Genetic and molecular markers of proteinuria and glomerulosclerosis

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Resistance to proteinuria development in anti-Thy-1 glomerulonephritis is governed by both renal and bone marrow-derived factors

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Abstract

Proteinuria is a common feature of many renal diseases, and its development is influenced by both genetic and environmental factors. Intrinsic renal, systemic, and bone marrow-derived factors are thought to influence either susceptibility or resistance to the development of proteinuria. We investigated whether renal or bone marrow-derived factors determine resistance to development of proteinuria in anti-Thy-1 glomerulonephritis by performing kidney transplants and bone marrow transfers between a proteinuria-susceptible rat strain (Lewis/Maastricht) and proteinuria-resistant rat strain (Lewis/Møllegard). The transfer of kidney or bone marrow from Lewis/Maastricht to Lewis/Møllegard rats did not convey susceptibility. Also, the transplantation of a single Lewis/Møllegard kidney to a bilaterally nephrectomized Lewis/Maastricht recipient rat did not lead to proteinuria after the induction of anti-Thy-1 glomerulonephritis. The transfer of Lewis/Møllegard bone marrow into Lewis/Maastricht rats resulted in decreased proteinuria compared to syngeneic bone marrow reconstitutions, accompanied by a decreased number of glomerular microaneurysms. From this study, we conclude that both renal and bone marrow-derived factors contribute to resistance development of proteinuria in anti-Thy-1 glomerulonephritis. Moreover, our data suggest that susceptibility to the development of proteinuria in Lewis/Maastricht rats is governed by genes expressed in the kidney, by bone marrow-derived cells, and by systemic factors. Identifying the factors involved in resistance of proteinuria may lead to better treatments for proteinuria in renal disease and result in limiting the progression to end-stage renal disease.

Introduction

Proteinuria is an independent risk factor for progression to end-stage renal disease and the development of cardiovascular disease.[1-3] Proteinuria is a common indicator for both immune- and non-immune-mediated glomerular damage. In most cases, proteinuria is the result of a loss of permselectivity in the glomerular filtration barrier. Immune-mediated glomerular damage, such as that seen in IgA nephropathy and lupus nephritis, is initiated by antibody or immune complex formation or deposition.[4;5] In glomerulonephritis, immune complex accumulation leads to complement activation, the release of chemoattractants, and an influx of inflammatory cells. This initial phase determines glomerular damage and the subsequent development of proteinuria. The level of proteinuria differs between individual patients, and its development is likely influenced by intrinsic renal, bone marrow-derived, and systemic factors, such as complement components.[6-9] However, it is unknown whether intrinsic factors or bone marrow-derived factors are the key determinants of development of proteinuria in immune-mediated glomerulonephritis.

Genetic factors contribute to the development and severity of renal disease and proteinuria in both humans and experimental models.[10-12] Genes involved in susceptibility to and protection against development of proteinuria have been described in animal models.[13;14] These genetic factors could be expressed by both the kidney and bone marrow-derived cells.[15,16] The influence of renal and extra-renal factors on development of proteinuria can be investigated in animal models. A model with different susceptibility to proteinuria after mesangial damage is the anti-Thy-1 glomerulonephritis (antiThy1GN) Lewis substrains. AntiThy1GN is induced by the injection of antibodies against Thy1.1, a glycosylphosphatidylinositol-anchored protein present on mesangial cells.[17] The injection results in antibody binding to mesangial cells, activation of the classical complement pathway, platelet aggregation, an influx of polymorph nuclear neutrophils and monocytes, mesangial lysis, and apoptosis. These events are followed by mesangial cell proliferation and expansion of the mesangial matrix.[18] Strain-related differences in development of proteinuria have been identified in this model; some develop high levels of proteinuria, whereas others do not develop proteinuria at all.[19] Lewis/Maastricht (Lew/Maa) rats develop high levels of proteinuria, reaching a maximum at day 7, whereas Lewis/Møllegard (Lew/Moll) rats do not develop proteinuria. The initial phase of antibody binding and complement activation in these substrains appears to be similar.[20] On the other hand, microaneurysms are present in glomeruli at day 7 in Lew/Maa, whereas glomeruli in Lew/Moll kidneys do not develop microaneurysms. In addition, macrophages from Lew/Maa rats produce more nitric oxide after stimulation than

macrophages from Lew/Moll,[20] indicating that bone marrow-derived cells may influence the course of antiThy1GN in Lewis substrains. However, genes expressed in the kidney, not those expressed by bone marrow-derived cells, determine glomerulosclerosis development in this model.[21] Transplanting Lew/Maa kidneys into Lew/Moll rats results in development glomerulosclerosis in the Lew/Molls.[21] Crosses of Lew/Maa and Lew/Moll demonstrate a dominant negative effect of Lew/Moll. These animals do not develop proteinuria and glomerulosclerosis in the context of antiThy1GN. In backcrossed animals development of proteinuria was not always followed by glomerulosclerosis.[11] Different genetic regions influence proteinuria and glomerulosclerosis. Therefore, different factors and different compartments may play roles in the development of acute glomerular injury and subsequent proteinuria compared to factors that contribute to progression or repair in this model.

The present study investigated whether genes expressed by the kidney or bone marrow-derived cells determine development of proteinuria or convey resistance to its development in antiThy1GN.

Materials and methods

Animals

All rats used in this study were female. The Lew/Maa rats were provided by the University of Maastricht (Maastricht, the Netherlands). The Lew/Moll rats were obtained from Taconic M&B Breeding Centre (Ry, Denmark). Animal care and experimentation were in accordance with the legislation on animal experiments as determined by the Dutch Veterinary Inspection and were approved by the Animal Experiments Committee of Leiden University Medical Center, Leiden, the Netherlands.

Kidney Transplantation

Kidney transplantation was performed as described previously.[17;21] Briefly, the left kidney without the adrenal gland but with a patch of the aorta, a cuff of the inferior vena cava, and the ureter were removed and transplanted in the heterotopic position. The donor ureter was anastomosed to the ureter of the recipient bladder.

Bone Marrow Chimera Generation

The generation of bone marrow chimeras was based on a previously described protocol.[36] Briefly, all animals, weighing between 160 and 200 g, were pre-treated by lethal irradiation (8 Gy per animal in 50 fractions) using an x-ray generator. No anesthesia was administered. Twenty-four hours after total-body irradiation, the rats

received adult bone marrow transplants. The dose of irradiation was based on pilot dose-response experiments and preceding reports. In our hands, an increase of the irradiation dose up to 8.5 Gy, followed by bone marrow reconstitution, was lethal to all recipient Lewis rats within 5 days.[21] Bone marrow cells were collected by flushing femur bone shafts with Hanks buffered medium. The cells were sieved through 50-μm sieves and washed twice with ice-cold Hanks buffered medium. Subsequently, cells were resuspended in ice-cold Hanks buffered medium at a concentration of 5 x 10^7 cells/ml. Rats received 5 x 10^7 bone marrow cells by intravenous injection directly after the isolation of bone marrow cells.

Anti-Thy-1 Glomerulonephritis

AntiThy1GN was induced by the intravenous injection of 2 mg/kg ER4 antibody. This monoclonal anti-Thy-1 antibody was affinity purified from culture supernatants of hybridoma ER4[19] on protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden). The antibody was subsequently depleted from possible contamination with endotoxin by running it batch-wise over Detoxy-Gel (Pierce, Rockford, IL). Urine was collected before antiThy1GN induction and on day 7 after induction. On day 7, kidney biopsies were performed. Three weeks after antiThy1GN induction, the rats were killed and the kidneys were removed and histologically examined.

Experimental Design

To determine whether renal cells convey resistance or susceptibility to the development of proteinuria, kidneys were exchanged between the two substrains by heterotopic transplantation of one kidney; the other kidney was left *in situ* (four animals per group). To exclude an additional role of substrain-dependent rejection on glomerular morphology, kidney transplantations were also performed between the Lewis substrains without inducing antiThy1GN. Immunosuppressive therapy after transplantation was omitted in this study. Because the two Lewis substrains have identical MHC haplotypes, rejection was not expected, nor was it observed. However, some transient tubulointerstitial infiltrate was observed, even in syngeneic transplants. Because no additional effect of the transient tubulointerstitial infiltrate was observed over the course of the antiThy1GN, the presence of this infiltrate was not considered further. To investigate the effect of the transplantation procedure on the course of antiThy1GN in Lew/Maa and Lew/Moll, syngeneic transplantations were performed.

Because Lew/Maa rats develop proteinuria with one kidney of their own, we investigated the development of proteinuria in antiThy1GN when a single Lew/Moll kidney was present in Lew/Maa rats after bilateral nephrectomy (n=4). The first

nephrectomy was performed during the transplantation procedure and the second 7 days later. However, this may lead to glomerular hyperfiltration, a feature known to influence proteinuria in Wistar rats with antiThy1GN.[22] To investigate the effect of hyperfiltration induced by unilateral nephrectomy on the course of antiThy1GN in Lew/Moll rats, we induced antiThy1GN in Lew/Moll 1 day or 7 days after unilateral nephrectomy (n=2 per time point).

To determine whether bone marrow conveys resistance or susceptibility to the development of proteinuria, bone marrow chimeric rats were generated by the lethal irradiation of each substrain, followed by reconstitution with bone marrow cells derived from the other substrain (n=7 per group). Four weeks after recovery, chimeric rats have a reconstituted immune system.[37] Four weeks after bone marrow transplantations, antiThy1GN was induced and monitored in these rats. As a control, antiThy1GN was induced in rats that received syngeneic bone marrow. To exclude the effect of radiation, bone marrow chimeric rats were generated and examined during an equivalent period of time as the experimental group but without inducing antiThy1GN.

Proteinuria

The animals were housed in metabolic cages for 24 hours to obtain urine samples from day -1 to 0 and from day 6 to day 7. The 24-hour urine protein excretion was measured by the biuret standard method.[38]

Immunohistochemistry

Kidney biopsies were performed by lateral incision on day 7 after the induction of antiThy1GN. Renal tissue sections (4 μm) from all rats were stained with periodic acid-Schiff (PAS) followed by hematoxylin counterstaining. Renal biopsy tissue was stained for ED-1, a marker of macrophages and monocytes,[39] and PL-1, which stains platelets.[25] After deparaffinization, endogenous peroxidase activity blocking, and antigen retrieval with citrate buffer, sections were incubated with ED-1 monoclonal Ab IgG1 (Leiden University Medical Centre, Pathology; diluted 1:100) overnight at room temperature. Primary antibodies were detected using monoclonal rabbit anti-mouse IgG1/HRP (MONO 5053, Monosan), and diaminobenzidine was used as substrate. Tissues were counterstained with hematoxylin. For PL-1 staining, sections were treated with proteinase K for 15 min before incubation with monoclonal mouse Ab IgG1 PL-1 (Leiden University Medical Centre, Pathology).

The percentage of glomeruli with microaneurysms was determined in at least 15 glomerular cross-sections per rat using PAS-stained sections.[27] The average area (calculated as a percentage) positive for PL-1 per glomerular cross-section was scored

by computer-based image analysis.[40] At least 20 randomly chosen glomeruli were photographed at 200x magnification with a Zeiss Axioplan microscope equipped with a Sony DXC-950P 3CCD color camera (Sony Corporation, Tokyo, Japan), and the average area percentage for PL-1 per glomerulus was measured using KS-400 image analysis software version 3.0 (Zeiss-Kontron, Eching, Germany).

Statistical Analyses

All data are expressed as mean ± SD. Statistical analyses were performed using two-tailed unpaired *t* tests. Statistical significance was defined as $P<0.05$.

Results

Renal Transplantation from Lew/Maa into Lew/Moll Does Not Result in Proteinuria

When a single kidney was replaced, Lew/Maa rats developed proteinuria after the induction of antiThy1GN, irrespective of whether they received a Lew/Maa or Lew/Moll kidney (Figure 1). Conversely, no proteinuria was detected in Lew/Moll rats when one kidney was replaced with a Lew/Moll or Lew/Maa kidney. Kidney transplantation without inducing antiThy1GN did not result in proteinuria (data not shown).

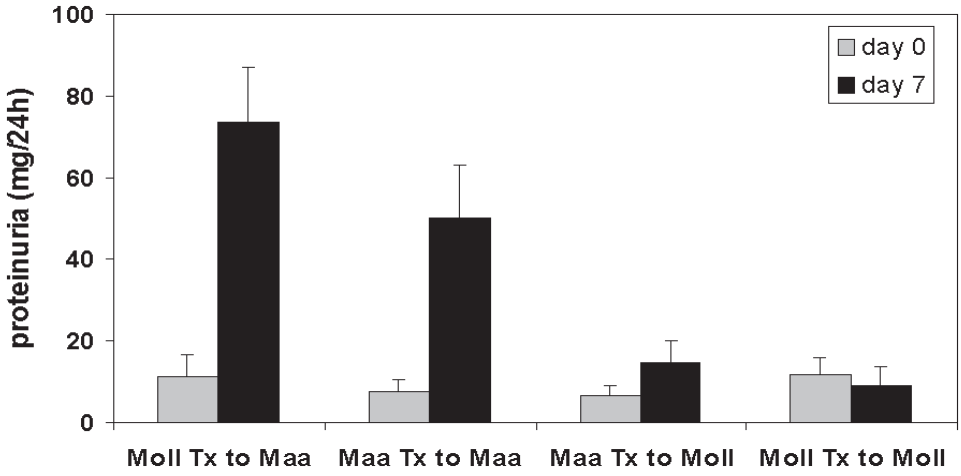

Figure 1. Proteinuria in Lew/Maa and Lew/Moll rats after kidney transplantation and unilateral nephrectomy. No proteinuria was seen in any of the groups before induction of antiThy1GN (day 0). Lew/Maa rats developed proteinuria regardless of whether they received a Lew/Maa (Maa Tx to Maa) or Lew/Moll kidney (Moll Tx to Maa). Lew/Moll rats did not develop proteinuria regardless of whether they received a Lew/Moll (Moll Tx to Moll) or Lew/Maa kidney (Maa Tx to Moll). N=4, Tx = transplantation.

Renal Transplantation from Lew/Moll into Lew/Maa after Bilateral Nephrectomy Does Not Result in Proteinuria

To investigate the effect of Lew/Moll kidneys in Lew/Maa rats without homologous kidneys, we induced antiThy1GN in Lew/Maa who received one Lew/Moll kidney after bilateral nephrectomy. Because bilateral nephrectomy induces hyperfiltration, a feature known to influence the severity of proteinuria and glomerulosclerosis,[22] we first examined the role of hyperfiltration in Lew/Moll rats by nephrectomizing one kidney and inducing anitThy1GN. Lew/Moll kidneys were resistant to proteinuria when antiThy1GN was induced 1 or 7 days after unilateral nephrectomy (data not shown).

Because hyperfiltration in Lew/Moll rats did not result in proteinuria, we induced AntiThy1GN in bilaterally nephrectomized Lew/Maa rats with one Lew/Moll transplant. These rats did not develop proteinuria (data not shown).

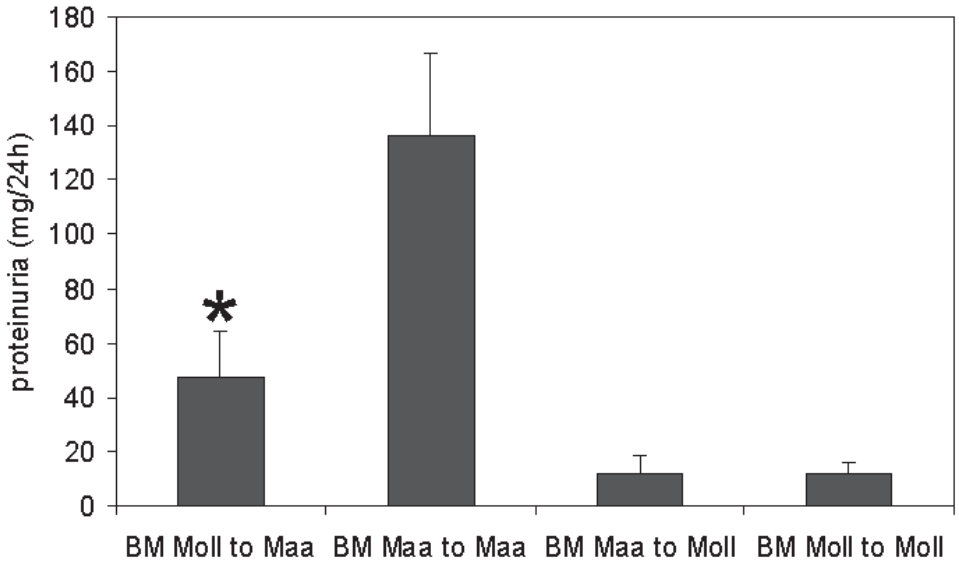

Figure 2. Proteinuria in bone marrow chimeras 7 days after the induction of antiThy1GN. Lew/Maa rats developed proteinuria regardless of whether they received Lew/Maa (Maa BM to Maa) or Lew/Moll bone marrow (Moll BM to Maa). However, the level of proteinuria was lower in Lew/Maa rats that received bone marrow from Lew/Moll rats. Lew/Moll rats did not develop proteinuria, regardless of whether they received Lew/Moll (Moll BM to Moll) or Lew/Maa bone marrow (Maa BM to Moll). N=7; BM= bone marrow * $P<0.001$ compared to Maa BM to Maa.

Transfer of Lew/Moll Bone Marrow to Lew/Maa Decreases Proteinuria

To investigate whether bone-marrow derived cells convey resistance or susceptibility to development of proteinuria, bone marrow chimeras were generated (Figure 2). The Lew/Maa rats, irrespective of whether they received Lew/Maa or Lew/Moll bone marrow, developed proteinuria after antiThy1GN induction. However, the level of proteinuria was significantly lower in Lew/Maa rats that received bone marrow from Lew/Moll compared to those that received marrow from Lew/Maa (46.9 ± 17.2 mg/24h vs. 136.1 ± 30.4 mg/24h, respectively; $P<0.001$). Lew/Moll rats that received bone marrow from Lew/Maa rats or homologous bone marrow did not develop proteinuria.

Decreased Proteinuria Is Accompanied by Fewer Glomerular Microaneurysms

Because proteinuria was significantly lower in Lew/Maa rats that received Lew/Moll bone marrow, the histology on day 7 was compared to the syngeneically transplanted rats. No differences were found in the average number of macrophages (ED1) or thrombocytes (PL-1) per cross-section area percentage (Figure 3). A significantly higher number of glomeruli were found to have microaneurysms in the Lew/Maa rats that received syngeneic transplants compared to the chimeric Lew/Maa rats (72.4 ± 9.9% vs. 50.6 ± 9.3%, respectively; $P=0.002$). Representative glomeruli from Lew/Maa rats with Lew/Maa or Lew/Moll bone marrow are shown in Figure 4.

Discussion

The development of renal disease is thought to be the result of a dynamic interaction between intrinsic renal factors and extra-renal factors, such as bone marrow-derived cells.[15;23;24] Susceptibility to development of proteinuria is regarded as multigenic and multifactorial and not all patients develop it. Much research is focused on development of proteinuria in several human renal diseases and experimental models. In order to identify factors involved in proteinuria, we separated renal factors from bone marrow-derived factors to establish which contribute to its development or resistance in antiThy1GN.

Three major conclusions can be drawn from this study. First, neither the transfer of bone marrow, nor kidney transplantation from the proteinuria-susceptible Lew/Maa rat to the proteinuria-resistant Lew/Moll rat transferred proteinuria into Lew/Moll rats. Moreover, intrinsic renal factors from Lew/Moll protect against proteinuria development because the transplantation of a Lew/Moll kidney into a

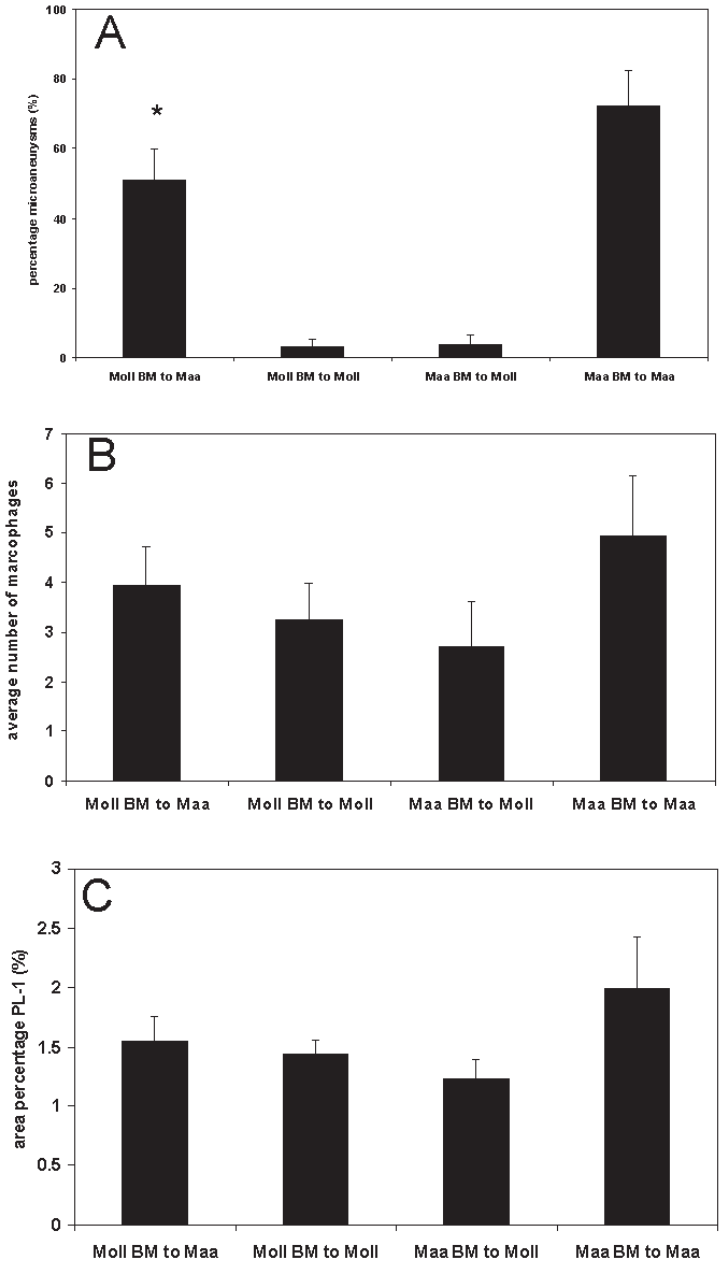

Figure 3. Histological features of bone marrow chimeras on day 7 after antiThy1GN induction. (A) Percentage of glomeruli with microaneurysms. (B) Average number of macrophages per glomerulus (C) Area percentage of PL-1. Lew/Maa rats with Lew/MoII bone marrow (MoII BM to Maa) exhibited significantly fewer glomeruli with microaneurysms compared to Lew/Maa rats with Lew/Maa bone marrow (Maa BM to Maa). N=7, $P<0.01$ compared to Maa BM to Maa. PL-1=platelet factor 1; BM= bone marrow.

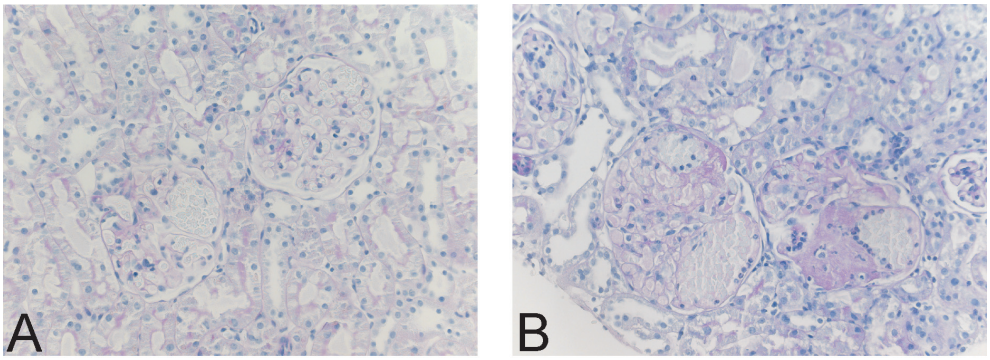

Figure 4. Examples of glomeruli with microaneurysms in Lew/Maa bone marrow chimeras. Fewer microaneurysms were found in the kidneys of Lew/Maa rats with bone marrow from Lew/Moll (B) compared to the kidneys of Lew/Maa rats with bone marrow from Lew/Maa (A).

Lew/Maa rat did not result in proteinuria after antiThy1GN was induced. Finally, bone marrow-derived factors from Lew/Moll suppressed proteinuria development after inducing antiThy1GN; bone marrow transfer from Lew/Moll to Lew/Maa suppressed proteinuria levels in Lew/Maa. This decrease was accompanied by fewer glomerular microaneurysms on day 7.

Bone marrow transfer from Lew/Maa to Lew/Moll did not result in proteinuria. Several studies have shown that inflammatory cells influence development of proteinuria in experimental and human renal diseases.[15;23;24] This view is also supported by the observation that several proteinuric renal diseases respond to immunosuppressive therapy. In addition, the transfer of bone marrow from Wistar-Kyoto rats to resistant Lewis rats results in increased proteinuria levels and glomerular damage in nephrotoxic nephritis.[15] Our study shows that bone marrow-derived factors from Lew/Maa did not influence development of proteinuria. Interestingly, bone marrow transplantation from Lew/Moll to Lew/Maa appears to suppress proteinuria. Our results are supported by the previous finding that macrophages from Lew/Maa and Lew/Moll respond differently after stimulation with LPS.[20] Macrophages from Lew/Maa produce more inducible nitric oxide synthase (iNOS) compared to those from Lew/Moll.[20] Reduced nitric oxide levels in Lew/Maa rats results in reduced proteinuria levels.[25] The suppression effect of Lew/Moll bone marrow-derived factors on development of proteinuria may result from decreased macrophage iNOS production, which leads to less proteinuria.

In regards to the direct effect of macrophages on proteinuria, bone marrow-derived cells can contribute to the repair response to glomerular damage[26] and substitute damaged mesangial and endothelial cells.[27] The decreased proteinuria

we observed could be the result of less acute injury or accelerated endothelial and mesangial repair. Microaneurysm development is thought to be the result of mesangiolysis and the initial inflammatory response, which suggests that Lew/Moll bone marrow influences acute injury in antiThy1GN. The difference in microaneurysm development could not be explained by a difference in macrophage attraction or intraglomerular coagulation. Further research is needed to identify the bone marrow-derived factors. Other inflammatory responses, such as chemokines, or intrinsic responses to these inflammatory reactions could lead to decreased glomerular permeability.

Genetic factors involved in protecting against development of proteinuria are also described in other proteinuric experimental models. For example, in Dahl rats, a strain that develops hypertension-induced proteinuria, a genetic locus on chromosome 11 has been linked to decreased proteinuria in the F2 population.[13] A similar gene effect was seen in Sabra rats that develop proteinuria without hypertension.[14] In addition, we have found a suggestive locus on chromosome 6 that is linked to proteinuria in the Lew/Maa and Lew/Moll backcross.[11] This locus may also represent a gene involved in suppression of proteinuria instead of promotion.

Kidney transplantation between Lew/Maa and Lew/Moll showed that renal genes are not sufficient to cause proteinuria. However, the fact that a Lew/Moll kidney in a Lew/Maa rat does not development proteinuria, suggests that intrinsic renal factors are required for the development of proteinuria in Lew/Maas. Though, as for bone marrow-derived factors, we cannot exclude that renal intrinsic factors from Lew/Moll inhibit development of proteinuria. Several researchers have shown that intrinsic renal factors do influence development of proteinuria.[15;24] For example, the development of proteinuria and glomerular crescents is influenced by renal genes in nephrotoxic nephritis.[15] The observation that kidney transplantation itself does not lead to proteinuria suggests that additional factors are required for susceptibility.

Circulating permeability factors not derived from bone marrow-derived cells may influence proteinuria in antiThy1GN. For example, mutations in the soluble urokinase receptor have been identified as a cause of proteinuria in primary focal segmental glomerulosclerosis (FSGS).[7;28] In these patients, the same circulating factor leads to recurrent FSGS.[29] We have investigated the role of plasma transfer in development of proteinuria in Lew/Moll rats. The transfer of serum from Lew/Maa to Lew/Moll for three days (before, day 1, and day 2) combined with the induction of antiThy1GN did not lead to proteinuria in Lew/Moll rats (data not shown). However, this pilot experiment does not definitively rule out a role for plasma factors in development of proteinuria. Plasma transfer from Lew/Moll to Lew/Maa might even suppress proteinuria in AntiThy1GN.

Renal neuronal factors are also thought to contribute to renal inflammation and proteinuria. Recently, Veelken *et al.* [30] described the role of renal innervation in the inflammation of antiThy1GN. Bilateral denervation of the kidneys resulted in decreased levels of proteinuria, less glomeruli with microaneurysm, less inflammation, and less glomerulosclerosis. Interestingly, as in our study, they did not find a difference in the number of glomerular macrophages.[30] In our renal transplantations of Lewis substrains, the innervation of the kidney was interrupted in the renal allografts. No functional reinnervation has been described for the first 9 months after kidney transplantation in rats.[31] Therefore, renal denervation could influence our kidney transplantation results. Future experiments of bilateral renal denervation in Lewis substrains may provide insight into the role of renal innervation in development of proteinuria in antiThy1GN.

Previous research from our group has shown that progressive glomerulosclerosis in Lew/Maa is determined by renal genes; thus, we were surprised to find that proteinuria was not transferred with the kidney. Several investigations have provided evidence that proteinuria and progressive glomerulosclerosis are linked in human renal disease.[32-34] Proteinuria severity is linked to accelerated renal disease progression. [35] However, not all patients with proteinuria develop progressive renal disease and not all proteinuric experimental models lead to glomerulosclerosis.[19] Our data suggest that, in antiThy1GN, proteinuria and progressive renal disease are determined by differential factors. This conclusion is supported by the fact that linkage analysis has revealed different loci for proteinuria and glomerulosclerosis in Lew/Maa and Lew/Moll.[11] We hypothesize that renal and bone marrow-derived factors from Lew/Moll suppress proteinuria in antiThy1GN, whereas renal factors in Lew/Maa promote glomerulosclerosis. Notably, a drawback of the Lew/Maa and Lew/Moll antiThy1GN model is that the proteinuria is transient, which could also explain why the development of proteinuria and glomerulosclerosis are not linked in this model.

In humans, the treatment of many renal diseases, such as membranoproliferative glomerulonephritis or IgA nephropathy, is initially focused on anti-inflammatory drugs and reduction of proteinuria in the progressive phase. Unfortunately, many patients still progress to end-stage renal disease. Based on our and others' observations, we suggest that the differences in therapeutic response may be dependent, in part, on both renal and bone marrow-derived factors. Also, protective factors rather than disease-promoting factors may predict severity and outcome in renal disease and should therefore, be taken in account.

In conclusion, proteinuria in antiThy1GN results from acute glomerular damage. Proteinuria follows antibody binding, complement activation, mesangiolysis,

macrophage recruitment, and development of microaneurysms. This study shows that, at least, intrinsic renal and bone marrow-derived factors influence proteinuria in antiThy1GN, both by promoting and suppressing glomerular damage. Therefore, the identification of factors involved in protecting against proteinuria may be a promising new treatment focus.

Acknowledgments

None

Statement of competing financial interests

None

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