

Cyclosporine A Slows the Progressive Renal Disease of Alport Syndrome (X-Linked Hereditary Nephritis): Results from a Canine Model

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Abstract. Alport syndrome refers to a hereditary disorder characterized by progressive renal disease and a multilaminar appearance to the glomerular basement membrane (GBM). In a small group of patients with Alport syndrome, cyclosporine A was reported to decrease proteinuria and maintain stable renal function over 7 to 10 yr of follow-up. The present study examined the effect of cyclosporine A on GBM structure and the progression to renal failure in a canine model of X-linked Alport syndrome. Affected male dogs and normal male dogs treated with cyclosporine A underwent serial renal biopsies. Body weight, serum concentrations of creatinine and albumin, and GFR were sequentially determined. Controls consisted of untreated dogs that developed end-stage renal failure by 8 mo of age. Renal biopsies were assessed for glomerulosclerosis and the percent of multilaminar GBM as measured by image

analysis. Significant differences were found between treated and untreated affected dogs for weight, serum creatinine, and GFR. There was a significant delay in the progression of multilaminar change to the GBM, although treated affected dogs at termination had attained approximately 100% split GBM as did untreated affected dogs. A significant difference in the number of sclerotic glomeruli was also noted; treated dogs rarely developed obsolete glomeruli during the period studied. Interstitial fibrosis was *not* significantly affected by cyclosporine A treatment. These findings indicate that cyclosporine A is beneficial in slowing, but not stopping, the clinical and pathologic progression of Alport syndrome. At least part of this beneficial effect comes from a delayed deterioration of GBM structure, which in turn may be related to glomerular hemodynamics altered by cyclosporine A.

Alport syndrome is a hereditary disorder of type IV collagen characterized by progressive nephropathy, ocular abnormalities, and high tone sensorineural deafness (1–4). Patients usually present with hematuria, and most subsequently develop proteinuria. Kidney function is near normal at birth, but deteriorates over time leading to end-stage renal disease by the end of the third decade, especially in male patients (1–4). About 80% of affected families show an X-linked inheritance pattern, and the remainder are autosomal dominant or recessive (5,6).

The pathogenesis of Alport syndrome has been linked to mutations in genes for type IV collagen. Type IV collagen is assembled from a family of distinct α -chains designated $\alpha 1$ to $\alpha 6$, which are encoded by six different genes, *COL4A1* to *COL4A6*, respectively (7). Over 300 mutations have been found in the *COL4A5* gene in the X-linked form of Alport syndrome (4,8). Mutations have been identified in the *COL4A3*

and *COL4A4* genes in patients with the autosomal recessive form (9,10). All these mutations result in an abnormal composition of type IV collagen α -chains in the glomerular basement membrane (GBM); in most patients with Alport syndrome, the GBM contains the $\alpha 1$ and $\alpha 2$ chains rather than the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains of the normal GBM (11–14). As a consequence, there is progressive damage to the GBM, which ultrastructurally begins as bilaminar and progresses to multilaminar splitting and eventually involves all capillary loops. As the glomerular disease advances, there is increasing sclerosis of glomeruli, tubular atrophy, and fibrosis of the interstitium. Indicators of poor prognosis include massive proteinuria, an extensive multilaminar appearance of GBM, interstitial fibrosis, and obsolete glomeruli (3,4,15,16).

No curative therapy yet exists for Alport syndrome, and most treatments are aimed at slowing the progression of the disease. Beneficial effects have been reported for human Alport patients and in animal models using low-protein diets (17) and angiotensin-converting enzyme inhibitors (18,19). More recently, cyclosporine A was reported to be beneficial to patients with Alport syndrome in a small noncontrolled study (20,21). Massive proteinuria decreased or disappeared, whereas creatinine clearance remained stable throughout treatment. Changes in renal pathology were reported to be nonprogressive, but assessment was qualitative.

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The availability of animal models for hereditary nephritis provides an opportunity to perform a controlled study to evaluate the benefits of cyclosporine A in the treatment of this disease. The renal disease occurring in a family of Samoyed dogs is a well-characterized model of the X-linked form of Alport syndrome, that results from a nonsense mutation in the *COL4A5* gene and closely mimics the human form of the disease (22–25). In this model, the progression to end-stage renal disease occurs predictably, starting at 4 to 5 mo of age and resulting in terminal renal failure by 10 mo of age. The purpose of the present study was to investigate the effects of cyclosporine A on the progression to end-stage renal disease in this model and to determine the effects on renal pathology. We hypothesized that improvement in the clinical progression of the disease might correlate with changes at the tissue level, either glomerular or extraglomerular. The former would suggest developing specific therapies for Alport syndrome might be justified, whereas the latter would suggest nonspecific therapies might be equally beneficial (15).

Materials and Methods

Subjects

Dogs used in this study were related through a line of Samoyed dogs affected with X-linked hereditary nephritis and were cared for under identical circumstances in a single institution. Dogs were maintained according to the guidelines of the Canadian Council for Animal Care. Carrier females were randomly bred to mixed breed males to maintain the colony. Twelve affected male dogs and five normal dogs were given cyclosporine A and subjected to clinical testing to determine the progression of their renal disease. Also included in the study were nine affected male dogs and five normal dogs that did not receive cyclosporine A. All dogs were fed free choice of a commercial puppy chow until 6 mo of age and then an adult maintenance diet. Cyclosporine A (Neoral; Novartis Pharma Canada Inc, Dorval, Quebec) was given orally twice a day to affected and normal dogs, beginning at 1 mo of age with a daily dose of 20 mg/kg. This dose was adjusted to maintain trough concentrations (16 h after the last dose) between 100 and 400 ng/ml. By 6 mo of age, the desired trough concentrations were attained at the recommended daily dose rate of approximately 10 mg/kg (26). Cyclosporine A was administered throughout the duration of the study. The concentration of cyclosporine A was determined weekly by RIA (Cyclo-Trac; Incstar, Stillwater, MN) using EDTA-anticoagulated whole blood.

Clinical Biochemical Analysis

Blood for serum creatinine ($\mu\text{mol/L}$), total protein (g/L), and albumin (g/L) concentrations were collected once weekly by venipuncture. Routine urine dipstick and sediment analysis, urine Ponceau S protein (mg/L) and creatinine (mg/L) concentrations were determined routinely throughout the study. The half time plasma clearance of sodium sulfanilate (min) was used as a measure of GFR. Specific gravity was determined by refractometry. Urine protein/creatinine ratios were calculated. All testing was performed as described previously (23) apart from creatinine, which was done enzymatically (Randox Laboratories Canada Ltd., Mississauga, ON) on an automated chemistry analyzer (Hitachi 911; Boehringer Mannheim Corp, Indianapolis, IN). For urine electrophoresis, urine samples were first concentrated approximately $10\times$ by ultrafiltration (15,000-dalton MW cutoff, Minicon Concentrator-B15; Amicon Canada Ltd,

Oakville, Ontario). Concentrated urine samples were subjected to agarose gel electrophoresis with a barbital buffer (Titan Gel Serum Protein System; Helena Laboratories, Beaumont, Texas).

Renal Morphometry

Renal biopsies were taken at various timepoints ranging from 1 to 10 mo of age. All 31 dogs underwent renal biopsies: three times for 8 dogs, twice for 7 dogs, and once for the remaining dogs. Dogs were given general anesthesia and biopsies were obtained by laparotomy using a disposable single-action biopsy 18-gauge needle (E-Z Core; Products Group International Inc., Lyons, CO). Samples were fixed in 4% formaldehyde/1% glutaraldehyde, post-fixed in 2% osmium tetroxide, dehydrated in graded acetone, and embedded in Epon. Thick ($1\text{-}\mu\text{m}$) sections were stained with toluidine blue, and blocks containing glomeruli were selected for ultrastructural examination. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a JEM1230 (JEOL, Tokyo, Japan). Images of capillary loops were captured electronically at a magnification of $\times 12000$ and transferred to a morphometric analysis program (Scion Image, Frederick, MD). A total of 351 images were analyzed in a blinded fashion. The length of GBM with multilaminar splitting was expressed as a percentage of the total length of GBM examined. Tangentially cut portions of the GBM were not included in the measurements. The proportions of normal, segmentally obsolete, and globally obsolete glomeruli were determined from toluidine blue-stained thick sections. The interstitial area was measured adapting the approach of Kashtan *et al.* (15). Instead of a grid-counting system, images of cortex were captured electronically at a magnification of $\times 20$ using an Olympus light microscope and transferred to Scion Image for direct measurement. A total of 52 images were analyzed in a blinded fashion. The interstitial area was obtained by subtracting the area of the tubules, glomeruli, and vessels from the total cortical area, and expressed as a fraction of interstitial area over total cortical area.

Statistical Analyses

Data were expressed as individual values observed in each dog. Relationships between age, dog status and treatment, and body weight, serum concentrations of creatinine and albumin, sodium sulfanilate half-time, percent split GBM, and proportion of obsolete glomeruli were assessed using least squares linear regression. Differences in the slopes of regression lines were assessed by the F test. Where indicated, the Wilcoxon rank sum test was used to assess treatment differences (Statistix 7; Analytical Software, Tallahassee, FL). Differences with $P \leq 0.05$ were considered significant.

Results

Body Weight

Affected male and normal male dogs treated with cyclosporine A were indistinguishable for the duration of the study (Figure 1). The rate of weight gain was significantly less in untreated affected male dogs; they gained no weight after approximately 4 mo of age. Regression lines calculated for untreated and treated affected male dogs up to 4 mo of age showed a significant difference in slope ($P = 0.007$).

Renal Function

Serum proteins were examined as an indirect measurement of GBM integrity. The serum albumin concentrations in normal dogs remained between 25 and 35 g/L throughout the duration of the study and were unaltered by cyclosporine A

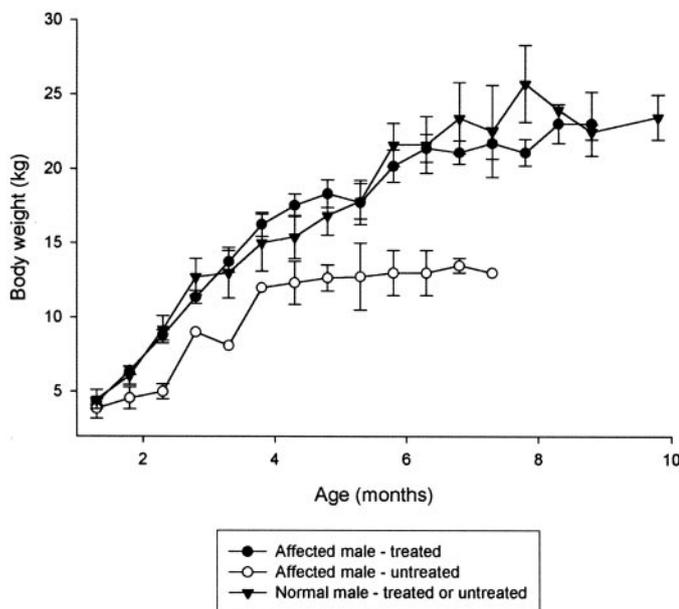


Figure 1. Body weight (kg) versus age (mo) in affected male dogs treated with cyclosporine A (●, $n = 12$), untreated affected male dogs (○, $n = 9$), and normal male dogs with or without cyclosporine A (▼, $n = 5$). Treatment with cyclosporine A commenced at 1 mo of age. Data points represent the mean \pm SE. Untreated male dogs did not gain weight after 4 mo of age. Rates of gains in treated dogs were not significantly different from normal dogs.

treatment (data not shown). In affected dogs, the serum albumin concentration decreased from a mean of 30 g/L to a mean of approximately 20 g/L at 4 mo of age, reflecting loss of protein in the urine as detected by urine dipstick analysis. Cyclosporine A treatment of affected male dogs had no significant sparing effect on the serum albumin concentration ($P = 0.38$). Total serum protein concentrations and albumin to globulin ratios were indistinguishable between treated and untreated affected male dogs (data not shown).

Urinary protein/creatinine ratios were determined because it is difficult to do 24-h urine collections in animals. Cyclosporine A treatment had no significant effect on the urinary protein/creatinine ratio in normal or affected dogs up to 4 mo of age, but ratios were significantly increased as a result of cyclosporine A treatment in both normal and affected dogs older than 4 mo (Table 1). On average for all groups of dogs, ratios doubled

with cyclosporine A treatment. In normal dogs, cyclosporine A treatment decreased the urine specific gravity, but the effect was only significant in older dogs (Table 2). In contrast, treatment of affected dogs was associated with significantly improved concentrating ability in both age groups. Urine agarose gel electrophoresis showed that albumin comprised between 38 to 46% of the protein present in affected dogs, regardless of whether the animals received cyclosporine or not (results not shown).

The serum creatinine concentration/body weight ratio was used to minimize the influence of muscle mass on the serum creatinine concentration, whereas the plasma half-time of sodium sulfanilate was used as a direct measurement of GFR. The serum creatinine concentration/body weight ratio results are presented in Figure 2. Cyclosporine A treatment had no effect on the ratio of serum creatinine concentration to body weight in normal dogs, thus the data from treated and untreated normal dogs were pooled. The ratios in the treated affected dogs were indistinguishable from those in normal dogs for the duration of the study. In untreated affected male dogs, the ratios increased progressively from 3 mo of age until euthanasia. The difference between the slopes of the regression lines generated for treated affected and untreated affected groups was significant ($P < 0.0001$).

The results for plasma $\tau^{1/2}$ of sodium sulfanilate are shown in Figure 3. Cyclosporine A treatment had no effect on the sodium sulfanilate half-times in normal dogs; thus the data from treated and untreated normal dogs were pooled. Between 3 and 4 mo of age, the half-time clearance of sodium sulfanilate began to increase in affected dogs, both treated and untreated. However, the increases were significantly lower in treated affected dogs ($P = 0.02$).

Glomerular Histology

Splitting of GBM in untreated affected dogs commenced at approximately 1 mo of age and rapidly progressed to the point where there was near 100% splitting by 3 mo of age (Figure 4). This same rate of progression has been noted in earlier studies (22,25,27). For treated affected dogs, the onset of splitting was delayed by about 1 to 1.5 mo, and the GBM did not develop 100% splitting until around 8 mo of age. The difference between the onset of splitting for the treated and untreated affected groups was found to be statistically significant ($P = 0.004$). Normal dogs showed essentially no splitting; cyclo-

Table 1. Effect of cyclosporine A treatment on urinary protein/creatinine ratio^a

Status	Treatment	No. of Dogs	Mean Ratio (min to max, n)	
			Age, 1 to ≤ 4	Age, >4 to 8 mo
Normal	No	5	0.56 (0.17 to 1.69, 12)	0.15 (0.08 to 0.2, 12)
	Yes	5	1.0 (0.7 to 1.5, 3)	0.64 (0.23 to 1.20, 3) ^b
Affected	No	3	1.7 (0.59 to 3.7, 5)	11.2 (1.2 to 21.1, 8)
	Yes	10	3.81 (0.3 to 28.9, 21)	23.5 (8.8 to 37.9, 8) ^b

^a n , the number of determinations. The effect of cyclosporine A treatment was assessed for each age and status grouping.

^b Significant treatment effect.

Table 2. Effect of cyclosporine A treatment on urinary concentrating ability^a

Status	Treatment	No. of Dogs	Mean Urine-Specific Gravity (min to max, <i>n</i>)	
			Age, 1 to ≤4	Age, >4 to 8 mo
Normal	No	5	1.025 (1.008 to 1.056, 15)	1.039 (1.015 to 1.057, 18)
	Yes	5	1.020 (1.017 to 1.022, 2)	1.025 (1.021 to 1.029, 3) ^b
Affected	No	3	1.016 (1.008 to 1.024, 9)	1.012 (1.006 to 1.022, 8)
	Yes	10	1.026 (1.018 to 1.034, 5) ^b	1.020 (1.014 to 1.033, 7) ^b

^a *n*, number of determinations. The effect of cyclosporine A treatment was assessed for each age and status grouping.

^b Significant treatment effect.

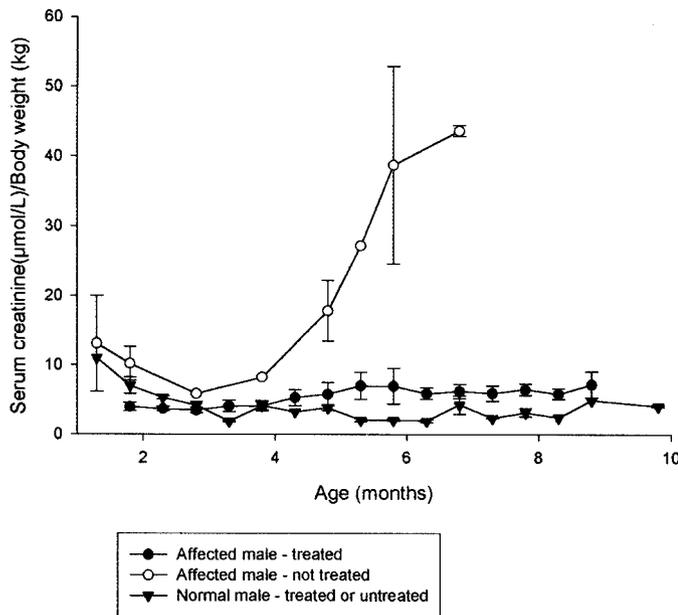


Figure 2. Serum creatinine ($\mu\text{mol/L}$)/body weight (kg) ratio versus age (mo) in affected male dogs treated with cyclosporine A (\bullet , $n = 12$), untreated affected male dogs (\circ , $n = 9$), and normal male dogs with or without cyclosporine A (\blacktriangledown , $n = 10$). The latter two treatment groups were pooled because there were no significant differences due to cyclosporine A treatment in normal male dogs. Treatment with cyclosporine A commenced at 1 mo of age. Data points represent the mean \pm SE. The rise in serum creatinine in affected dogs was significantly reduced by cyclosporine A treatment ($P < 0.0001$). The serum creatinine concentration/body weight ratios in treated affected male dogs were not significantly different from normal male dogs.

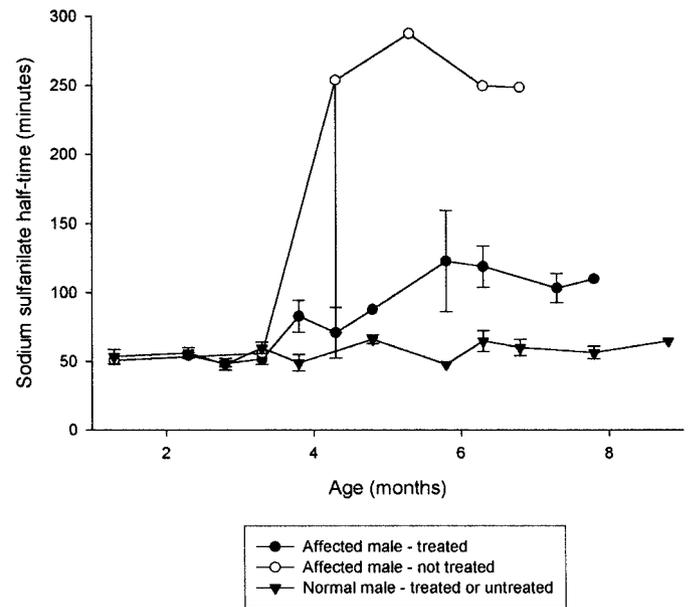


Figure 3. Sodium sulfanilate half-time (min) versus age (mo) in affected male dogs treated with cyclosporine A (\bullet , $n = 12$), untreated affected male dogs (\circ , $n = 9$), and normal male dogs with or without cyclosporine A (\blacktriangledown , $n = 10$). The latter two treatment groups were pooled because there were no significant differences due to cyclosporine A treatment in normal male dogs. Treatment with cyclosporine A commenced at 1 mo of age. Data points represent the mean \pm SE. Affected dogs showed significant increases in clearance times compared with normal dogs. Treated affected dogs showed a significant improvement in clearance time compared with untreated affected dogs ($P = 0.02$).

spore A treatment of normal dogs did not significantly alter the GBM ultrastructure.

Glomerular damage was also assessed by the extent of sclerosis. Normal dogs had no segmental or global glomerulosclerosis for the duration of the study. Untreated affected dogs showed about 20% of glomeruli with segmental sclerosis and about 10% global sclerosis by 3 mo of age (Figure 5). At this time, treated affected dogs had no sclerosis. Untreated affected dogs continued to develop glomerulosclerosis with over 60% global sclerosis by 9 mo of age, whereas treated dogs still only rarely developed global sclerosis, although there was an increase in the number of glomeruli with segmental sclerosis

such that approximately 40% of glomeruli were involved. Segmental sclerosis was significantly decreased in younger dogs treated with cyclosporine A ($P = 0.0011$), and global sclerosis was significantly decreased in older dogs treated with cyclosporine A ($P < 0.0001$).

Interstitial Area

As affected dogs grew older, there was a subjective increase in the degree of interstitial fibrosis apparent by microscopic examination. A similar change was not noted in normal male dogs over time. As might therefore be expected, by morphometry there was a significant difference in interstitial cortical

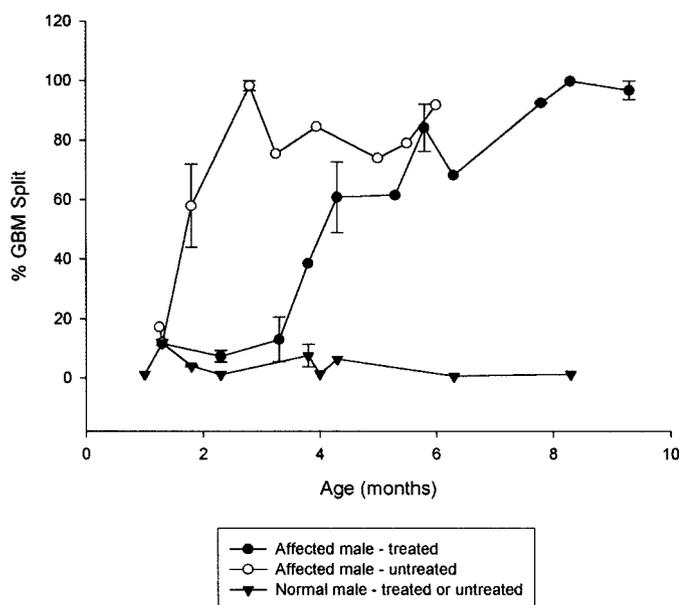


Figure 4. Extent of abnormal glomerular basement membrane (GBM, % split) versus age (mo) in affected male dogs treated with cyclosporine A (●, $n = 12$), untreated affected male dogs (○, $n = 9$), and normal male dogs with or without cyclosporine A (▼, $n = 10$). Treatment with cyclosporine A commenced at 1 mo of age. Data points represent the mean \pm SE. The onset of development of GBM splitting was significantly delayed by cyclosporine A treatment ($P = 0.004$).

area between normal male dogs and affected male dogs ($P = 0.005$; Figure 6). In contrast, there was no significant difference between normal dogs treated with cyclosporine A and untreated normal males ($P = 0.21$). Similarly, there was no significant difference between treated and untreated affected male dogs ($P = 0.99$).

Discussion

Cyclosporine A has been used in transplantation and immune-mediated disease for many years; however, its therapeutic effects in the treatment of Alport syndrome has only recently been examined (20,21). In a small and uncontrolled study, cyclosporine A decreased or eliminated proteinuria and halted the progression of renal disease. Alport syndrome is a heterogeneous disorder in which the progression to renal failure varies greatly depending on the individual family and the specific mutation involved (1–4). The course of renal disease in Alport syndrome can span decades; therefore, an animal model offers many advantages to study the potential benefits of specific therapies; only a single mutation is involved, the progression to renal failure is predictable, and it occurs over a much shorter time span. Our animal model has been well characterized with respect to clinical and pathologic progression (22–25).

In the present study, affected and normal dogs treated with cyclosporine A were compared with untreated dogs. Cyclosporine A treatment of affected dogs had significantly beneficial effects on weight gain, renal function, and renal morphom-

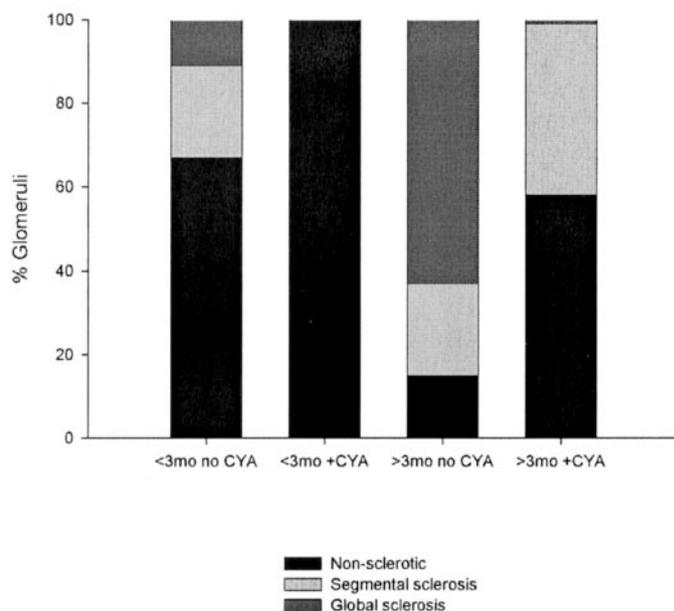


Figure 5. Glomerulosclerosis in male dogs affected with X-linked Alport syndrome for untreated dogs ($n = 116$ glomeruli from 19 biopsies for dogs <3 mo of age; $n = 165$ glomeruli from 15 biopsies for dogs >3 mo of age) and dogs treated with cyclosporine A ($n = 39$ glomeruli from 8 biopsies for dogs <3 mo of age; $n = 121$ glomeruli from 23 biopsies for dogs >3 mo of age). Glomeruli with segmental or global sclerosis were scored separately. Bar height corresponds to mean % of glomeruli. Over time, segmental sclerosis became global in untreated affected dogs. Treated affected dogs only rarely developed global sclerosis during the duration of the study. Cyclosporine A treatment was associated with significant decreases in segmental sclerosis ($P = 0.011$) and global sclerosis ($P < 0.0001$) in dogs <3 mo of age and dogs >3 mo of age, respectively.

etry. Weight gain, urine-specific gravity, serum creatinine concentration/body weight ratio, and GFR were all significantly improved. In fact, weight gain and serum creatinine concentration/body weight ratio were indistinguishable from normal dogs. Although all treated and untreated affected dogs eventually attained 100% splitting of GBM, the onset and rate of progression of multilaminar splitting were significantly delayed in treated dogs. Additionally, cyclosporine A treatment was significantly associated with decreased glomerulosclerosis. In no instances, however, were the dogs cured of their disease; the relentless progression to renal failure was only delayed, not halted.

One of the most striking results in human Alport patients receiving cyclosporine A was a marked reduction or elimination of proteinuria (20,21). Protein balance in the present study was assessed by determining serum protein concentrations and the urinary protein/creatinine ratio. The latter is useful in dogs because the ratio is highly correlated with 24-h protein loss in the urine, and timed urine collections are logistically difficult in animals. Serum albumin levels were reduced in affected dogs compared with normal dogs, implying these animals had persistent proteinuria. The levels were comparable to normal dogs until about 2 mo, which corresponds with the onset of

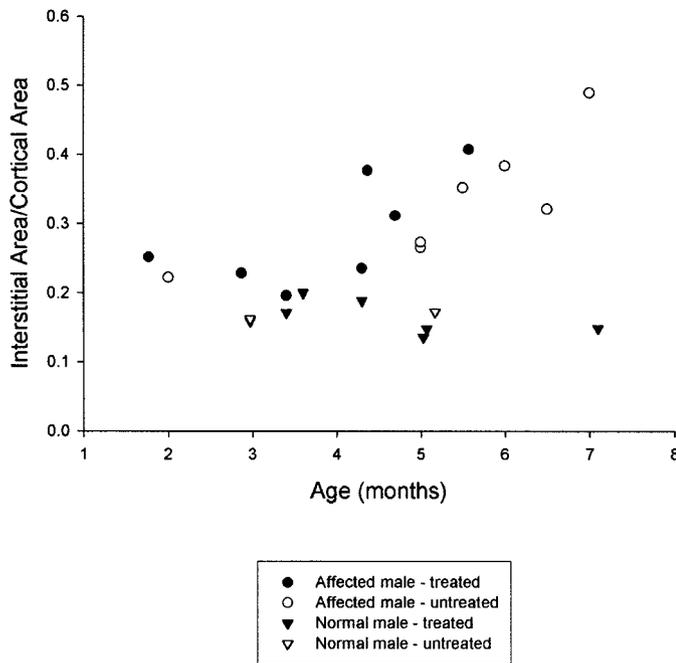


Figure 6. Interstitial area in affected male dogs treated with cyclosporine A (●, $n = 14$), untreated affected male dogs (○, $n = 14$), normal male dogs treated with cyclosporine A (▼, $n = 12$), and untreated normal male dogs (▽, $n = 6$). Values are expressed as the fraction of area occupied by the interstitium over the entire area of cortex measured. Data points represent the mean of duplicate determinations. The difference in interstitial area was significantly greater in affected male dogs compared with normal dogs. ($P = 0.005$). Over time, there was an increase in the interstitial area in affected dogs, but this was not significantly altered by cyclosporine A treatment ($P = 0.99$). Normal dogs did not develop interstitial area increases, and this was not significantly changed by cyclosporine A treatment ($P = 0.21$).

proteinuria in affected animals. Furthermore, treatment of affected dogs was associated with *increased* urinary protein/creatinine ratios. The increased ratios could theoretically result in part from decreased urinary creatinine, except that muscle mass was increased in treated dogs and GFR improved, hence urinary creatinine would be unlikely to decrease. The increased ratio would then indicate increased or at least persistent proteinuria, with cyclosporine A. Urine protein loss was still elevated after urine protein concentrations were adjusted for a standardized urine creatinine concentration (data not shown), supporting our conclusion that a reduced creatinine did not account for the higher ratios. Additional studies, likely utilizing timed urine collections, would be needed to resolve the magnitude of the urine protein loss. We can nevertheless conclude that, unlike the protein-sparing effect of cyclosporine A treatment demonstrated in human patients with Alport syndrome, for this canine model of Alport, cyclosporine A treatment does not have any protein-sparing effect. This lack of protein sparing is not related to preexisting GBM damage; dogs were started on treatment at 1 mo of age, when morphologic evidence for GBM damage is just starting. Urine electrophoresis

confirmed that the amount of albumin spilled in the urine is comparable in treated and untreated affected dogs, indicating that the selective permeability of the GBM is not restored by cyclosporine A in our animal model and that the benefits of treatment come from other mechanisms.

One advantage of an animal model over human-based studies is the ability to follow sequential histologic changes. We hypothesized that if cyclosporine A reduces or eliminates proteinuria in humans and slows the decline of renal function in affected dogs and humans, this effect might be at the glomerular level and may manifest as improved glomerular histology. We had previously found that multilaminar splitting of the GBM begins around 1 mo of age and progresses steadily until virtually 100% is affected by about 4 mo of age (22,27). In the present study, affected dogs receiving cyclosporine A showed a significant 1-mo delay in the onset of multilaminar splitting but then developed this change at about the same rate as untreated affected dogs. This suggests that at least part of the beneficial effect of cyclosporine A treatment was associated with a delay in glomerular deterioration. We did not find, however, that cyclosporine A halted progression of renal pathologic changes, as was found in the human study (21). In that study, no morphometric assessment of biopsies was performed, so progression of changes at the ultrastructural level may have gone undetected. Alternatively, the rate of ultrastructural change in human Alport syndrome is likely considerably slower than that in our canine model.

The measurement of the total number of obsolete glomeruli was based on work by Kashtan *et al.* (15) in which this parameter was found to correlate with a decline in GFR and renal outcome. In both human and canine Alport syndrome, as glomeruli become progressively damaged, they develop segmental and global sclerosis. In untreated affected dogs, by 3 mo of age, segmental sclerosis was well-established and there was global sclerosis in a minority of glomeruli, but this increased such that almost all glomeruli are globally sclerotic by the time the dog reaches end-stage renal disease. Treatment with cyclosporine A had the dramatic effect of reducing the extent of global sclerosis to almost zero over the same time period. Sclerosis still progressed in treated animals, but it remained predominately segmental for the duration of the study. The functional and morphometric data support that cyclosporine A treatment is associated with a delay in glomerular deterioration. In patients receiving cyclosporine A, detailed counts of obsolete glomeruli were not performed, but two of eight patients were reported to show segmental sclerosis on biopsy, which was nonprogressive over 7 to 10 yr. Of note, all the patients in the cyclosporine A study were under 25 yr of age (21). Kashtan *et al.* (15) found that global sclerosis was present in about 50% of patients by age 25, suggesting the possibility that the patients in the cyclosporine A study may have been affected with a milder form of Alport syndrome.

The later stages of renal damage common to most glomerular diseases include tubular atrophy and interstitial fibrosis. In Alport syndrome, there is a strong correlation between increased cortical interstitial volume and deterioration in renal function (15). An increase was rarely seen in the first decade of

life, but approximately 40% of patients with Alport syndrome had increased cortical interstitial volumes after 10 yr of age (15). In our canine model, increasing interstitial fibrosis was noted over time in affected male dogs with the result that there was a significant difference in interstitial area compared with normal male dogs. Although cyclosporine A itself can also result in interstitial fibrosis, we noticed no effect of cyclosporine A on the interstitial area in either normal dogs or affected male dogs. This may be a reflection of the drug levels maintained in this study; these values have been shown to be safe for use in dogs (26). Similarly, the patients with Alport syndrome who received cyclosporine A were reported to show no evidence of toxicity on biopsy (21), although no morphometry was done. From our results, it can also be stated that cyclosporine A did not reduce the amount of interstitial fibrosis. We noted increased concentrating ability in affected dogs treated with cyclosporine A. Assuming this reflects tubulointerstitial function, we cannot attribute this improvement to better preservation of the tubulointerstitial component of the kidney. Perhaps this improvement reflects increased blood flow to tubules because there is better preservation of glomeruli with cyclosporine A treatment.

Although most patients with Alport syndrome are treated with dialysis and/or renal transplantation, other therapies have been tried before cyclosporine A. An earlier study in this family of dogs showed that a modified diet low in protein, lipid, calcium, and phosphorus delayed the onset of renal failure, increased life span by about 50%, and delayed the progression of the multilaminar appearance of the GBM (17). Drug therapy in canine and human Alport syndrome has included the use of angiotensin-converting enzyme inhibitors (ACE-I) (18,19,28). Patients on this therapy showed a decrease in the urinary protein/creatinine ratio, stabilization of the decline of creatinine clearance (18), and decreased proteinuria in about one third of subjects, although serum creatinine continued to increase (28). We previously studied the therapeutic benefit of an ACE-I in our family of dogs with Alport syndrome (19) and found this drug increased the lifespan of the affected dogs by about 35% and reduced the rise of serum creatinine concentration, the degree of proteinuria, and the urinary protein/creatinine ratios. There was a significant delay in the extent of multilaminar splitting of the GBM. Overall, ACE-I was as effective as cyclosporine A in improving the renal outcome in this family of Alport dogs, and both drugs were associated with a delay in glomerular deterioration. It is unknown whether combined therapy using more than one drug or adding dietary modification would be synergistic in the treatment of Alport syndrome.

Multimodal therapy is more likely to succeed if the individual therapies work through different mechanisms. The mechanism of action of cyclosporine A in canine or human Alport syndrome is not well understood. On the basis of animal studies and treatment of other glomerular diseases with proteinuria in humans, suggested mechanisms of action have included alterations in (1) renal hemodynamics, (2) GBM permselectivity, and (3) lymphokine secretion. Changes in renal hemodynamics could account for many of the changes we

observed in the present study. In rats, cyclosporine A has been shown to reduce renal blood flow and increase renal vascular resistance mainly at the afferent glomerular level, leading to reduced filtration pressure and a lowered GFR (29–32). Reduced filtration pressure would decrease the amount of protein passing through the GBM (*i.e.*, decreased proteinuria) and reduce mechanical stress on the GBM. The latter could account for the delay in multilaminar splitting seen in treated affected dogs. However, we did not find a decrease in proteinuria, and GFR was actually improved in affected dogs on cyclosporine A. This in turn may be related to the improved glomerular morphology.

In the normal GBM, there are two networks of collagen type IV, one containing the $\alpha 1$ and $\alpha 2$ chains and the other containing the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains (33). The $\alpha 3\alpha 4\alpha 5$ network is more highly cross-linked and is believed to confer added structural integrity to the GBM. In canine X-linked Alport syndrome (25) and most cases of human Alport syndrome (11–13), the GBM contains only the $\alpha 1$ and $\alpha 2$ chains and lacks the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains. The Alport GBM is likely structurally weaker than the normal GBM and deteriorates progressively under the continued exposure of normal glomerular filtration pressures. This deterioration is reflected at the ultrastructural level as a multilaminar appearance that is not present at birth (25) but gradually increases in extent over time (22). It follows that a reduction in glomerular filtration pressure might delay this deterioration, as we observed. The mechanism by which cyclosporine A lowers glomerular filtration pressure is also not understood. Some have suggested that this effect is mediated through angiotensin II (30), but others have refuted this (29). Our previous study using ACE-I would support the latter (19). We found that administration of ACE-I duplicated many of the effects of cyclosporine A in affected dogs. ACE-I would block the effects of angiotensin II; it therefore follows that the effects of cyclosporine A are not likely mediated through the action of angiotensin II.

Other possible actions of cyclosporine A should be considered. Glomerular diseases treated with cyclosporine A also showed a reduction in proteinuria that was attributed to a restoration of the permselectivity of the GBM (34). Whether improved GBM permselectivity is an important effect of cyclosporine A in Alport syndrome, human or experimental, is unknown. Cyclosporine A also affects the expression of other inflammatory mediators and nonstructural proteins that can affect the glomerulus. Transforming growth factor- β (TGF- β) has been implicated as playing a role in the progression of glomerular disease in murine Alport syndrome (35), and blockade of TGF- β was associated with improved glomerular function and GBM morphology (36). The beneficial effects of cyclosporine A are *not* likely mediated through blockade of TGF- β because cyclosporine A increases TGF- β expression (37,38), and the toxicity of cyclosporine A may be the result of increased TGF- β expression (39). This would suggest, however, that combining cyclosporine A with drugs that block of TGF- β might have synergistic benefits in Alport syndrome.

It has also been shown that the GBM in Alport syndrome is more susceptible to proteolysis than the normal GBM (40).

This raises the possibility that, *in vivo*, proteolysis is contributing to the progressive deterioration of the GBM, either from activation of MMP-2 and MMP-9 or decreased activation of TIMP. Cyclosporine A has been shown to increase the expression of TIMP (39,41) and decrease the expression of MMP-2 (42) and MMP-9 (39). Hence, some of the beneficial effects of cyclosporine A observed in canine and human Alport syndrome may be the result of suppression of proteolysis of the GBM *in vivo*. A molecular genetic approach may provide a more detailed and informative dissection of the mechanisms of action of cyclosporine A in Alport syndrome. Newer technologies utilizing DNA microarrays would allow for screening of large numbers of genes with respect to identifying those with differential expression upon exposure to cyclosporine A.

In conclusion, we found that cyclosporine A had beneficial effects in improving renal function and delaying the onset of renal failure in canine X-linked Alport syndrome. At least part of this effect was mediated through improved glomerular morphology. Postulated mechanisms of action include a decrease in mechanical damage of the GBM from reduced glomerular filtration pressures and reduced matrix degradation from altered expression of metalloproteinases and their inhibitors. In these ways, cyclosporine A could effect an improvement in glomerular function and morphology. It must be borne in mind, however, that proteinuria was unchanged by cyclosporine A, and this may ultimately contribute to the renal failure that ensues despite drug treatment. Proteinuria can mediate damage in both glomerular and tubulointerstitial components; therefore, a more detailed analysis of the latter would also be informative.

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