RESEARCH ARTICLE

Inflammation augments the development of experimental glomerulonephritis by accelerating proteinuria and enhancing mortality

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ABSTRACT. Proteinuria represents a parameter for a damaged filtration capacity of the kidney. We investigated how inflammation influences the development of experimental, immune complex-mediated glomerulonephritis by monitoring proteinuria. Mice pre-treated with LPS or TNF, one day before induction of glomerulonephritis, excreted high levels of protein in the urine immediately after the induction of glomerulonephritis, in contrast to non-treated mice where proteinuria increased steadily after day 3. Protein levels in the urine of pre-treated mice remained elevated over the 15-day observation time. The severity of proteinuria at later times correlated with the degree of tissue pathology and mortality in individual mice. Pre-treatment with inflammatory agents accelerated the development of proteinuria and induced more severe kidney damage.

Key words: kidney, inflammation

Most forms of glomerulonephritis (GN) result from immunologically-mediated glomerular injury, which leads to proteinuria and renal dysfunction. This process is associated with a variety of glomerular histological changes, including leukocyte infiltration, fibrin and platelet deposition, proliferation of intrinsic glomerular cells, mesangial sclerosis, and glomerular basement membrane (GBM) thickening. Injection of rabbit antibodies directed against proteins of the murine GBM, into mice or rats that had previously been immunized with rabbit Ig, provides an excellent model of proliferative GN [1].

Inflammatory agents such as bacterial lipopolysaccharide (LPS) induce the production of tumor necrosis factor (TNF) within minutes. TNF is a potent inflammatory cytokine and an important mediator of inflammatory tissue damage (reviewed in [2]). It is one of the first mediators released upon inflammatory stimulation and starts a series of other pro-inflammatory reactions. Using the experimental model of immune complex-induced GN, we analyzed the impact of pre-treatment of mice with the inflammatory stimuli LPS or TNF, before the induction of GN. Such pre-treatment accelerated the development of proteinuria, induced more severe pathological changes of kidney tissue, and enhanced mortality, suggesting that inflammation induces an early onset of renal failure, and sensitizes for enhanced organ damage.

METHODS

Mice

C57BL/6 mice were purchased from Charles River, Sulzfeld, Germany. Mice were housed under SPF-like conditions in the animal facility of the University of Regensburg and handled in accordance with institutional guidelines. All experiments were performed in compliance with the federal guidelines for animal experimentation.

Induction of GN and collection of urine, serum, and kidneys

Nephrotoxic serum was prepared by immunizing rabbits with mouse glomerular basement membrane (GBM), as described previously [1]. In brief, Chinchilla rabbits were immunized with a preparation of homogenized glomeruli isolated from mouse kidneys (kindly provided by R. Witzgall, Regensburg, Germany) to generate nephrotoxic serum containing rabbit anti-mouse GBM antibodies. Male, 7-18 week-old mice were immunized subcutaneously with rabbit IgG (0.2 mg, Jackson Immuno Research, Suffolk, UK) in complete Freund's adjuvant (Sigma-Aldrich, Munich, Germany). Six days later, mice were injected intravenously with 0.25 mL of the nephrotoxic serum. Urine samples were collected from the mice once a day before immunization and up to day 15 after injection of the nephrotoxic serum. Mice were killed by cervical dislocation on day 15, and both kidneys were removed, paraffin-fixed, sectioned and stained with both hematoxylin & eosin, and PAS.

Quantification of protein and creatinine in urine and serum

Protein concentrations in urine were measured according to the method of Bradford using albumin as standard (BCA Protein Assay Kit, Thermo Scientific, Schwerte, Germany). Urine samples were diluted in PBS. Creatinine

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concentrations were measured in urine and serum in order to determine glomerular damage. The assay was carried out in accordance with the QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, USA). Urine and serum sample measurements were performed twice.

Pre-treatment of mice

Mice were pre-treated one day before injection of the nephrotoxic serum with either LPS (0.05 mg/mouse, *E. coli* 0127:B8, Sigma-Aldrich) or recombinant human TNF (0.1 mg/mouse, *E. coli*-expressed) in PBS (0.2 mL) by intraperitoneal injection.

Statistics

Two-way ANOVA analysis with the Bonferroni *post hoc* test was used in experiments with two or more experimental groups. $p<0.05$ was accepted as significantly different. All statistics were performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, USA).

RESULTS AND DISCUSSION

The classical and well-studied model of immune complex-induced GN used in this study consisted of challenging mice, previously immunized with rabbit Ig, with rabbit antibodies directed against proteins of the mouse GBM. The developing GN was characterized by recruitment and accumulation of inflammatory cells into the glomerulus, and capillary damage followed by regeneration with crescent formation [1]. Early after induction, acute inflammatory changes in the glomeruli occurred such as deposition of antibodies, activation of complement, granulocyte influx, intravascular coagulation, and necrosis. This was accompanied by proteinuria within six hours, depending on the dose of the heterologous antibodies injected.

Development of proteinuria and kidney damage

We detected little increase in proteinuria during the so-called, heterological early phase after injection of the nephrotoxic serum (*figure 1A*). From day 6 after induction of GN however, the mice showed a continuous increase in protein secreted in the urine. After injection of the nephrotoxic serum, the creatinine concentrations in urine were slightly enhanced on day 15, and the ratios of the protein to creatinine values were also increased on days 7 and 15 (*figure 1B*). With the exception of one mouse that had a very high protein level in the urine on day 9 (>30 mg/mL) and died on day 14, all other animals survived the observation period of 15 days. The degree of tissue pathology in the histological studies correlated, in individual mice, with the severity of the proteinuria at later time points (*figure 1D*). Glomerular damage typical of crescentic or mesango-proliferative glomerulonephritis was seen, i.e. a large crescent-shaped zone enclosing the glomerular capillaries caused by massive extra-capillary hyper-cellularity, clustering of cells in the capillaries, and the Bowman's capsule showing signs of hyper-cellularity and broadening.

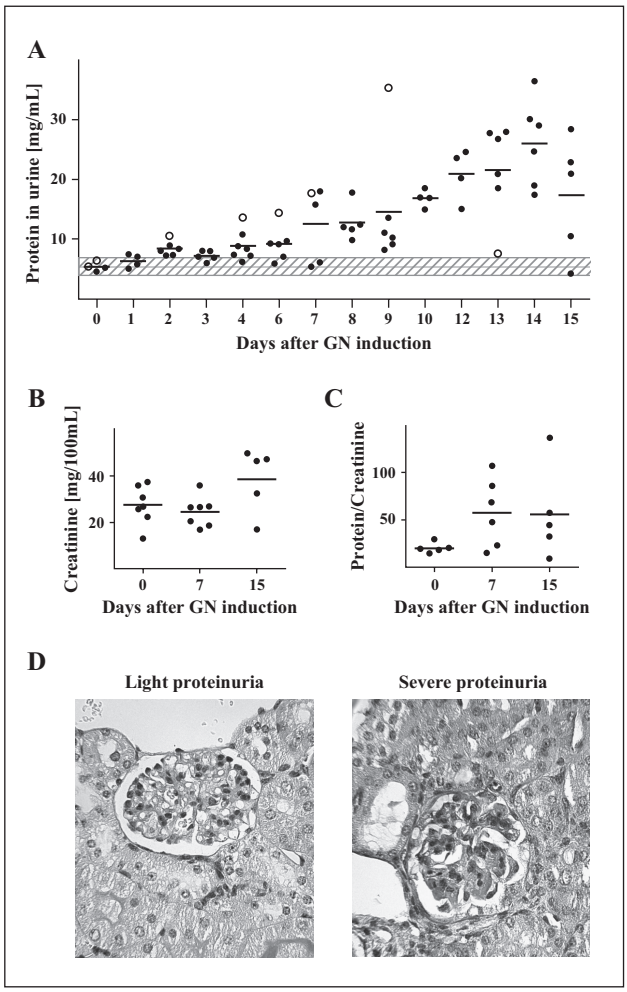

Figure 1

Development of GN.
A) Proteinuria was determined in mice (n=7) for 15 days, after injection of the nephrotoxic serum. The protein levels in urine from individual mice are given as symbols with mean, on the respective day after GN induction by the nephrotoxic serum. One mouse died on day 14 after the injection of the nephrotoxic serum (open symbols). The shaded area represents the range of protein concentrations measured in non-treated mice ±1 SD. **B)** Creatinine levels in urine and **(C)** ratios of protein to creatinine values with the mean, in urine on days 0, 7, and 15 after the injection of the nephrotoxic serum are shown. **D)** Representative pictures of PAS-stained kidney sections from mice 15 days after the injection of the nephrotoxic serum with slight proteinuria (5-12 mg/mL, left) and severe proteinuria (27 mg/mL, right) are shown.

Inflammatory pre-treatment enhanced early proteinuria in GN

In order to test for the effect of acute, short-term inflammatory stimuli, previously immunized mice were pre-treated with LPS, a potent inducer of TNF, or recombinant human TNF, 24 hours before the induction of GN. Pre-treatment with the inflammatory stimuli significantly and greatly enhanced proteinuria during the early, heterologous phase of GN, and protein levels remained elevated during the course of the GN as seen in *figure 2A*. These findings are support earlier findings in rats where administration of LPS or the inflammatory cytokines TNF or Interleukin-1 shortly before the initiation of GN, accelerated the early release of protein into the urine demonstrating the dependence of the development of GN on inflammation, especially the early phase [3].

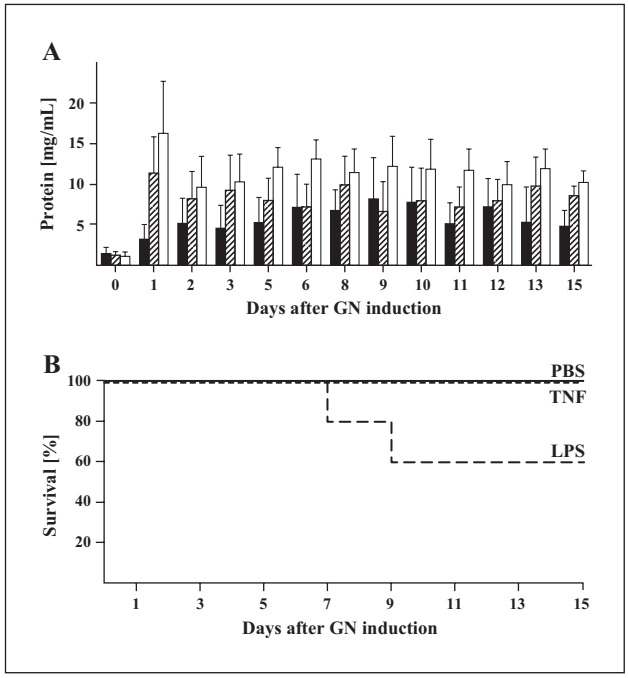

Figure 2
Development of proteinuria in mice after pre-treatment with inflammatory agents.
A) One day before the injection of the nephrotoxic serum, mice (n=10 per group) received either LPS (0.02 mg/mouse) (dashed line), recombinant human TNF (0.02 mg/mouse) (dotted line) or no stimulant (PBS, black line) in 0 2 mL PBS. Proteinuria was determined for 15 days after the injection of the nephrotoxic serum. Mice treated with LPS showed significantly increased levels of proteinuria on days 1, 3, 5, 6, 11 and 13 compared to mice treated with PBS. Mice treated with rhTNF showed significantly increased levels of proteinuria on day 1 and 3 compared to mice treated with PBS. Data are shown as mean with standard deviation as error bars. **B)** Survival curve of the experiment shown in (A).

Pre-treatment with LPS also enhanced mortality in our model as four out of 10 mice in this group died during the 15 days of GN, in comparison to no mortalities in the control group (*figure 2B*). The mice dying on day 7 or day 9 had urine protein levels between 11 and 17 mg/mL. This is in line with the results reported by Tomosugi *et al*. where injection of small doses of LPS or TNF into rats markedly amplified the severity of injury caused by subsequent injection of heterologous, anti-GBM antibodies [3]. The observation that pre-treatment with TNF aggravated the course of GN development to a lesser extent compared with LPS, could possibly be due to the fact that human TNF only activates the mouse TNF receptor type 1 and not TNF receptor type 2. The critical role of TNF in GN had been however, previously confirmed by TNF neutralization experiments: passive immunization against TNF [4, 5], as well as neutralization of endogenous TNF [6, 7], provided protection from LPS-enhanced glomerular injury in nephrotoxic nephritis in rats. Even when TNF blockade was delayed until the peak of crescent formation, renal function was still preserved, indicating that TNF not only mediates inflammatory injury in the glomerulus, but also subsequent tubulointerstitial fibrosis [5]. TNF-deficient mice showed delayed development of proteinuria, reduced formation of crescents and influx of PMN, while the deposition of immune complexes in glomeruli was comparable to that found in wild type mice [8]. TNFR1-deficient mice also developed less proteinuria and glomerular injury, with fewer renal leukocyte infiltrates at early time points after GN induction. This is associated with a reduced antibody response to nephrotoxic rabbit IgG, demonstrating that TNFR1-mediated effects are critical for the development of GN.

These findings clearly demonstrate that inflammatory stimulation, such as that provided by LPS or TNF in particular, correlates with the initiation of GN and renders mice more susceptible to greater kidney damage at later time points.

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REFERENCES

1. Assmann KJ, Tangelder MM, Lange WP, Schrijver G, Koene RA. Anti-GBM nephritis in the mouse: severe proteinuria in the heterologous phase. *Virchows Arch A Pathol Anat Histopathol* 1985; 406: 285-99.

2. Hehlgans T, Pfeffer K. The intriguing biology of the tumour necrosis factor/tumour necrosis factor receptor superfamily: players, rules and the games. *Immunology* 2005; 115: 1-20.

3. Tomosugi NI, Cashman SJ, Hay H, *et al*. Modulation of antibody-mediated glomerular injury in vivo by bacterial lipopolysaccharide, tumor necrosis factor, and IL-1. *J Immunol* 1989; 142: 3083-90.

4. Karkar AM, Koshino Y, Cashman SJ, *et al*. Passive immunization against tumour necrosis factor-alpha (TNF-alpha) and IL-1 beta protects from LPS enhancing glomerular injury in nephrotoxic nephritis in rats. *Clin Exp Immunol* 1992; 90: 312-8.

5. Khan SB, Cook HT, Bhangal G, Smith J, Tam FW, Pusey CD. Antibody blockade of TNF-alpha reduces inflammation and scarring in experimental crescentic glomerulonephritis. *Kidney Int* 2005; 67: 1812-20.

6. Karkar AM, Tam FW, Steinkasserer A, *et al*. Modulation of antibody-mediated glomerular injury in vivo by IL-1ra, soluble IL-1 receptor, and soluble TNF receptor. *Kidney Int* 1995; 48: 1738-46.

7. Karkar AM, Smith J, Pusey CD. Prevention and treatment of experimental crescentic glomerulonephritis by blocking tumour necrosis factor-alpha. *Nephrol Dial Transplant* 2001; 16: 518-24.

8. Vielhauer V, Stavrakis G, Mayadas TN. Renal cell-expressed TNF receptor 2, not receptor 1, is essential for the development of glomerulonephritis. *J Clin Invest* 2005; 115: 1199-209.