Subsequently, the slides were incubated with a 1:200 dilution of the secondary antibody, rabbit anti-PCNA (Santa Cruz Biotechnology Inc) at 4 C overnight. After that, the slides were incubated with an alkaline phosphatase 1:200 dilution of the secondary antibody (Cappel
Laboratories, West Chester, Pennsylvania), then developed with NBT/BCIP (nitro-blue tetrazolium chloride/5-bromo-4-chloro-3’-indolyolphosphate p-toluidine salt) solution in the dark for 15 mins. Finally, the slides were counterstained with methyl green.

Human GNS

The institutional review board at the National Defense Medical Center (Taipei, Taiwan) approved the study. All patients were older than 20 years and capable of providing informed consent. In brief, patients were recruited for this study when they had currently active GN. These patients were required to have kidney biopsy evidence of Ab or non-Ab GN and major renal pathologic findings on hematoxylin-eosin, IF, and EM. The types of Ab GN disease collected in the study included MN, IgAN, and LN; non-Ab GN consisted of FSGS and MCD. At least 3 cases of each GN type were obtained when patients underwent routine renal biopsies. Kidney tissues were collected to confirm the experimental findings. Kidney tissues from patients with GN were compared with those from healthy controls.

RESULTS

Blood and Urine Biochemistry in Mice Models of MN and FSGS

The MN group developed overt proteinuria, termed FBMN, around week 4, similar to that seen in our previous studies, whereas FSGS mice showed overt proteinuria at about day 5. Both groups of mice were euthanized and underwent routine blood and urine examinations at disease onset. As shown in Table 1, FBMN and FSGS mice both showed nephrotic patterns, including proteinuria, hypoalbuminemia, and hyperlipidemia. Compared with the NC, both the FBMN and FSGS mice had significantly lower serum albumin and higher proteinuria levels (Table 1), and the disease described by these 2 parameters was of greater severity in MN than in FSGS mice. The total cholesterol level in both FBMN and FSGS mice was significantly higher than in the NC mice, but there was no difference between FBMN and FSGS mice. Blood urine nitrogen and creatinine levels showed no variation between the FBMN, FSGS, and NC mice.

Histopathology in Mice Models of MN and FSGS

Light Microscopy.—The results of the periodic acid–Schiff stain (Figure 1, A through C) for tissue glycogen showed basement-membrane thickening in connective tissues and basal laminae in FBMN, but in FSGS, it revealed a well-delineated, rounded glomerulosclerosis that adhered to the Bowman capsule, with the appearance of a small fibrosis. The results of the colloidal iron stain (Figure 1, D through F) for evaluation of glomerular polyanions showed diminished blue staining along the glomerular capillary walls in both the FBMN and FSGS groups, indicating that both FBMN and FSGS mice had lost glomerular charge selectivity.

Immunofluorescence Staining.—Further examination of the disease course was performed by IF staining of the immunoproteins. As seen in Figure 1, G through I, IgG was expressed as strong, granular strings along the glomerular capillary walls in FBMN mice, whereas it was weakly expressed in the sclerotic area in FSGS mice and very blurred in the NC mice. For other immunoproteins, staining for C3 was grade 4+ for IgG, grade 1+ for IgM, grade 1+ for IgA, was negative in FBMN mice, and there was no detectable staining in FSGS mice (data not shown).

Electron Microscopy.—Full-blown MN mice showed severe, irregular thickening of the lamina rara, the interna, and the externa of the GBM, with layered and more confluent, subepithelial deposits, whereas the FSGS mice showed collapsed basement membranes with severe effacement of the foot processes (Figure 1, J through L). This suggests that both models lost size selectivity.

K5 Expression in Mice Models

Examination of K5 in the kidney tissue of GN mice was performed on frozen renal tissue. A significant expression of K5 was observed along the capillary walls of the glomeruli in the FBMN mice (Figure 2, B). In contrast, there was very little expression of K5 in the FSGS (Figure 2, C) and NC (Figure 2, A) mice, suggesting that the recruitment of K5 into the diseased kidney was MN specific. To confirm this finding, K5 in the whole kidney cortex was quantitatively examined by Western blotting. A significant level of K5 expression was again demonstrated in FBMN, but not in either the FSGS or NC mice (Figure 2, D and E), implying that K5 might involve the pathogenesis of MN.

Pathogenic Relationship Among K5, Immune Complexes, and the Alteration of GEC in the MN Mouse Model

Because MN is known to be mediated by antibodies, we investigated the relationships between K5 and immunoproteins during the course of the MN disease. Results showed that the influx of IgG into the kidney cortex started 2 weeks before FBMN, then peaked at the onset of FBMN, and remained at high levels for the rest of the study period. The influx of complement protein C3 started

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**Table 1. Comparison of Blood and Urine Biochemistry From Normal Control (NC), Full-Blown Membranous Nephropathy (FBMN), and Focal Segmental Glomerulosclerosis (FSGS) Mouse Groups**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NC, mean (SD)</th>
<th>FBMN, mean (SD)</th>
<th>FSGS, mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BUN, mg/dL</td>
<td>32.2 (3.6)</td>
<td>36.3 (4.8)</td>
<td>34.8 (2.5)</td>
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<td>Cr, mg/dL</td>
<td>1.0 (0.2)</td>
<td>1.2 (0.1)</td>
<td>1.0 (0.3)</td>
</tr>
<tr>
<td>T. chol, mg/dL</td>
<td>140.6 (17.6)</td>
<td>322.7 (24.2)</td>
<td>329.2 (28.8)</td>
</tr>
<tr>
<td>Alb, g/dL</td>
<td>3.4 (0.4)</td>
<td>1.7 (0.2)</td>
<td>2.6 (0.5)</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPL, UP:UCr</td>
<td>0.5 (0.1)</td>
<td>1.4 (0.3)</td>
<td>0.9 (0.1)</td>
</tr>
</tbody>
</table>

Abbreviations: Alb, albumin; BUN, blood urea nitrogen; Cr, creatinine; DPL, daily protein loss quantified by measuring the ratio of urine Alb excretion to urine Cr excretion; T. chol, total cholesterol; U, urine.

$^a$ 10 mice per group.

$^b$ $P < .001$ versus NC.

$^c$ $P = .003$ versus NC.

$^d$ $P < .001$ versus FBMN.

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a week later than the IgG, gradually becoming prominent during the onset of FBMN, and then achieving a plateau in the week following the onset of FBMN. The influx of K5 occurred last, and complete induction of K5 appeared only a week after the onset of FBMN (Figure 3, A through L, and 4). On the other hand, concerning the alteration of GEC, the expression of CD31 was along the capillary wall as shown in the NC group (Figure 5, A through E), and the intensity decreased obviously as the disease progressed. However, the number of PCNA-positive cells in the

Figure 1. Histopathologic findings of glomeruli in normal controls (A, D, G, and J), full-blown membranous nephropathy (B, E, H, and K), and focal segmental glomerulosclerosis (C, F, I, and L) mice models (periodic acid–Schiff [A through C], colloidal iron stain [D through F], and immunofluorescence stain [G through J], original magnifications ×400; electron microscopy [J through L], original magnifications ×3000).
glomeruli showed no significant change at the different time points. The PCNA and CD31 results imply that there is no significant cell proliferation in the glomeruli, particularly in endothelial cell.

Taken together, K5 might be associated with inhibiting GEC growth in the MN mouse model.

K5 in Human Subjects With GN

In our study, anti-mouse K5 was used to detect the expression of human K5. The amino acid sequence of K5 between human and mouse was compared with ClustalW2 software (European Molecular Biology Laboratory, European Bioinformatics Institute, Hinxton, Cambridge).

Figure 2. Protein expression of plasminogen K5. Immunofluorescence stain of glomeruli for kringle domain 5 (K5) in normal controls (NC; A), full-blown membranous nephropathy (FBMN; B), and focal segmental glomerulosclerosis (FSGS; C) mice models. D, Western blot for K5. In the representative immunoblot, K5 has a prominent intense band in FBMN. The amount of K5 was normalized against the amount of actin, and results are presented as ratios compared with the NC group (E). *P < .001 versus NC, and P = .01 versus FSGS (original magnifications ×400).

Figure 3. Temporal relationships among kringle domain 5 (K5), immunoglobulin G (IgG), and C3 in the disease course of the membranous nephropathy (MN) mouse model. C, G, and K, Full-blown MN (FBMN). A, E, and I, Two weeks before FBMN onset. B, F, and J, One week before FBMN onset. D, H, and L, One week after FBMN onset (immunofluorescence stain for IgG, original magnifications ×400 [A through D]; immunofluorescence stain for C3, original magnifications ×400 [E through H]; immunofluorescence stain for K5, original magnifications ×400 [I through L]).

Figure 4. Semiquantitative evaluation of fluorescence. Only the mean is shown.

Figure 5. Temporal relationship of CD31 and proliferating cell nuclear antigen (PCNA) in the disease course of the membranous nephropathy (MN) mouse model with double staining. Positive staining of CD31 is red (arrow), and positive staining of PCNA is blue (star). A, Normal control. B, Two weeks before onset of full-blown MN (FBMN). C, One week before FBMN onset. D, FBMN. E, One week after FBMN onset (original magnifications ×400).
United Kingdom), and 85% sequence identity was found. It has been suggested that anti-mouse K5 can recognize a human specimen, but the intensity of the K5 expression in human specimens may be weaker than in mouse tissue. Table 2 shows that 5 types of patients with GN, including those with MN, IgAN, LN, MCD, and FSGS, were enrolled in this study. All cases were idiopathic (except LN) and had firm diagnoses based on renal pathologic examinations (data not shown). In accordance with our previous findings, the antiangiogenic K5 was again found to be highly expressed in the renal glomeruli of patients with MN (Figure 6, A), IgAN (Figure 6, B), and LN (Figure 6, C) but not in those with MCD and FSGS (data not shown). As seen before, the cases with Ab-mediated GN all showed strong K5 expression in the glomeruli (with the intensity of K5 expression being LN > IgAN > MN). The deposition of K5 in the diseased kidneys was located along the capillary walls in MN and LN, and in the mesangial areas in IgAN, showing that K5 is colocalized with immune deposits.

COMMENT

Two mice models of GN with suitable human MN and FSGS patterns were established as in previous studies.23,24 Using these models, in the mechanistic study, we have, for the first time to our knowledge, presented evidence that the antiangiogenic factor plasminogen kringle domain 5 might be involved in the pathogenesis of GN. In the kinetic study, the connections among K5, CD31, and MN were established. In the clinical study, the significant expression of K5 in Ab-mediated GN was proven in clinical subjects. The first major finding of this study is that K5, from mice models, is expressed in MN, but not in FSGS. In clinical subjects, K5 was present exclusively in Ab-mediated GN. Taken together, we suggest that K5 might be related to the pathogenesis of Ab-mediated GN. Regarding the role of angiogenesis in the pathogenesis of MN and FSGS, the mounting evidence from animal studies29 has suggested that the visceral glomerular epithelial cell (podocyte) is the primary target of injury. Thus, the differences in the resulting cell types injured on the pathogenesis of GN could also be examined with this simple method. Membranous nephropathy and FSGS have similar clinical manifestations but have different disease pathogeneses. The former is initiated by an antigen–antibody reaction, followed by podocyte damage, but the latter results from direct podocyte injury. As for

<table>
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<th>Subjects</th>
<th>Age, y/Sex</th>
<th>Urine:Creatinine (Up:UCr)</th>
<th>BUN, mg/dL</th>
<th>Cr, mg/dL</th>
<th>Alb, g/dL</th>
<th>K5 Intensity Score</th>
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Abbreviations: Alb, albumin; BUN, blood urea nitrogen; Cr, creatinine; FSGS, focal segmental glomerulosclerosis; IgAN, Immunoglobulin A nephropathy; LN, lupus nephritis; MCD, minimal change disease; MN, membranous nephropathy; Up:UCr, the ratio of urine protein to urine albumin, representing daily protein loss.

Figure 6. Expression of plasminogen K5 in human patients with glomerulonephritis. The representative immunofluorescence stain of glomeruli is shown. Kringle domain 5 is expressed in membranous nephropathy (A), immunoglobulin A nephropathy (B), and lupus nephropathy (C). The semiquantitative analysis of fluorescence is shown in Table 2 (original magnifications ×400).
involvement of angiogenesis, a literature review suggests that angiogenic factors have a role in both models: vascular endothelial growth factor and thymidine phosphorylase in MN and vascular endothelial growth factor in FSGS. However, the role of antiangiogenic factors has yet to be determined. As is evident in our animal and human experiments, indirect or direct podocyte injury–mediated GN, represented by MN and FSGS, respectively, are distinguishable by examining the presence of plasminogen kringle domains in diseased kidneys, implying that K5 might potentially be used not only for differentiating GN but also for providing insight into the pathogenic mechanisms of Ab-mediated GN.

The second major finding of our study is that the spatiotemporal deposition of K5 fits into the course of development of the glomerular injury in MN mice, and it follows the influx of immune proteins into glomeruli. Based on a literature review, we propose 3 possible mechanisms for Ab-induced K5 production. First, Ab deposition can stimulate endothelial cells to produce tissue factor and, subsequently, activate the coagulation cascade. The activation of coagulation, if not properly counterbalanced by fibrinolysis, may be a major factor in the late development of fibrotic changes in glomeruli. As an antifibrotic effect, fibrinolytic factors, such as plasminogen, may be increased. Second, immune complexes can activate protease, so the enzymes (ie, the matrix metalloprotease or elastase) required for K5 cleavage from plasminogen may be activated, leading to an increase in the expression of K5. Third, immune complexes may elicit high expression of specific receptors reacting with K5, such as glucose-regulated protein 78 and voltage-dependent anion channels. However, K5 is a relatively small peptide, so nonspecific K5, trapped in immune complex deposits and/or combined with C3, cannot be ruled out. Further studies are needed to support the K5 interaction with endothelial cells in the disease process and to identify where K5 is deposited in the glomerular capillary wall.

The third major finding of this study is that the glomerular capillary of the MN mouse model showed an obvious decrease in the expression of CD31, which represents angiogenesis. Lu et al also suggested that idiopathic MN subjects have a partial or complete loss of CD31, but the mechanism is unknown. K5 has the antiangiogenic ability to inhibit endothelial cell growth. In our study, we established K5 and CD31 as higher and lower expression, respectively, in the MN mouse model. This may imply that K5 plays a role in MN antiendothelial cell growth.

Both K5 and immune proteins mediate progressive deterioration in renal function. As discussed previously, glomerular injury is initiated by the deposition of immune complexes in subepithelial, subendothelial, and mesangial areas, where the membrane attack complex of complement colocalizes to propagate renal injury. However, the detailed mechanism of K5 interacting with immune proteins, such as IgG and C3, to mediate renal injury is still essentially unknown. As for the possible role of K5 in biologic processes, the evidence suggests that K5 not only possesses antiangiogenic properties but also has the ability to act as an immune modulator through its ability to recruit neutrophils and T lymphocytes. From our present data, we are still unable to determine the role of K5 in glomeruli during massive injury subsequent to the deposition of Abs.

In this study, we have for the first time, to our knowledge, presented evidence that K5 may be involved in the pathogenesis of GN, particularly in Ab-mediated GN. In addition, K5 may associate with the alteration of GEC in MN.

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References

Prepare Now for the CAP ’11 Abstract Program

Plan now to submit abstracts and case studies for the College of American Pathologists (CAP) 2011 meeting, which will be held September 11th through the 14th in Grapevine, Texas. Submissions for the CAP ’11 Abstract Program will be accepted from:

Monday, January 31, 2011, through Friday, April 1, 2011.

Accepted submissions will appear in the September 2011 issue of the Archives of Pathology & Laboratory Medicine. Visit the ARCHIVES Web site at www.archivesofpathology.org and also the CAP ’11 Web site at www.cap.org/cap11 for additional abstract program information as it becomes available.