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Renal Disease in Carrier Female Dogs with X-linked Hereditary Nephritis

Implications for Female Patients with this Disease

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Male dogs with X-linked hereditary nephritis (HN) serve as a model for studying male patients with this disease. In the present study, carrier female dogs were found to resemble female patients in showing a broad range of renal dysfunction. Of 37 carrier female dogs studied, all were healthy up to 5 years of age; however, all had proteinuria develop at 2 to 3 months, and focal segmental glomerulosclerosis (FSGS) was detected after 7 months. After 5 years, 4 of 13 dogs remained healthy and showed mild FSGS on renal biopsy; 4 had mild renal dysfunction develop and their kidneys showed extensive FSGS; 5 died prematurely of renal failure with end-stage kidneys. By immunofluorescence, using antibody to the NC1 domain of collagen type IV, segmental staining of glomerular basement membranes (GBM) was seen in all dogs before 3 to 4 years, and lesions of FSGS were negative. Thereafter, a transition to global staining of GBM was noted and lesions of FSGS became positive. Lens capsule and basement membranes in lung and choroid plexus showed discontinuous staining in two young carrier female dogs and continuous staining in one older carrier female dog. By electron microscopy, multilaminar splitting of some GBM was seen up to 4 years, and thereafter, splitting took on a compressed appearance, with the layers becoming apposed though still detectable. The authors conclude that: 1) carrier female dogs with X-linked HN are mosaics for an abnormality in the NC1 domain of GBM and other basement membranes; 2) FSGS develops in all carrier female dogs in glomerular capillary loops that possess an abnormal NC1 domain, and progresses to a variable extent in different dogs; and 3) the abnormality of NC1 in GBM of carrier female dogs appears to diminish with age, but this does not prevent progression of renal disease. Similar conclusions may apply to females with X-linked HN. (Am J Pathol 1991, 139:751–764)

Human hereditary nephritis (HN) consists of a group of inherited glomerular diseases that often progress to end-stage renal failure and may be associated with sensorineural hearing loss, referred to as Alport syndrome, and lens abnormalities, particularly anterior lenticonus.[1,2] HN can be grouped into a number of subtypes, depending on the age of development of renal failure, the presence or absence of extrarenal clinical manifestations, and the genetics of inheritance.[3,4] A diagnosis of HN is confirmed by electron microscopy (EM), which shows multilaminar splitting of glomerular basement membranes (GBM).[5–8] The abnormality of GBM that produces splitting has not been fully elucidated, but is believed to involve the carboxy terminus (NC1 domain) of one or more collagen type IV chains.[9,10] The collagen type IV molecule is arranged in a helix of three α chains, which are joined via the NC1 domain to an adjacent molecule.[11,12] Five different α(IV) chains have been described,[13,14] but their proportions in collagen type IV molecules are unknown. The Goodpasture antigen is believed to reside mainly in the NC1 domain of the α3(IV) chain.[15–17] When isolated from the helical portion of the molecule, the NC1 domain forms a hexamer derived from two adjacent molecules of collagen type IV, and by SDS-PAGE can be separated into dimers of about 50 kd and monomers of about 24–28

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kd.[13] Under these conditions, the NC1 domains of the α3(IV) and α4(IV) chains yield 28 kd monomers,[18] both of which have been reported to be absent from GBM of some male patients with X-linked HN.[9] However, immunologic abnormalities have also been documented in the 26 kd monomer band of the isolated NC1 hexamer,[10] which is probably derived from both the α1(IV) and α5(IV) chains.[14] Furthermore, the gene for the α5(IV) chain is located on the X-chromosome,[14] and has recently been shown to be abnormal in some cases of human X-linked HN.[19,20]

We have been studying a spontaneously occurring glomerular disease in a family of Samoyed dogs, referred to previously as "Samoyed hereditary glomerulopathy," and in this study as "canine X-linked HN," as a model for human X-linked HN.[21] Pedigree analysis has shown that canine X-linked HN is transmitted by an X-linked gene,[22] similar to HN in most families.[3,4] Affected male dogs with X-linked HN rapidly have renal failure develop and die within the first year of life,[23] reminiscent of the early development of renal failure seen in many male patients.[1,2] The splitting of GBM seen by EM in affected male dogs is identical to that seen in most human males with HN.[5–8] All GBM of affected male dogs did not stain by immunofluorescence (IF) microscopy, using serum obtained from a patient with Goodpasture syndrome[24] or a plasmapheresis fluid obtained from a patient with anti-GBM nephritis, which was shown to contain antibody to the NC1 domain of collagen type IV (referred to hereafter as human anti-NC1 antibody).[25] Absence of staining of GBM has been observed in renal biopsies of most males with X-linked HN, using serum obtained from patients with Goodpasture syndrome.[26–29] Studies of the NC1 domain isolated from GBM have shown abnormalities in both male patients and affected male dogs with X-linked HN.[9,10,25]

X-linked HN has not been as well characterized in female patients as in male patients. In a previous study of young carrier female dogs with X-linked HN, the only biochemical evidence for renal dysfunction was proteinuria in all of these dogs and microscopic hematuria in some of them.[23] In contrast, female patients with HN show a broad spectrum of abnormalities, ranging from normal renal function to end-stage renal failure.[30] By EM, foci of multilaminar splitting of GBM were seen in carrier female dogs, but splitting did not become as extensive over the same time period as it did in affected male dogs.[31] Similarly, in one study, female patients with HN showed less splitting of GBM than did male patients.[32] By IF, segmental staining of GBM was seen in carrier female dogs, using the human anti-NC1 antibody.[33] GBM of female patients with HN have shown various results by IF staining with anti-NC1 antibodies, ranging from negative[26–29] to segmental[34–36] or global[26–29] positivity.

In the present study, we examined young and older carrier female dogs clinically, and characterized the evolution of renal dysfunction and the morphologic changes in their kidneys and a number of extra-renal basement membranes. We found that the NC1 domain of collagen type IV was abnormal in several basement membranes of young carrier female dogs, and that some older carrier female dogs progressed to end-stage renal damage, even though the abnormality of the NC1 domain appeared to diminish with age. These findings may provide insight into X-linked HN in human females.

Materials and Methods

Dogs

Adult carrier female dogs with X-linked HN[23] were mated to mixed-breed males in the Laboratory Animal Facility, Ontario Veterinary College, University of Guelph. Their offspring were examined regularly by a veterinarian, and records were kept of their growth, development and behavior. Renal biopsies were performed at 6 to 8 weeks of age, and GBM were examined by IF and EM, allowing the dogs to be classified as unaffected, affected males, or carrier females.[33] Between 1977 and 1990, 37 newborn dogs were found to be carrier females. Twenty-four of these were kept at Guelph for up to 5 years, and some were used in previous studies.[26,27,33,35] Renal biopsies were performed at various times and examined by IF and EM. Eighteen of these dogs were killed at various times up to 7 months, whereas the remaining six were killed between 7 months and 5 years of age. The timing of sacrifice was purely random, since all dogs were healthy. At sacrifice, samples of kidney were taken for examination by LM, IF, and EM. Samples of lens, lung, and choroid plexus were taken for IF from two dogs, both 8 months old. Thirteen dogs selected at random were retained for longer than 5 years. These included two dogs who are being maintained at Guelph for breeding and 11 who were sold as pets. The latter dogs were seen by a veterinarian if they became ill, and those that could not be successfully treated were killed. Samples of postmortem kidney were available from six of these dogs for examination by LM, IF, and EM. Samples of lens, lung, and choroid plexus were available for IF from one of these dogs, who was 9 years old.

Clinical Biochemistry

Serum creatinine of the carrier female dogs kept at Guelph was monitored every 2 months, using a KDA an-

alyzer (American Monitor Corp., Downsview, Ontario). The normal level in our clinical biochemistry laboratory is 60 to 110 μmol/L. Serum creatinine of the privately owned carrier female dogs was determined when they were brought to a veterinarian. Urinary protein was quantitated using 20% sulfosalicylic acid as in a previous study.[22] The plasma clearance of sodium sulfanilate, which has been employed extensively in dogs to estimate glomerular filtration rate (GFR),[37,38] was determined in normal female dogs at 7 months of age and in carrier female dogs at various ages, as previously described.[23] This method was used, rather than creatinine clearance, to avoid the necessity of catheterizing the dogs. The half-life ($\tau^{1/2}$) in minutes of sodium sulfanilate clearance was converted to GFR in mL/min/1.73 m^2 as previously described.[39]

Tissue Processing

Renal biopsies were performed as in a previous study.[33] Only biopsies containing at least six glomeruli were considered to be adequate for evaluation by LM and IF. Of the carrier female dogs kept at Guelph, 9 underwent biopsies once, 15 underwent biopsies twice, and 5 underwent biopsies three times. Biopsies were done before 3 years of age in some dogs, and after 3 years of age in others. One carrier female dog underwent a biopsy at 6 months and 3.5 years of age. One half of the biopsy was snap-frozen in liquid nitrogen and stored at −70°C. Cryostat sections were cut at 5 μm and used for IF. In addition, one of these sections was stained with periodic acid-Schiff (PAS), and examined by LM. The second half of the biopsy was fixed in a 4% paraformaldehyde–1% glutaraldehyde solution, embedded in an Epon-Araldite mixture, sectioned at 50 nm, and stained with uranyl acetate and lead citrate. Grids were examined on a Philips 201 electron microscope.

Samples of kidney obtained at the time of sacrifice were fixed in 10% buffered formalin and processed for LM. At least 50 glomeruli were examined, and the percentage of segmentally sclerosed glomeruli was quantitated using sections stained with PAS. Samples of kidney obtained for EM and IF were processed as described earlier. Renal biopsies and postmortem kidneys were obtained from normal female dogs of various ages for examination by LM, IF, and EM.

Samples of choroid plexus and lung were placed in cryomolds containing OCT compound, and snap-frozen in liquid nitrogen for examination by IF. The lens capsules were removed from the eyes using a dissecting microscope, snap-frozen as mentioned and examined by IF.

Immunofluorescence

IF was performed as described previously,[24] using (a) a rabbit anti-NC1 antibody, which by Western blotting binds to the 26 kd monomer band of dog and human NC1, and by IF stains GBM of normal and affected male dogs with X-linked HN;[24,25] (b) a human anti-NC1 antibody obtained from a patient with anti-GBM nephritis, which by Western blotting binds mainly to the 28 kd monomer band of human NC1 and to the 24 kd and 26 kd monomer bands of dog NC1, and by IF stains GBM of normal dogs but not affected male dogs with X-linked HN;[25] and (c) a dog anti-NC1 antibody that was raised by immunization of an affected male dog with NC1 domain isolated from normal dog GBM. By Western blotting, this antibody binds to the 26 kd monomer band of dog and human NC1, and by IF stains GBM of normal dogs but not affected male dogs with X-linked HN (Thorner, unpublished observations). A 1:10 dilution of the rabbit anti-NC1 antibody and a 1:3 dilution of the human anti-NC1 and dog anti-NC1 antibodies were used to stain frozen sections of kidney and lens capsule, whereas a 1:5 dilution of the rabbit antibody and a 1:2 dilution of the human and dog antibodies were employed with the other tissues. Fluorescent-labelled anti-human and anti-dog IgG were obtained from Dako (Copenhagen, Denmark) and Cappel (Organon Teknika Corp., West Chester, PA) respectively. Tissue sections were first treated with acid urea to expose hidden NC1 domain determinants, as described previously.[40] In some experiments, kidney and lens capsule sections were double-stained as follows: the dog anti-NC1 antibody was applied, followed by fluorescein-labelled anti-dog IgG; then the rabbit anti-NC1 antibody was applied, followed by rhodamine-labelled anti-rabbit IgG. As negative controls, the human, dog and rabbit anti-NC1 antibodies were either omitted or substituted with normal human, dog, or rabbit serum, respectively.

Morphometric Studies

GBM were evaluated by electron microscopic examination of renal biopsies and kidneys obtained at autopsy from the carrier female dogs of various ages. To quantitate splitting, 12 capillaries with split GBM were photographed at a magnification of 25,000X. The film negative of each glomerular capillary was projected onto a monitor that provided a further magnification of 42X. The length of normal and split GBM was determined, using an Interactive Image Analysis System (IBAS) (Kontron, Etching, West Germany), by tracing out normal and split GBM on a magnetized tablet and storing measurements in sepa-

rate channels of the IBAS. The negative was then advanced to bring a new field of GBM into view, and the measurements were repeated until the entire length of GBM around the capillary was assessed. The length of normal and split GBM was expressed as a percent of the total length. In a preliminary experiment, the length of normal and split GBM around 12 capillaries was measured in each of four glomeruli of a 5-month-old carrier female dog, and there was little variation from glomerulus to glomerulus (data not shown). Hence, the first 12 capillaries that showed splitting by EM were photographed, irrespective of the total number of glomeruli present on an EM grid. The relationship between splitting of GBM and age of the carrier female dogs was determined by linear regression, and the significance of the regression was tested by analysis of variance.

The thickness of the entire width of GBM (lamina rara interna, lamina densa, and lamina rara externa) was determined, using the IBAS, by measuring the distance between the fenestrated cytoplasm of glomerular endothelial cells and foot processes of visceral epithelial cells apposed to GBM. No measurements of thickness were made of GBM cut in a tangential manner. Thickness of GBM was assessed in one each of normal female and carrier female dogs at various ages. In both the normal and carrier female dogs, thickness was determined for completely normal GBM (i.e., no splitting of GBM) around five capillaries, and an average of 20 measurements were made per capillary. In the case of the carrier female dogs, thickness was also determined of GBM around five capillaries that showed both nonsplit and split foci, amounting to 10 to 20 measurements for each per capillary. Thickness was measured for multilaminar split foci only. Values were expressed as mean ± SEM. Student's *t*-test was used to test the significance of differences between means. P values < 0.05 were considered to be significant.

Results

Assessment of Clinical Status and Renal Function

All 37 carrier female dogs were healthy at birth and showed normal growth, development, and behavior for up to 5 years. Serum creatinine was normal; $\tau^{1/2}$ for plasma clearance of sodium sulfanilate and GFR were not significantly different from the values observed in normal female dogs ($\tau^{1/2}$ of 68 ± 7 minutes and GFR of 110.5 ± 5.9 ml/min/1.73 m^2) ($P > 0.05$) (Table 1). However, proteinuria > 0.3 g/l was present in all carrier female dogs after 2 to 3 months of age.

After 5 years of age, a variable clinical picture was seen in 13 carrier female dogs (Table 1). Four dogs (group I) are alive at 6 to 11 years of age (mean, 8 years) and have remained in good health with normal serum creatinine; $\tau^{1/2}$ for plasma clearance of sodium sulfanilate, measured in one dog at 6.5 years, was 80 minutes, giving a GFR of 92.2 ml/min/1.73 m^2. Four dogs (group II) were killed at 6.5 to 13.5 years (mean, 10.5 years) because they developed various non-renal diseases, namely arthritis, diabetes mellitus, mastitis, and skin infections, none of which could be successfully treated. All of these dogs had an elevated serum creatinine at time of sacrifice (160–315 μmol/L), and $\tau^{1/2}$ for plasma clearance of sodium sulfanilate, measured in one dog at 9 years, was 216 minutes, giving a GFR of 42.2 ml/min/1.73 m^2.

Table 1. *Clinical Status and Renal Function in Carrier Female Dogs of Various Ages wtih X-linked Hereditary Nephritis*

Age	Number	Clinical status	Serum creatinine (μmol/l)*	$\tau^{1/2}$ in minutes for sodium sulfanilate clearance†	GFR in ml/min/ 1.73 m^2‡
Birth	37	Normal	Normal	ND	ND
Up to 7 months	18	Normal	Normal	74 ± 4‖	105.2 ± 6.4‖
7 months to 5 years	6	Normal	Normal	62 ± 2¶	119.0 ± 8.2¶
Older than 5 years					
Group I	4	Normal	Normal	80#	92.2
Group II	4	#1 skin infections	315	ND	ND
		#2 arthritis	160	216**	42.2
		#3 diabetes mellitus	260	ND	ND
		#4 mastitis	260	ND	ND
Group III	5	Terminal renal failure	775, 820, 900, 985, 1250	ND	ND

* Serum creatinine in normal dogs is 60–110 μmol/l.
† $\tau^{1/2}$ for sodium sulfanilate clearance in normal female dogs is 68 ± 7 minutes.
‡ GFR in normal female dogs is 110.5 ± 5.5 ml/min/1.73 m^2.
‖ Mean ± SE obtained in four dogs at 7 months.
¶ Mean ± SE obtained in three dogs at 2, 2, and 4.5 years.
# Observed in one dog at 6.5 years.
** Observed in one dog at 9 years.
ND = not determined.

Table 2. *Renal Morphology in Carrier Female Dogs of Various Ages with X-linked Hereditary Nephritis*

Age	Number of dogs	Number of renal specimens*	LM of kidneys	IF of GBM†	EM of GBM
Birth	5	5B	Normal	Segmental	Normal
Up to 7 months	18‡	41B → 18K	Normal	Segmental	Some appear normal; others show foci of bilaminar and multilaminar splitting
7 months to 5 years	6‖	6B → 6K	Lesions of FSGS appear, increasing in frequency and size with age; no tubulo-interstitial damage	Segmental up to 3–4 years; global thereafter	Some appear homogeneous and thickened; others show multilaminar splitting and thickening
Older than 5 years					
Group I	4	7B	FSGS; no tubulo-interstitial damage	Global	Some appear homogeneous and thickened; others show compressed multilaminar splitting
Group II	4	3K	Large lesions of FSGS and hyalinosis with tubulo-interstitial damage	Global	Same as group I
Group III	5	4K	End-stage renal damage	Global	Thickened and wrinkled

* B = biopsy, K = kidney at postmortem.
† IF was performed with the human and dog anti-NC1 antibodies.
‡ These dogs underwent 41 renal biopsies during life and their kidneys were obtained at the time of sacrifice.
‖ These dogs underwent six renal biopsies during life and their kidneys were obtained at the time of sacrifice.
FSGS = focal segmental glomerulosclerosis; ND = not determined.

Five dogs (group III) had signs of renal failure (lethargy, depression, decreased appetite, weight loss, intractable vomiting) at 5 to 9 years of age (mean 7.25 years) and were killed, at which time their serum creatinine was markedly elevated (775–1250 μmol/l).

Examination of Kidneys by LM

All renal biopsies[41] and postmortem kidneys[18] obtained from the 18 carrier female dogs appeared normal by LM up to 7 months of age (Table 2). In the case of the six carrier female dogs whose kidneys were examined between 7 months and 5 years of age (six biopsies and six postmortem kidneys), small lesions of focal segmental glomerulosclerosis (FSGS) were first detected at 8 months in < 1% of glomeruli (Figure 1a); these became progressively larger and more frequent (< 5% of glomeruli) up to 5 years. No interstitial fibrosis or tubular atrophy was seen in these kidneys. After 5 years, seven kidney biopsies performed on five group I carrier female dogs continued to show FSGS, but there was no tubulointerstitial damage. Kidneys obtained from three of the four group II carrier female dogs at the time of sacrifice

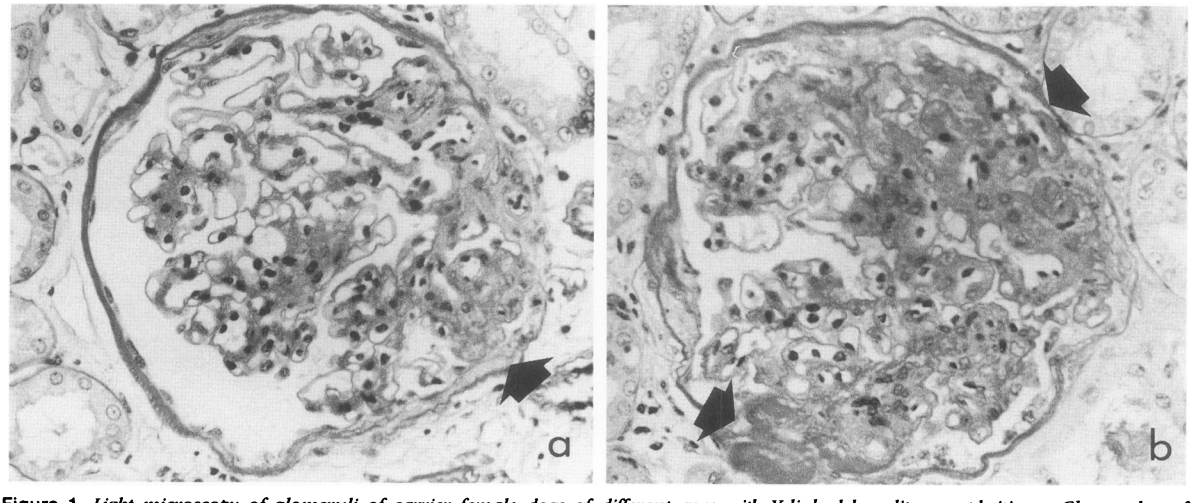

Figure 1. *Light microscopy of glomeruli of carrier female dogs of different ages with X-linked hereditary nephritis.* **a**: *Glomerulus of 8-month-old carrier female dog, showing small segmentally sclerotic lesion (arrow);* **b**: *Glomerulus of 9-year-old group II carrier female dog, showing large segmentally sclerotic lesion with hyalinosis (arrows). Both slides were stained with the periodic acid-Schiff stain, both ×400.*

showed large lesions of FSGS with hyalinosis in > 50% of glomeruli and moderate tubulointerstitial damage (Table 2, Figure 1b). Kidneys obtained from four of the five group III carrier female dogs at the time of sacrifice showed end-stage renal damage with global sclerosis in virtually 100% of glomeruli, extensive tubular atrophy, interstitial fibrosis, and interstitial inflammation.

Examination of GBM and Lesions of FSGS by IF

By IF, there was global staining (i.e., all GBM) of normal female dogs at all ages, using either the human or dog anti-NC1 antibodies (Figure 2a). In the 42 renal biopsies and 20 postmortem kidneys obtained from 20 carrier female dogs < 3 years of age, segmental staining of GBM was observed (i.e., staining of 25–75% of GBM in most glomeruli and < 25% or > 75% of GBM in an occasional glomerulus) (Table 2, Figure 2b). The GBM that did not stain using the human or dog anti-NC1 antibodies showed positive staining with the rabbit anti-NC1 antibody. The proportion of negatively staining GBM tended to decrease as the dogs approached 3 years of age. Global staining of GBM was seen in the three renal biopsies and three postmortem kidneys obtained from three carrier female dogs between 3 to 5 years of age (Figure 2c). The one carrier female dog who had renal biopsies done at 6 months and 3.5 years of age showed conversion of the staining pattern of GBM seen by IF from segmental at the younger age to global at the older age. After 5 years, GBM seen on seven renal biopsies that were completed on the five group I carrier female dogs and on one postmortem kidney from a group II and group III carrier female dog continued to show global staining.

By IF, two 8-month-old carrier female dogs whose kidneys showed early FSGS, and one 9-year-old carrier female dog whose kidneys showed extensive FSGS, showed staining of the sclerotic lesions using the rabbit anti-NC1 antibody (Figure 3a). When the dog anti-NC1 antibody was used, the sclerotic lesions did not stain in the case of the 8-month-old carrier female dogs (Figure 3b), but did stain in the case of the 9-year-old carrier female dog (Figure 3c). No staining of GBM or sclerotic lesions was observed when IF was performed in the absence of anti-NC1 antibody, or when normal serum was substituted for immune serum, other than nonspecific staining of areas of hyalinosis.

Examination of Extrarenal Basement Membranes by IF

By IF, the lens capsule of normal female dogs at all ages showed homogeneous staining, using the dog anti-NC1 antibody (Figure 4a). In the case of the two 8-month-old carrier female dogs, alternating areas of positive and negative staining were seen in the lens capsule, producing a striped pattern (Figure 4b). In sections stained with

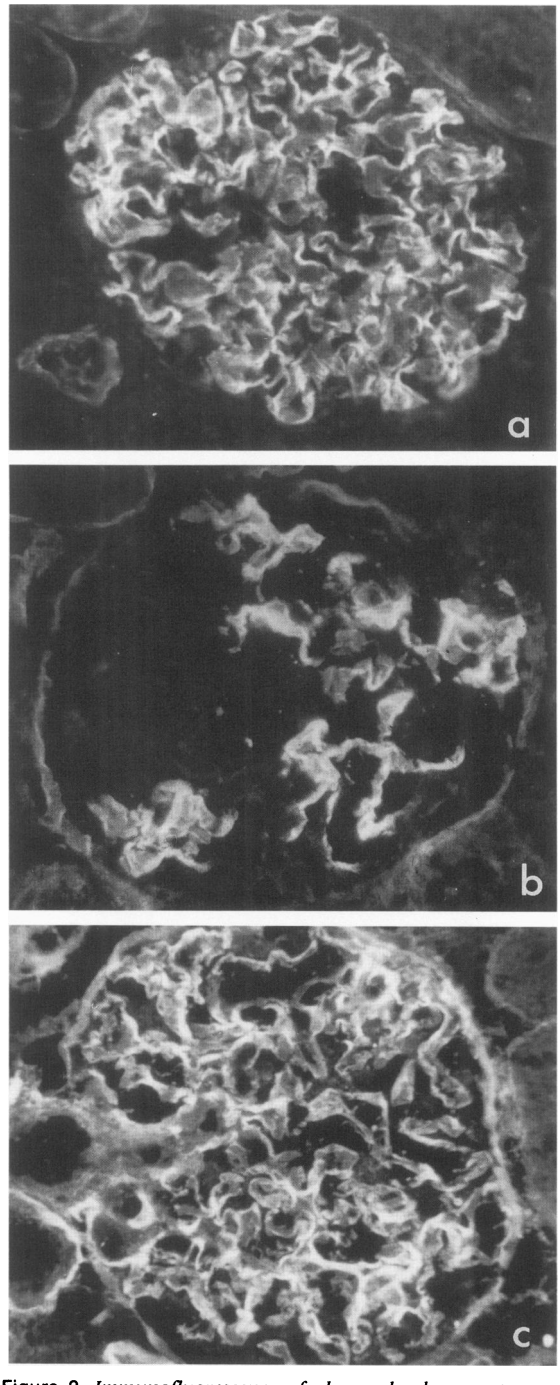

Figure 2. *Immunofluorescence of glomerular basement membranes (GBM) of normal female and carrier female dogs of different ages with X-linked hereditary nephritis. Immunofluorescence was performed using a dog anti-NC1 antibody.* **a**: *2-month-old normal female dog, showing global staining of all GBM, and* **b**: *2-month-old carrier female dog, showing segmental staining of some GBM but not others;* **c**: *5-year-old carrier female dog, showing global staining of GBM, all ×400.*

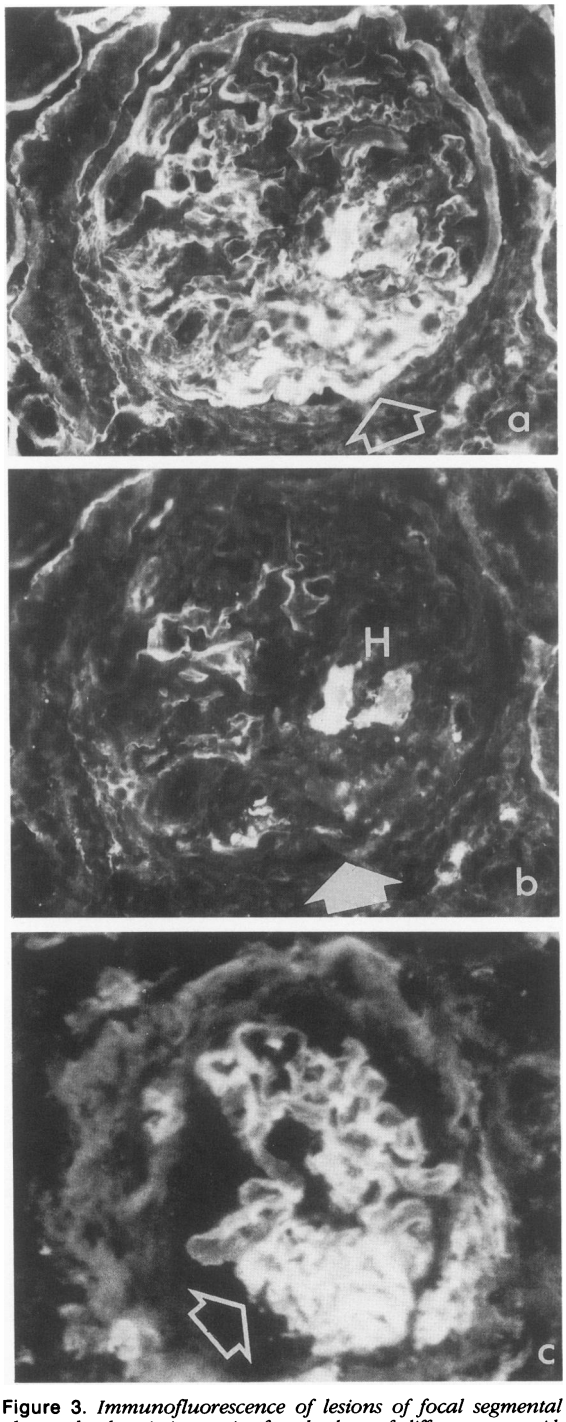

Figure 3. *Immunofluorescence of lesions of focal segmental glomerulosclerosis in carrier female dogs of different ages with X-linked hereditary nephritis. Sections were doublestained for IF using rabbit and dog anti-NC1 antibodies.* **a**: *Glomerulus of 8-month-old carrier female dog showing staining of a segmentally sclerotic lesion (arrow) using the rabbit anti-NC1 antibody and rhodamine-labeled anti-rabbit IgG.* **b**: *Same glomerulus showing no staining of the segmentally sclerotic lesion (arrow) using the dog anti-NC1 antibody and fluorescein-labeled anti-dog IgG. The irregular foci of staining* (H) *are areas of hyalinosis in the region of sclerosis.* **c**: *Glomerulus of a 9-year-old carrier female dog showing staining of the segmentally sclerotic lesion (arrow) using the dog anti-NC1 antibody, all ×375.*

both the dog and rabbit anti-NC1 antibodies, the striped pattern seen with the dog antibody was replaced by a homogeneous pattern using the rabbit antibody (data not shown). The lens capsule of the 9-year-old group II carrier female dog showed homogeneous staining, similar to that seen in normal dogs, with a suggestion of the striped pattern in a few areas (Figure 4c). The lungs of normal female dogs at all ages showed diffuse staining of all alveolar basement membranes (Figure 4d). Lungs of the two 8-month-old carrier female dogs showed regions of positive and negative staining of alveolar basement membranes (Figure 4e), whereas lungs of the 9-year-old group II carrier female dog showed staining of all alveolar basement membranes (Figure 4f). The choroid plexus of normal female dogs at all ages showed continuous staining of the epithelial basement membranes (Figure 4g). Choroid plexus of the two 8-month-old carrier female dogs showed discontinuous staining of the epithelial basement membranes (Figure 4h). However, continuous staining of these basement membranes was seen in the 9-year-old group II carrier female dog (Figure 4i).

Examination of GBM by Electron Microscopy

By EM, GBM seen on renal biopsies of five carrier female dogs examined at birth and up to 30 days of age appeared normal (Table 2). Thereafter, GBM around some capillaries of 36 renal biopsies and 18 postmortem kidneys obtained from 18 carrier female dogs up to 7 months of age continued to appear normal (Figure 5a), whereas other GBM showed foci of bilaminar splitting of the lamina densa, which eventually became multilaminar (Figure 5b) and increased in extent with age (Figure 6). Between 7 months and 5 years of age, GBM seen in six renal biopsies and six postmortem kidneys obtained from six carrier female dogs became progressively thicker (Table 3); some had a homogeneous appearance (Figure 5c), similar to GBM of unaffected female dogs (data not shown), whereas multilaminar splitting persisted in others. After 5 years, some GBM seen on seven renal biopsies performed on four group I carrier female dogs, and on three postmortem kidneys obtained from three of the four group II carrier female dogs appeared thick and homogeneous (Table 3), similar to GBM of normal dogs of the same age. In other GBM, foci of compressed multilaminar splitting were seen, with apposition of the split layers (Figure 5d) such that the thickness was less than that measured in young carrier female dogs (Table 3). The length of the split foci could not be easily measured since it was difficult to differentiate split from nonsplit foci. Hence, measurements were not taken in dogs beyond about 300 days of age (Figure 6). EM of GBM in four

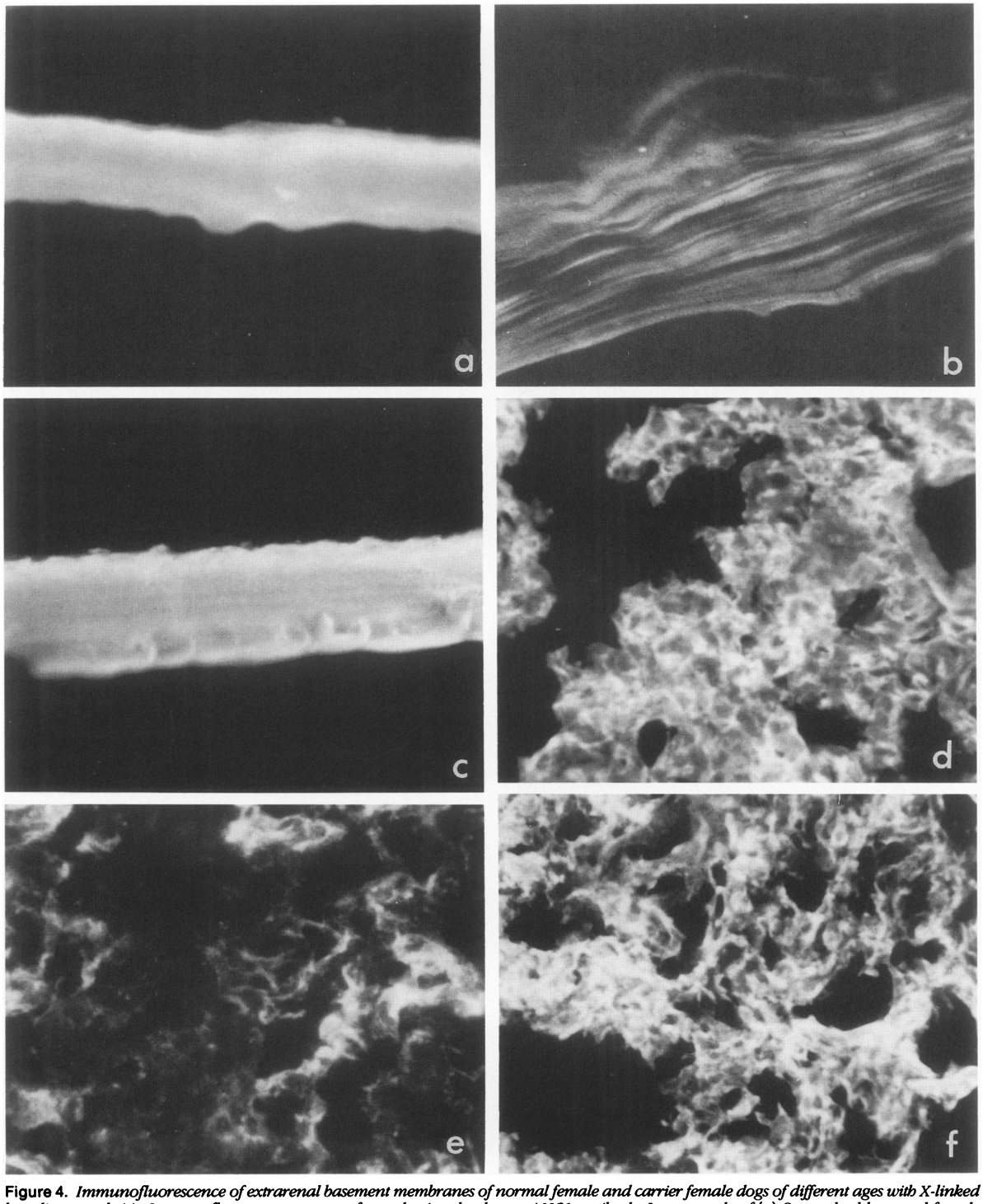

Figure 4. *Immunofluorescence of extrarenal basement membranes of normal female and carrier female dogs of different ages with X-linked hereditary nephritis. Immunofluorescence was performed using the dog anti-NC1 antibody. Lens capsule of* **(a)** *8-month-old normal female dog showing homogeneous staining (×450);* **(b)** *8-month-old carrier female dog showing striped pattern of staining (×450);* **(c)** *9-year-old carrier female dog showing a more homogeneous pattern of staining with some residual striped pattern (×450). Lung of* **(d)** *8-month-old normal female dog showing diffuse staining of all alveolar basement membranes (×300);* **(e)** *8-month-old carrier female dog showing regions of positive and negative staining of alveolar basement membranes (×300);* **(f)** *9-year-old carrier female dog showing staining of all alveolar basement membranes (×300). Choroid plexus of* **(g)** *8-month-old normal female dog showing continuous staining of epithelial basement membrane (×450);* **(h)** *8-month-old carrier female dog showing areas of positive (open arrow) and negative (white arrows) staining of epithelial basement membrane (×450);* **(i)** *9-year-old carrier female dog showing continuous staining of epithelial basement membrane, ×450.*

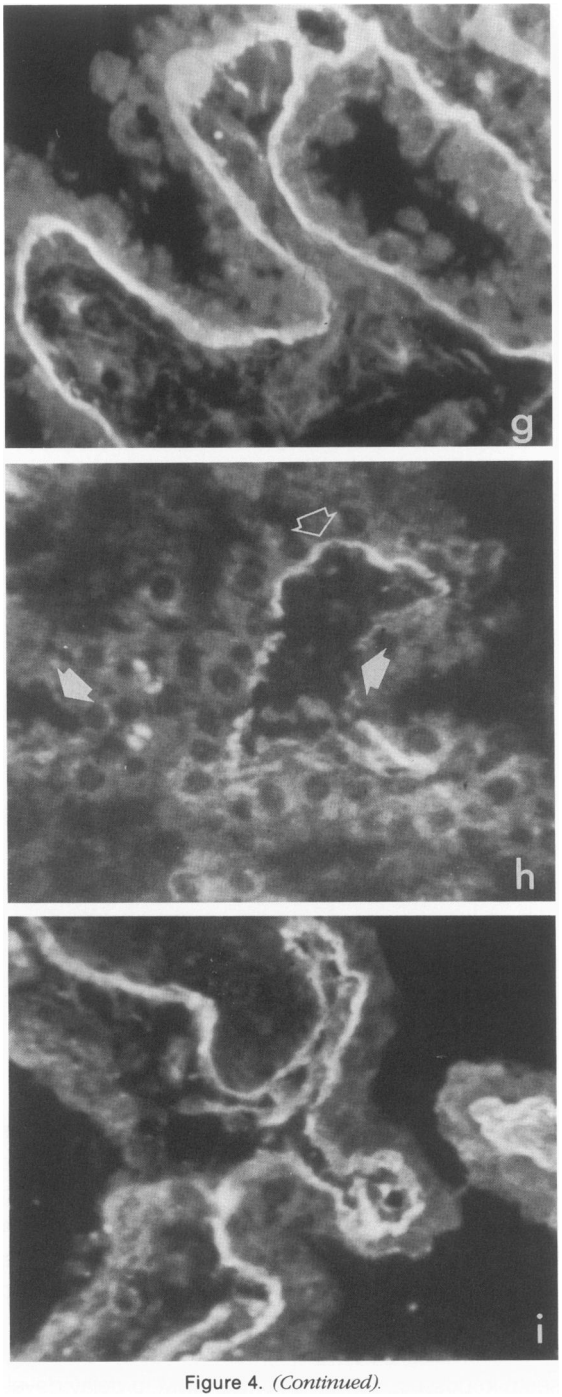

Figure 4. *(Continued).*

postmortem kidneys obtained from four of the five group III carrier female dogs showed collapsed and obliterated capillary loops, with thickened and wrinkled GBM.

Discussion

We have previously shown that X-linked HN in Samoyed dogs is an excellent model for studying human X-linked HN, based on clinical and genetic similarities between affected male dogs and male patients and identical ultrastructural and immunohistologic changes in their GBM.[23–25,31,33] The canine disease most closely resembles subtype II of human HN (i.e., X-linked, onset early in life, and abnormalities of eye and ear),[4] although these abnormalities have so far been detected in dogs only by IF.[41] An apparent discrepancy between the canine and human diseases is that dogs present with proteinuria rather than hematuria.[23] Studies are being performed to determine why hematuria is not a prominent feature of canine X-linked HN.

We initially reported that carrier female dogs developed proteinuria early in their lives (2–3 months of age), but showed no further evidence of renal disease.[23] However, this conclusion was based on results obtained in young carrier female dogs, and only one dog had been followed to 5 years of age. We have additional data on older carrier female dogs that document the development of renal disease, thereby allowing us to draw a closer parallel between them and female patients with HN.

After 5 years, the spectrum of renal disease diverged in carrier female dogs: 31% (group I) maintained normal renal function and their kidneys showed a few small lesions of FSGS but no tubulointerstitial damage; 31% (group II) had mild impairment of renal function with more extensive FSGS and tubulointerstitial damage; and 38% (group III) progressed to terminal renal failure with end-stage renal damage. Thus, the clinical picture in these older carrier female dogs resembles that seen in female patients with X-linked HN, who also show variable renal dysfunction.[30] This contrasts with the picture seen in affected male dogs with X-linked HN, all of whom die of renal failure within the first year of life,[23] and in many male patients with this disease, who have renal failure develop in the first decades of life.[1,2]

The human anti-NC1 antibody has been previously shown to produce global staining of GBM of normal dogs, no staining of affected male dogs with X-linked HN,[26] and a segmental pattern of staining in young carrier female dogs.[33] In the present study, the dog anti-NC1 antibody also produced a segmental pattern of GBM staining by IF in young carrier females. We have attributed this segmental pattern to lyonization of an X-linked gene, which in mutant form results in an abnormal NC1 domain in GBM. This gene could be the recently described α5(IV) gene,[14,19,20] but this remains to be proven in dog HN. Thus, segments of GBM that are positive by IF would reflect a gene product from cells that have inactivated the mutant gene, and segments of GBM that are negative by IF would indicate a gene product from cells that have inactivated the normal gene.

In affected male dogs with X-linked HN, the single

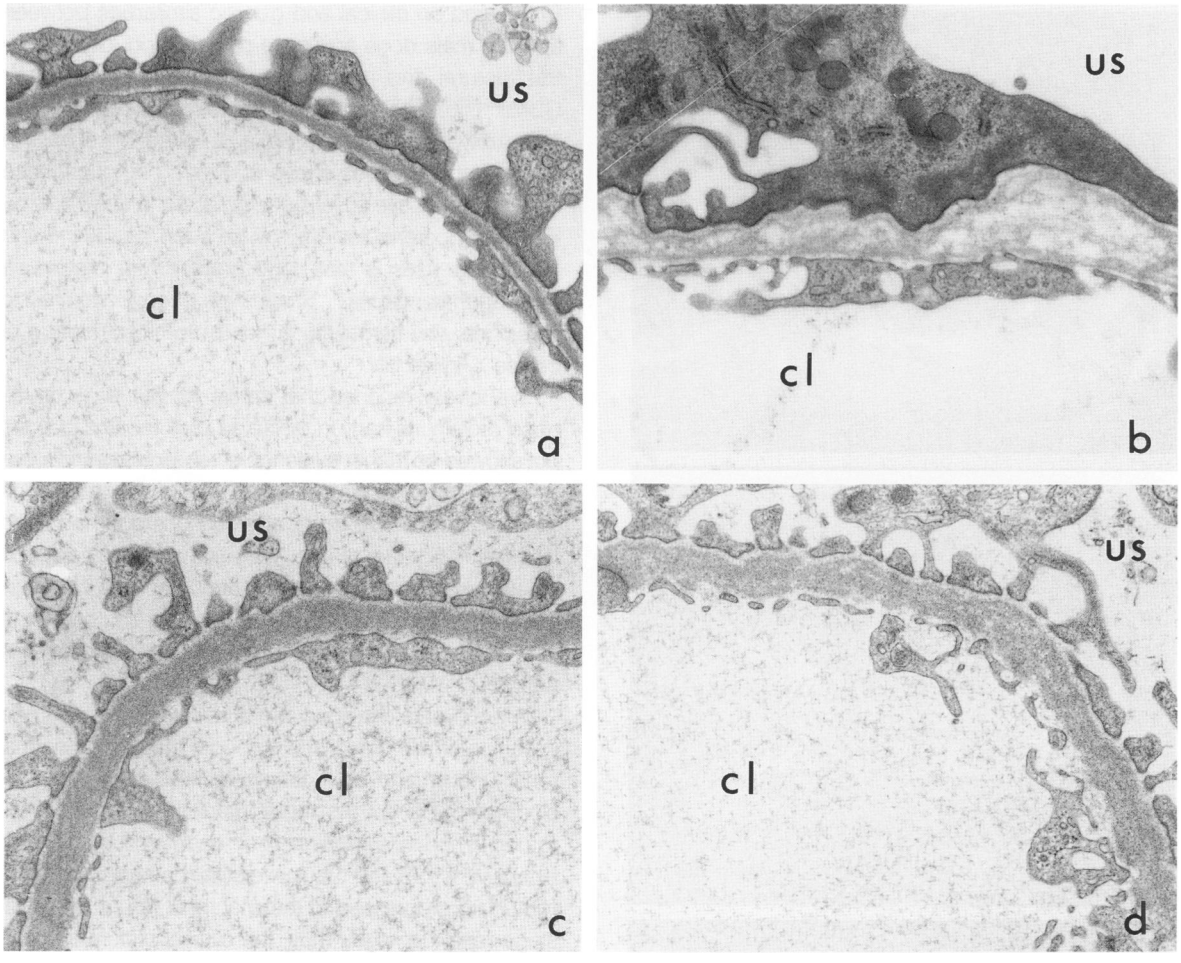

Figure 5. *Electron micrographs of glomerular basement membranes (GBM) of carrier female dogs of different ages with X-linked hereditary nephritis;* **(a)** *nonsplit GBM at 6 months showing normal trilaminar appearance;* **(b)** *GBM at 7 months showing multilaminar splitting;* **(c)** *GBM at 4 years showing a homogeneous thickened appearance;* **(d)** *GBM at 4 years showing compressed multilaminar splitting, all ×17,500; cl = capillary lumen; us = urinary space.*

X-chromosome in all cells possesses the mutant gene; this would result in production of only abnormal GBM, leading to the rapid development (i.e., within 1 year) of renal failure in all of these dogs. In carrier female dogs, however, each cell contains both a normal and mutant X-chromosome, one of which is randomly inactivated early in embryonic development. Those carrier female dogs who inactivate the normal gene predominantly might be expected to have renal disease develop at an earlier age and of greater severity than those dogs who inactivate the mutant gene predominantly. Although this might account for the variation in severity of renal disease seen in older carrier female dogs, the situation is not so straightforward since carrier female dogs never manifest the rapid deterioration of renal function seen in affected male dogs, and only have renal failure develop after 5 years. The most likely explanation is that each glomerulus is derived from several embryonic stem cells, each of which undergoes random inactivation of the X-chromosome. The result would be that each glomerulus contains a mixture of normal and abnormal GBM, a conclusion borne out by our IF studies with the human and dog anti-NC1 antibodies, in which a completely negative or positive glomerulus was almost never observed. Thus, although carrier female dogs are mosaics for normal and abnormal GBM, this mosaicism is present within each glomerulus, with the result that all carrier female dogs seem to possess sufficient normal GBM to delay development of renal failure until at least 4 years later than seen in affected male dogs. A similar situation could exist for female patients with X-linked HN.

A segmental pattern of staining by IF has recently been reported by us and others in GBM of female patients with HN.[34–36] Before these reports, GBM of female patients were stated to show either negative or positive staining,[26–29] the latter presumably global since it is likely that segmental staining would have evoked comment. The absence of segmental staining could have resulted

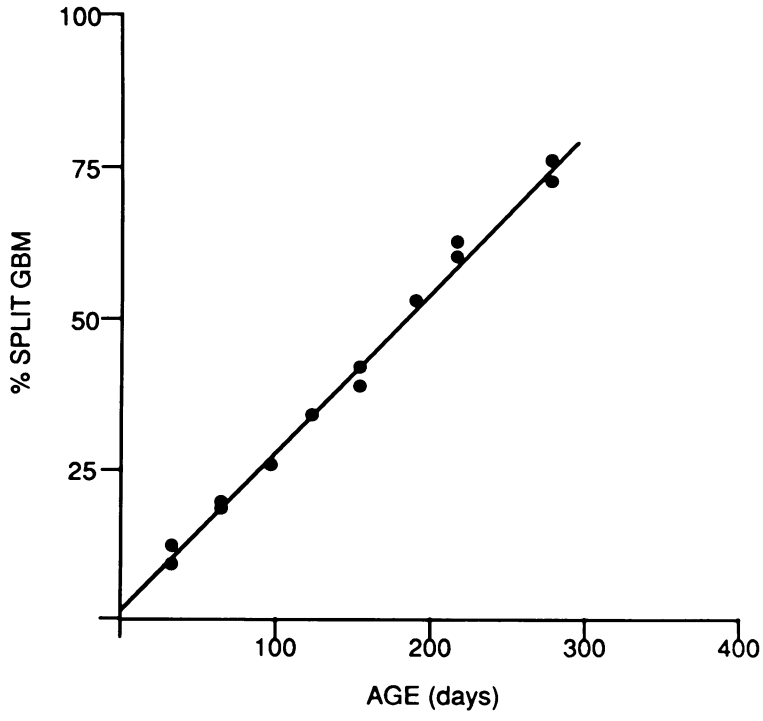

Figure 6. *Length of split glomerular basement membranes (GBM) in young carrier female dogs of different ages with X-linked hereditary nephritis. GBM showing both nonsplit foci and foci of splitting (i.e., bilaminar and/or multilaminar) were evaluated by electron microscopy, using the Interactive Image Analysis System (IBAS) as described in Materials and Methods. Measurements were made on GBM around 12 capillaries and values are expressed as percentage splitting versus age of the dogs. Each point represents the percentage of GBM, which was split in a single dog. The solid line represents the regression line* $Y = 0.62 + 0.26X$, $P < .001$, *correlation coefficient* $= 0.99$.

from the fact that different sera containing anti-NC1 antibody were used in these studies. However, another possibility has arisen as a result of our IF studies in older carrier female dogs. After 3 to 4 years, all carrier female dogs showed a global pattern of staining using either the human or dog anti-NC1 antibodies. Since the younger dogs were all healthy and were sacrificed at random, it cannot be concluded that segmental staining is a marker for those destined to develop renal disease, and global staining a marker for a separate group of dogs destined to live longer. In fact, all dogs who died of renal disease showed a global pattern of staining. We interpret our results, therefore, to reflect actual conversion of the staining pattern of GBM by IF from segmental to global. This conversion occurs around 3 to 4 years of age, and was shown in one dog who underwent a biopsy at 6 months of age and again at 3.5 years. If a similar conversion occurs in female patients, the pattern of IF staining in their GBM may depend on the patient's age at the time of renal biopsy.

A similar change from a mixture of positive and negative staining of basement membranes by IF to only positive staining, using the human and dog anti-NC1 antibodies, was seen in a number of extrarenal sites, specifically lens capsule, lung and choroid plexus. This cannot be explained simply on the basis of examining, by chance, tissues from a healthy older carrier female dog since the one older dog examined was in group II and

Table 3. *Thickness of Glomerular Basement Membranes (GBM) of Carrier Female Dogs of Various Ages with X-linked Hereditary Nephritis*

Age of dogs (yr)	Thickness (nm) of GBM		
	Normal	Abnormal	
		Nonsplit foci	Split foci
0.5	180 ± 6	192 ± 4	640 ± 21
2	231 ± 5	203 ± 4	562 ± 25
4	389 ± 9	395 ± 11	503 ± 16
6	354 ± 9	397 ± 12	393 ± 21
8	365 ± 12	371 ± 16	342 ± 10

Thickness of GBM was determined as outlined in Materials and Methods. Electron microscopy showed normal GBM around some capillaries and abnormal GBM around others, consisting of both nonsplit foci and foci of splitting (i.e., bilaminar and/or multilaminar). Values shown are means ± SEM. The thicknesses of the normal GBM of the carrier female dogs were not significantly different from those of normal female dogs of comparable ages (data not shown).

had renal disease develop at the time of sacrifice. These results underline the systemic nature of the abnormality of the NC1 domain of collagen type IV in basement membranes in canine X-linked HN, even though dogs show no abnormalities by LM or EM[41] or clinically[31] at these sites. Since conversion of staining pattern occurred in these extrarenal sites of carrier female dogs in the absence of disease, the mechanism of conversion was not disease-driven, as might have been suspected from examining only the kidneys of carrier female dogs. Our results also provide insight into the embryologic development of lens, lung, and choroid plexus since the positively and negatively staining areas of basement membrane by IF are presumed to reflect separate precursor cells that have inactivated different X-chromosomes. Similar patchy staining of the basement membrane at the epidermal-dermal junction of skin has been observed in female patients with X-linked HN, using an anti-NC1 antibody obtained from a patient with Alport syndrome after renal transplantation.[45]

There are a number of possible explanations for the conversion of the staining pattern of GBM from segmental to global. First, it may reflect the process of age-related reactivation of an X-linked gene,[42] in this case the normal allele of the gene that is mutant in canine X-linked HN. Second, there may be activation of some other gene for a collagen type IV with a normal NC1 domain, in an attempt to replace the mutant gene. Third, there may be movement (exact mechanism unknown) of collagen type IV with a normal NC1 domain from glomerular segments that contain it into segments that do not; such movement has been suggested to occur with dystrophin in skeletal muscle in canine X-linked muscular dystrophy.[43] Fourth, there may be a selective growth advantage in cells that produce collagen type IV with a normal NC1 domain. A proliferative advantage for fibroblasts in which the mutant X-chromosome was inactivated has been described in carrier female patients with incontinentia pigmenti.[44] Whatever the mechanism, by 3 to 4 years of age, all carrier female dogs show a global staining pattern of GBM.

In addition to conversion of the IF staining pattern of GBM from segmental to global as carrier female dogs grew older, changes were seen by EM in the GBM of these dogs. Multilaminar splitting was initially detected in all of them after 30 days of age, the same time as it was seen in the GBM of affected male dogs.[31] However, in carrier female dogs, the splitting involved only some capillary loops and spared others, presumably corresponding to negative and positive segments, respectively, seen by IF using the human or the dog anti-NC1 antibodies. After 4 years, multilaminar splitting became less obvious as the layers of split GBM became apposed, giving rise to a compressed multilaminar appearance. It is tempting to relate this change by EM to the conversion of glomerular capillary loops from negative to positive staining by IF. This conversion could then reflect the gradual replacement over time of defective collagen type IV by normal collagen type IV in an attempt to change abnormal GBM, seen as negative staining by IF and multilaminar splitting by EM, to normal GBM, seen as positive staining by IF with a homogeneous appearance by EM. Four possible mechanisms for this have been considered earlier. However, the process did not completely correct defective GBM, since the abnormal morphology persisted in the form of compressed multilaminar splitting, and a number of the carrier female dogs eventually suffered renal failure. Although the changes seen by LM and EM in the kidney have been well documented in human HN,[5–8] only one attempt has been made to deal with the severity of GBM splitting in female patients.[32] Compressed multilaminar splitting of GBM has not been reported in human female patients with X-linked HN, possibly owing to the difficulty in distinguishing this change from adjacent normal GBM by EM.

By LM, kidneys of all carrier female dogs showed FSGS after 7 months, and both the incidence and size of the lesions increased with age. The dog model of X-linked HN has provided a basis for speculating on the pathogenesis of the lesion of FSGS that has been described in human HN.[46] FSGS is a nonspecific morphologic change that can be seen in a variety of renal diseases,[47] and reflects damage to glomerular capillary loops from many causes. In the present study, we have shown that lesions of FSGS failed to stain by IF in young carrier female dogs using the human and dog anti-NC1 antibodies, implying that these lesions develop in segments of glomeruli in which the GBM possess an abnormal NC1 domain. We suggest that, in female patients with X-linked HN, FSGS also begins in glomerular capillaries whose GBM possess an abnormal NC1 domain. However, in older carrier female dogs, lesions of FSGS were positive by IF using the human and dog anti-NC1 antibodies, implying that they had either converted to positive staining and/or incorporated normal GBM. Hyperperfusion is known to be associated with the development of FSGS in rats subjected to subtotal renal ablation, and has been believed to be responsible for the production of FSGS in many human renal diseases.[48] We speculate that hyperperfusion in surviving glomerular capillary loops may have been responsible for the progression of FSGS in older carrier female dogs with X-linked HN. However, why some carrier female dogs had renal failure develop and others did not remains unclear. It would be reasonable to expect that carrier female dogs who possess a larger proportion of GBM with a normal NC1 domain would fare better, although to prove this hypothesis would require morphometric studies,

which were not carried out in this study. Nevertheless, conversion of glomerular capillary loops from negative to positive staining by IF did not prevent the progression of FSGS. Either conversion occurred too late in the course of the disease, or the positively staining GBM were still not entirely normal. The persistence of the compressed multilaminar split appearance of GBM by EM in older carrier female dogs tends to support the latter possibility. In contrast to carrier female dogs and female patients with X-linked HN, it may be that other hemodynamic factors (eg, intraglomerular perfusion pressure) are more important in determining which capillary loops develop FSGS in affected male dogs and male patients with X-linked HN, in whom all GBM have an abnormal NC1 domain. Once initiated, the lesions of FSGS could be perpetuated in either male or female patients by hyperperfusion of remaining glomerular capillaries.

In conclusion, the present study has documented several aspects of X-linked HN in carrier female dogs that may be applicable to female patients with this disease. By long-term study of such dogs, we have demonstrated the evolution of the morphologic changes in their kidneys, and the antigenic changes seen in GBM and some extrarenal basement membranes. Mechanisms have been proposed for these changes. Further understanding of the evolution of this X-linked disease awaits detailed biochemical and molecular genetic studies of the NC1 domain in both young and older carrier female dogs. This information will contribute to our understanding of X-linked HN in particular and X-linked disease in general.

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