Genes expressed by the kidney, but not by bone marrow-derived cells underlie the genetic predisposition to progressive glomerulosclerosis after mesangial injury

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Abstract

Progressive renal failure is accompanied by uncontrolled accumulation of extracellular matrix in glomeruli and tubulointerstitium, eventually resulting in glomerulosclerosis. Although glomerulosclerosis occurs secondary to various renal diseases, the fact that not all patients develop progressive glomerulosclerosis suggests that genetic factors may underlie the tendency to progress, or not to progress. We have identified two Lewis sub-strains with small genetic differences but with considerable difference in resolution of glomerulonephritis after anti-Thy-1 administration. In the Lewis/Møllegard rat strain, anti-Thy-1 glomerulonephritis spontaneously resolves within 4 weeks. In contrast, Lewis/Maastricht rats develop progressive glomerulosclerosis after induction of this disease. Here we determine the involvement of bone marrow-derived cells and kidney cells in the development of glomerulosclerosis. In the first study, exchange of bone marrow between these sub-strains did not affect the course of anti-Thy-1 nephritis. Lewis/Møllegard rats recovered rapidly and Lewis/Maastricht rats showed progressive disease regardless of the genotype of the bone marrow they received. In the second study kidneys were exchanged between the sub-strains. After the transplantation anti-Thy-1 nephritis was induced and glomerular damage assessed at day 21. Severe damage was observed in Lewis/Maastricht glomeruli independent of whether the kidney had been transplanted or not. Similarly, Lewis/Møllegard glomeruli, whether transplanted or not, revealed no residual histopathological abnormalities. We conclude that the inherited differences between the two sub-strains with regard to their in susceptibility to develop progressive glomerulosclerosis after mesangial injury are governed by genes expressed by the kidney, but not by bone marrow-derived cells.
Introduction

Progressive renal failure is accompanied by uncontrolled accumulation of extracellular matrix in glomeruli and tubulointerstitium, eventually resulting in glomerulosclerosis and interstitial fibrosis. Glomerulosclerosis is a process that occurs secondary to various renal diseases such as diabetes mellitus, systemic lupus erythematosus, Wegener’s granulomatosis, IgA nephropathy, and essential hypertension. However, not all patients with these diseases develop progressive glomerulosclerosis and loss of renal function. Suggesting that patients can be subdivided into progressors and non-progressors. No predictive markers for progressive glomerulosclerosis or repair of glomerular damage have been discovered. Several studies describe a genetic predisposition to develop progressive glomerulosclerosis. These include studies on idiopathic and hypertension-induced focal and segmental glomerulosclerosis. In rodents, markers of several genes are associated with susceptibility to develop irreversible glomerulosclerosis. Identification of these markers would provide better understanding of the pathogenesis of glomerulosclerosis secondary to renal disease and might be an important step towards development of prevention and improved therapy. The natural genetic heterogeneity among individuals and the limited availability of tissue severely hampers the search for these markers in patients. We have turned to animal studies in the hope that identification of strains that are progressors and those that are not due to a genetic predisposition may lead to identification of genes also of relevance in human populations. Upon screening of different rat strains we found two inbred Lewis sub-strains, i.e., Lewis/Maastricht (Lew/Maa), and Lewis/Møllegard (Lew/Moll) that recover differently after induction of anti-Thy-1 nephritis. This is a self-limiting disease in most rat strains and represents a useful and frequently used model of renal tissue repair after acute immune-mediated injury. The injury is induced by the injection of antibodies against Thy1.1, a transmembrane protein present on mesangial cells. Antibody injection results in in situ immune complex formation on mesangial cells, classical pathway complement activation, platelet aggregation, influx of polymorph nuclear neutrophils (PMN’s) and monocytes, and mesangial lysis and apoptosis. This is followed by mesangial cell proliferation and expansion of the mesangial matrix. In most rat strains, including Lew/Moll, the mesangioproliferative lesion spontaneously resolves within 4 weeks after induction of the disease. In contrast, Lew/Maa rats do not show spontaneous repair of glomerular injury after induction of anti-Thy-1 nephritis, but develop progressive glomerulosclerosis. F1 hybrids of Lew/Moll and Lew/Maa rats show repair within 3 weeks, revealing a dominantly inherited trait (E. de Heer unpublished observation). Both strains were derived from Lewis inbred rats and have small genetic differences, thus providing a unique tool with which to identify genes involved in
development of progressive glomerulosclerosis or repair of glomerular damage. The development of glomerulosclerosis in this model is the result of a dynamic interaction of the immune system and the kidney responding to inflammatory damage. The goal of this study was to determine whether in this model disease progression is mediated by genes intrinsic to the kidney or by extrinsic factors determined by bone marrow-derived cells. Bone marrow and renal transplantation were performed between the two sub-strains. The data presented indicate that the inherited ability of Lew/Maa rats to develop progressive glomerulosclerosis in the course of anti-Thy-1 nephritis is governed by the phenotype of the kidney and not by the phenotype of the bone marrow.

Materials and Methods

Animals
Female Lewis/Maastricht (Lew/Maa) rats were provided by Frans Weekers from the University of Maastricht, Maastricht, The Netherlands. Female Lewis/Møllegard (Lew/Moll) rats were obtained from M&B Breeding Center, Ry, Denmark. Animal care and experimentation were in accordance with legislation on animal experiments as determined by the Dutch Veterinary Inspection.

Antibodies
Monoclonal anti-Thy-1 antibody was affinity-purified from culture supernatants from hybridoma ER4 on Protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden). It was subsequently depleted from possible contamination with endotoxin by running it batch-wise over Detoxy-Gel (Pierce, Rockford, IL, USA).

Generation of bone marrow chimeras
The generation of bone marrow chimeras was based on a previously described protocol. Briefly, all animals, weighing between 160 and 200 grams, were lethally irradiated with 8 Gy per animal in 50 fractions by the use of an X-ray generator without anesthesia. Twenty-four hours after total body irradiation rats received adult bone marrow transplantation. Bone marrow cells were collected by flushing bone shafts of femurs with Hanks buffered medium. 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 5x10^7 bone marrow cells by i.v. injection directly after isolation of the bone marrow cells. To exclude the effect of radiation and bone marrow transplantation, bone marrow chimeric rats were generated and examined during an
equivalent period of time as the experimental group but without inducing anti-Thy-1 nephritis. No additional histopathological abnormalities were observed in the glomeruli of the control rats.

**Kidney transplantation**

Kidney transplantation was performed heterotopically as described previously\(^1\). Briefly, the left kidney without the adrenal gland together with a patch of the aorta, a cuff of the inferior vena cava, the ureter, and the bladder dome, were removed and transplanted in the heterotopic position. The donor ureter together with a small piece of the donor bladder was anastomised to the dome of the recipient bladder. To exclude an additional role of sub-strain-dependent rejection on glomerular morphology, kidney transplantations were performed between the Lewis sub-strains without inducing anti-Thy-1 nephritis. Although the interstitium was infiltrated in some grafts, no histopathological abnormalities were observed in the glomeruli of the transplanted kidneys without anti-Thy-1 nephritis (data not shown).

**Histological examination**

Kidney tissue fixed in 4% neutral buffered formalin embedded in paraffin were cut into 4 µm sections and stained with periodic acid Schiff (PAS). Glomerular injury was quantified according to a protocol based on the method described by Bidani et al.\(^1\). Briefly, normal glomeruli were defined by the appearance of the glomeruli in the kidney of the control group. Abnormal glomeruli were defined by the appearance of micro aneurysms, mesangial cell proliferation, extra cellular matrix expansion, glomerular crescent formation, collapsed capillary loops with mesangial expansion and hyaline deposits. Forty glomeruli in each section were scored. No histopathological abnormalities were observed in extraglomerular tissue, which therefore was excluded from the analysis. Data are expressed as the mean per group animals of the percentage of abnormal glomeruli in forty analysed glomeruli.

**Statistics**

All data are expressed as means ± SD. Statistical analysis was performed using two-tailed unpaired Student’s T-test. Statistical significance was defined as P<0.05.

**Experimental design**

The natural course of anti-Thy-1 nephritis was monitored in both substrains. Therefore anti-Thy-1 nephritis was induced in the rats by intravenous injection of 2 mg/kg ER4 antibody; subsequently animals (n = 7 per group) were sacrificed 3, 7, 21, and 90 days after induction of the disease followed by the removal of the kidneys. Sections of the removed kidneys were
histologically analyzed. To determine whether the bone marrow expresses genes that are related to progressive glomerulosclerosis, bone marrow chimeric rats were generated by lethal irradiation of each substrain, followed by reconstitution with bone marrow cells derived from the other substrain (n = 7 animals per group). Four weeks after recovery chimeric rats have reconstituted their immune system. After recovery anti-Thy-1 nephritis was induced and monitored in these bone marrow-reconstituted rats by histological examination of renal biopsies and renal tissue taken at day 7 and day 21, respectively. In order to determine whether cells from the kidney express genes that are related to progressive glomerulosclerosis, kidneys were exchanged between the two sub-strains by heterotopic transplantation of one kidney, while the other kidney remained in situ (n = 4 animals per group). Anti-Thy-1 nephritis was induced in the transplanted rats by intravenous injection of 2 mg/kg ER4 antibody. After three weeks the rats were sacrificed and the autologous and the transplanted kidneys were removed and histologically examined. In both experiments syngeneically transplanted rats and transplanted rats without induction of anti-Thy-1 nephritis served as a control. All experiments were terminated at three weeks after disease induction because by this time disease was such that whether animals would recover completely or progress to chronic renal disease can be determined.

Figure 1. Histochemical demonstration of matrix expansion, mesangial cell proliferation and glomerulosclerosis by PAS stained kidney sections in Lew/Moll glomeruli (A to C) and Lew/Maa glomeruli (D to F) during anti-Thy-1 nephritis development at day 0 (A and D), day 7 (B and E) and day 21 (C and F). Note the complete remodeling of glomeruli from Lew/Moll rats at day 21 (C) compared to persistent extra cellular matrix expansion and hypercellularity in glomeruli from Lew/Maa rats (F).
Results

Anti-Thy-1 nephritis time course in Lew/Maa and Lew/Moll rats
Both sub-strains showed severe mesangial cell lysis, apoptosis and an influx of PMN’s and monocytes 3 days after induction of anti-Thy-1 nephritis (data not shown). Increased numbers of mesangial cells and extracellular matrix expansion were observed in both sub-strains at 7 days. After 21 days no histopathological abnormalities were seen in Lew/Moll rats kidneys. At 21 days however, glomeruli in Lew/Maa rats demonstrated mesangial proliferation, matrix expansion and glomerular crescent formation (Fig. 1). The numbers of abnormal glomeruli were quantified in kidney sections obtained 7 and 21 days after induction of anti-Thy-1 nephritis. Although the number of abnormal glomeruli was higher in Lew/Maa rats compared with Lew/Moll rats at day 7, the damage per glomerulus was identical within both substrains after 7 days (69% ± 15% versus 40% ± 8%). Significant less abnormal glomeruli was observed in Lew/Moll in comparison to Lew/Maa glomeruli at day 21 (57% ± 3% versus 9% ± 1% P< 0.001) (Fig. 2). After 3 months, Lew/Maa rats developed focal segmental glomerulosclerosis (Fig. 3). Preliminary experiments have shown that even after 6 months, progressive glomerulosclerosis persists (data not shown).

Bone marrow transplantation
To investigate whether the sub-strain-related differences are due to genes expressed by bone marrow-derived cells, anti-Thy-1 nephritis was monitored in bone marrow reconstituted rats. Lew/Moll kidneys irrespective of whether or not the rats received Lew/Maa or Lew/Moll bone marrow recovered completely within 3 weeks, while Lew/Maa recipients of bone marrow from Lew/Maa showed persistent, severe glomerular damage. Glomerular damage scores for the both control groups and both experimental groups receiving bone marrow transplant after total body irradiation and induction of anti-Thy-1 nephritis are shown in Figure 4.

Figure 2. Quantitative time score of glomerular damage during development of anti-Thy-1 nephritis in Lew/Maa (solid bars) and Lew/Moll (open bars) rats (7 animals per group). Glomeruli (n=40) were quantitatively scored in periodic acid-Schiff-stained kidney sections at day 7 and day 21 after induction of anti-Thy-1 nephritis. Values indicated are means ± SD.
Kidney transplantation

As shown in Figure 5, disease progression in response to anti-Thy-1-induced glomerulonephritis followed the genotype of the kidney and not of the recipient animal. When anti-Thy-1 nephritis was induced glomerular damage appeared in Lewis/Maa rat kidneys whether they were transplanted into Lew/Maa or into Lew/Moll recipients. Glomerular damage also persisted in the non-transplanted autologous Lew/Maa kidneys irrespective of whether or not the rats received a Lew/Maa or a Lew/Moll transplant. Glomeruli of Lew/Moll kidneys, either transplanted or autologous, revealed no residual histopathological abnormalities 21 days after induction of anti-Thy-1 nephritis (Fig. 5). To examine whether the transplantation procedure itself interfered with the development of anti-Thy-1 nephritis autologous kidney transplantations were performed followed by induction of anti-Thy-1 nephritis. In Lew/Maa rats both the transplanted and the non-transplanted kidney showed glomerular damage. Lew/Moll rats showed no histopathological abnormalities in the glomeruli both in the transplanted and in the non-transplanted kidney 21 days after induction of anti-Thy-1 nephritis (Fig. 5). Graphical representation of glomerular damage scores is presented in Figure 6 for the autologous and transplanted kidneys in the four transplant groups.
The aim of this study was to investigate whether genes involved in the development of progressive glomerulosclerosis are expressed by the kidney or by bone marrow-derived cells. Therefore, we studied the development of glomerulosclerosis in the anti-Thy-1 nephritis model in two Lewis sub-strains. The development of glomerulosclerosis in this model is the result of a dynamic interaction between factors intrinsic to the kidney, and extrarenal factors. In addition to the immune system other factors, extrinsic to the kidney, are related to the development of chronic renal disease. These include smoking, age, systemic hypertension, and diabetes. The present study indicates that, at least in these two Lewis sub-strains, factors intrinsic to the kidney and not extrinsic factors determine whether progressive glomerulosclerosis develops in the course of anti-Thy-1 nephritis. This represents an important step in identification of genetic factors, which predispose rats to progressive renal disease. We did not identify genes responsible for the development of progressive glomerulosclerosis or the specific cells within the kidney, which are expressing those progression-related genes. There is, however, increasing evidence for a pivotal role of glomerular mesangial cells, and

**Figure 5.** Histochemical demonstration of glomerular damage after kidney transplantation by PAS stained kidney sections in donor kidneys (Donor = A to D) and recipient kidneys (Recipient = E to H) 21 days after induction of anti-Thy-1 nephritis. Note the absence of glomerular damage in Lewis/Møllegard (Lew/Moll) glomeruli irrespective of whether the kidney was transplanted or not (donors = A and D; recipient = F and H). Lewis/Maastricht (Lew/Maa) glomeruli demonstrated severe glomerular damage irrespective of whether the kidney was transplanted or not (donors = B and C; recipient = E and G).
the biomolecules they produce, in the development of anti-Thy-1 nephritis and glomerulosclerosis as summarized by Floege and Johnson 17. Mesangial cells produce factors involved in the mesangial cell proliferation and mesangial matrix expansion that follows mesangial cell injury during anti-Thy-1 nephritis. Among them are growth factors platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and transforming growth factor-beta (TGF-beta). Administration of anti-bFGF antibodies resulted in a overall reduction in mesangial cell proliferation 18. Others showed that administration of an aptamer-based antagonist against PDGF in anti-Thy-1 nephritis resulted in an almost complete inhibition of matrix protein accumulation 19. Blockade of TGF-β reduces the disease severity of anti-Thy-1 nephritis whether neutralizing antibodies are injected, soluble TGF-β receptors are produced by gene transfection or the natural antagonist, decorin, is administered intravenously or by gene therapy 20-23. In addition, constitutive overexpression of growth hormone has been shown to promote the development of glomerulosclerosis 24. These publications show that growth hormones like bFGF, PDGF and TGF-β are involved not only in the development of anti-Thy-1 nephritis but also in glomerulosclerosis following this experimental disease.

In addition to growth factors, the chemokine monocyte chemoattractant protein –1 (MCP-1), which is among those produced by mesangial cells, also plays an important role in the development of anti-Thy-1 nephritis. This is confirmed by neutralization studies of MCP-1 in Wistar rats during anti-Thy-1 nephritis 25. Furthermore, MCP-1 has a fibrogenic effect through the stimulation of TGF-β 26. This indicates that MCP-1, at least in part, mediates glomerular matrix accumulation. However, in previous experiments using a kidney transplant approach similar to the one used here, we have shown that the MCP-1 expression after induction of anti-Thy-1 nephritis was not significantly different between Lewis and Fisher 344 rats, a strain which does not develop progressive sclerosis 27. Thus factors other than MCP-1 clearly modulate the fibrotic response in this model.

Furthermore, in Lew/Moll rats with a Lew/Maa kidney transplant (Maa → Moll) the donor kidney showed less damaged glomeruli in comparison with the recipient kidney (4% ± 1% versus 54% ± 9%, P < 0.001). In both control groups (Maa → Maa and Moll → Moll) no significant differences were observed between donor and recipient kidneys. Values indicated are means ± SD.

**Figure 6.** Representation of glomerular damage after kidney transplantation in donor kidneys (solid bars) and recipient kidneys (open bars). Glomeruli were quantitatively scored in PAS-stained kidney sections taken 21 days after induction of anti-Thy-1 nephritis. In Lew/Maa rats with a Lew/Moll kidney transplant (Moll → Maa) the donor kidney showed less damaged glomeruli in comparison with the donor kidney (4% ± 1% versus 54% ± 9%, P < 0.001). In both control groups (Maa → Maa and Moll → Moll) no significant differences were observed between donor and recipient kidneys. Values indicated are means ± SD.
In addition to the pivotal role of mesangial cells in anti-Thy-1 nephritis, the infiltration of macrophages in the glomerulus is also one of the hallmarks of anti-Thy-1 nephritis. Ketteler and co-workers found that LPS-stimulated macrophages isolated from Lew/Maa rats expressed more inducible nitric oxide synthase (iNOS)-mRNA and – NO activity per cell than those from Lew/Moll rats, while the numbers of infiltrating macrophages in Lew/Maa and Lew/Moll rats was equal during anti-Thy-1 nephritis 12. These studies suggest that not the number of infiltrating macrophages but the expression levels of iNOS in the infiltrating macrophages may contribute to the development of glomerulosclerosis.

Apart from the role of mesangial cells and macrophages in anti-Thy-1 nephritis there is increasing evidence for a pathogenetic involvement of glomerular endothelial cells. Johnson and colleagues demonstrated that endothelial cell proliferation is markedly increased during the first week after induction of anti-Thy-1 nephritis 28. In addition, glomerulosclerosis appears to be strongly associated with impairment of vascular regeneration 29. It has recently been suggested that the glomerular epithelial cell also plays a role in the progression of anti-Thy-1 nephritis 30.

Regarding our results the following considerations seem to be of special importance. First, in our study we exchanged the bone marrow of the two sub-strains by subjecting the rats to a total body irradiation followed by bone marrow transplantation. While it has been reported that total body irradiation followed by bone marrow transplantation by itself can cause renal toxicity and glomerulosclerosis, the earliest signs of renal toxicity after total body irradiation appear after three months 31, much later than the observation period in our study. Therefore, we believe that an additional effect of total body irradiation on the glomerular damage in our experiments can be excluded.

Secondly, after induction of anti-Thy-1 nephritis, all rats developed a full-blown disease at day 7 that is indistinguishable between the sub-strains, but only Lew/Maa rats developed progressive glomerulosclerosis. These results indicate that progressive disease can develop when the severity of disease at day 7 is similar.

Thirdly, the kidney exchange experiment described in this study shows that progressive glomerulosclerosis in the kidney of the recipient was not transferred systemically to the transplanted kidney. Thus kidney transplantation would be expected to be a successful therapy for the kind of genetic defect existing in the Lew/Maa rats. Finally, although we demonstrated that glomerulosclerosis will only develop in the kidney which is able to express genes that are responsible for the development of glomerulosclerosis, it is still possible that after transplantation and induction of anti-Thy-1 nephritis recipient derived cells infiltrate in the donor kidney and express these progression genes. This is supported by previous experiments describing bone marrow transplantation in combination with glomerulosclerosis, revealing
that glomerular mesangial cell progenitors are derived from the bone marrow. However, only 7% to 8% of mesangial cells are reported to be replaced by bone marrow cells after induction of anti-Thy-1 nephritis in rats. Due to this low number, it has been suggested that most of the glomerular regeneration should be provided by locally proliferating mesangial cells.

In conclusion, although the literature suggests that both intra- and extra-renal processes contribute to the development of anti-Thy-1 nephritis and glomerulosclerosis, our study demonstrates that, at least in Lew/Maa rats, the genetic predisposition to progressive glomerulosclerosis is governed by genes expressed by the kidney, but not by those expressed in bone marrow-derived cells. Identification of the genes responsible for the development of glomerulosclerosis by differential gene expression analysis is underway in our laboratory. It is hoped that this approach will identify genes of relevance to progression in humans.

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**References**


