Glomerular crescent-related biomarkers in a murine model of chronic graft versus host disease

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Abstract

Background. We examined the alterations in gene expression associated with the development of crescentic glomerulonephritis in murine chronic graft-versus-host disease, a model for human systemic lupus erythematosus.

Methods. The disease was induced in (C57BL/6 × DBA/2) F1 hybrids by injection of DBA/2 lymphocytes leading to deposition of auto-antibodies in the glomeruli, and a lupus type of nephritis morphologically. After extensive crescent formation at week 9 of disease, cDNA microarray analysis was performed and highly expressed genes were evaluated as molecular markers by real-time reverse transcription–polymerase chain reaction (RT–PCR), in situ hybridization, immunohistochemistry and immunoassay of urine proteins.

Results. Six genes, secreted acidic cysteine-rich glycoprotein (Sparc), thymosin beta 10 (Tmsb10), S100 calcium-binding protein A6 (S100a6), annexin A2 (Anxa2), osteopontin (OPN) and lipocalin 2 (Lcn2), were quantified by real-time RT–PCR in laser microdissected glomeruli in a time course manner. Sparc was detected early before the onset of proteinuria and continued to increase throughout the course of the disease. The expression of Tmsb10, S100a6 and Anxa2 coincided with heavy proteinuria. By week 9, OPN and Lcn2 were highly expressed. The expression of proteins encoded by these genes was predominant in the glomerular crescent. The protein levels of Sparc, OPN and Lcn2 in urine were significantly elevated.

Conclusions. These findings implicate these six genes in the development of glomerular crescents. More importantly, detection of Sparc, OPN and Lcn2 in urine may mean that these molecules could serve as important biomarkers for non-invasive diagnosis of glomerular crescents.

Keywords: Anxa2; crescentic lupus nephritis; laser microdissection; S100a6; Sparc; Tmsb10

Introduction

Crescentic glomerulonephritis is a syndrome associated with severe glomerular injury [1,2]. Regardless of the cause, the classic histological picture of crescentic glomerulonephritis is characterized by the presence of crescents in most of the glomeruli [2]. Crescentic lupus nephritis (CLN) falls under the group of immune complex-mediated diseases and is an extremely progressive form of lupus nephritis [2]. Recently, Aben et al. [3], using a rat model, demonstrated that genes expressed by the kidney, but not by bone marrow-derived cells, underlie the progression to glomerulosclerosis after mesangial injury. This was consistent with the clinical studies using real-time mRNA analysis of human kidney diseases [4,5]. In a preliminary study, we observed that there are extensive crescents associated with either collapsed glomerular tufts or early glomerulosclerosis in a murine chronic graft-versus-host disease model, consistent with CLN. The recently developed cDNA microarray hybridization methodology allows simultaneous monitoring of thousands of genes expressed in renal tissue. This prompted us to use a cursory scanning approach of global gene expression analysis of the cortical renal tissue and isolated glomeruli in this CLN model. Based on this profile, the upregulated genes and their relevance to the evolution of the crescentic lesion were examined further by time course studies.
Materials and methods

Induction of CLN model

We used 7- to 8-week-old (C57BL/6 × DBA/2J) F1 hybrid mice as recipients of DBA/2J donor lymphocytes as described previously [6], with minor modifications. Cell suspensions containing a mixture of donor cells from the thymus, spleen and lymph nodes (originating from the neck, axillary and inguinal regions) were injected intravenously (i.v.) three times at 3- to 4-day intervals. The experimental mice (six for group) were killed in the 3rd, 6th or 9th week after injection, corresponding to a setting of mild, moderate or severe proteinuria after the induction of the disease. Age-matched untreated (C57BL/6 × DBA/2J F1) hybrids were used as normal controls. Renal tissues, blood and urine samples were collected and saved until analysis, as described below.

Clinical and pathological evaluation

The collection and evaluation of blood and urine samples were performed as described previously [7].

Renal tissues were snap-frozen or fixed in 10% buffer formalin for routine histopathology evaluation, immunofluorescence (IF), in situ hybridization (ISH) or immunohistochemistry (IHC).

For histopathology, at least 100 glomeruli of each renal tissue section were examined for crescent formation. Crescentic glomeruli were expressed as a percentage of the total number of evaluated glomeruli.

For IF, the frozen sections were air-dried, fixed in acetone for 10 min at room temperature and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG, IgA, IgM or C3 (Cappel; Organon Teknika, Durham, NC) as described previously [7].

FISH, sense and antisense RNA probes were generated by in vitro transcription (AmpIiScript T7 and SP6 high yield transcription kits, Epicentre, Madison, WI). cDNA for mouse secreted acidic cysteine-rich glycoprotein (Sparc), thymosin beta 10 (Tmbs10), S100 calcium-binding protein A6 (S100ab), annexin A2 (Anxa2), osteopontin (OPN) and lipocalin 2 (Lcn2) was generated in the kidney by reverse transcription–polymerase chain reaction (RT–PCR). The primers used for the PCRs are shown in Table 1. The product from the PCR was cloned using a pGEM-T vector system (Promega, WI), and digoxigenin-labelled antisense and sense cRNA probes were synthesized with T7 RNA polymerase (Roche, Indianapolis, IN). Sections were then incubated with anti-digoxigenin antiserum conjugated to alkaline phosphatase, and histochemical detection was performed using NBT/BCIP solution (Roche). The same procedure performed with the sense cRNA probe served as negative control. Semi-quantitative evaluation was modified as described previously [7]. Forty or more glomeruli were examined on each slide and assigned values of staining intensity from 0 to 3+. The total intensity score was calculated for the three major components, including (i) crescent epithelial cells; (ii) podocytes; and (iii) mesangial cells, according to the following equation for each specimen: total intensity score = (% glomeruli intensity negative × 0) + (% glomeruli intensity trace intensity × 0.5) + (% glomeruli 1 + intensity × 1) + (% glomeruli 2 + intensity × 2) + (% glomeruli 3 + intensity × 3). The values ranged from 0 to a maximum of 300.

For IHC, a microwave heating procedure was used as described previously [8], followed by incubating with goat anti-Sparc (R&D, Minneapolis, MN), goat anti-S100ab (Santa Cruz, Santa Cruz, CA), rabbit anti-OPN antibodies (Assay Designs Inc., Ann Arbor, MI) or rabbit anti-Lcn2 (a generous gift from Professor Marit Nilsen-Hamilton, Iowa State University, IA) at 4°C overnight. A horseradish peroxidase-conjugated protein-G (Pierce, Rockford, IL) was then applied to the sections for 1 h. Reaction products were visualized using a colour solution that consisted of AEC (DAKO, Carpinteria, CA) for 2–3 min, and the slides were counterstained lightly by haematoxylin.

A semi-quantitative evaluation for glomerular staining was performed as described above for ISH.

Microarray analysis

Embryonic development cDNA microarray, which contains 15 000 different mouse cDNA clones (http://lgsun.grc.nia.nih.gov/cDNA/15k.html), provided by Biochip R&D Center, Tri-Service General Hospital, Taipei, Taiwan, was used. Total RNA was extracted with Trizol reagents (Life Technologies, Gaithersburg, MD) from the renal cortex of the CLN model at 9 weeks and F1 normal control, respectively, and was then annealed to oligo(dT) and reverse transcribed in the presence of Cy5- and Cy3-labelled dUTP, respectively. After concentration, the two cDNA probes were mixed with a hybridization solution of 1 μl of poly(dA40–60mer) (8 μg/ml), 1 μl of yeast tRNA (4 μg/ml), 10 μl of mouse Cot-1 DNA (1 ng/ml), 1 μl of 50 × Denhardt solution and 6 μl of 20 × SSC. This mixture was applied to the slide and sealed under a coverslip, and then placed in a humidified chamber and incubated at 65°C overnight. The slide was scanned with a GenePix 4000A scanner (Axon Instruments, Union City, CA).

Data normalization and analysis were performed as described previously [9]. The data were imported into Microsoft Excel

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Glomerular isolation by laser microdissection

This was performed for renal tissues from the F1 normal control and the CLN model sacrificed in the 3rd, 6th or 9th week after the induction, using a laser microdissection (LMD) system (Leica Microsystems, Wetzlar, Germany), according to the manufacturer’s instructions. Frozen sections of tissue (10 µm) were cut and mounted on glass slides covered with PEN foil (2.5 µm thick; Leica). For each sample, 150 glomeruli were harvested from at least two consecutive sections. The microdissected specimens were collected in a microcentrifuge cap containing 50 µl of extraction buffer (PicoPure RNA isolation kit; Arcturus, Mountain View, CA) to extract total RNA. The quality of RNA was confirmed by the detection of 18S and 28S bands after agarose-formaldehyde gel electrophoresis, and then subjected to real-time PCR analysis as described below.

RT–PCR and real-time PCR examination

RT–PCR was used to verify altered gene expression detected in the cDNA microarray analysis. For first-strand cDNA synthesis, 1.5 µg of total RNA was used in a single-round reverse transcription reaction, containing 0.9 µl of oligo(dT)12-18 primer, 1.0 mM dNTPs, 1× first-strand buffer, 0.4 mM dithiothreitol, 80 U of RNaseout recombinant RNase inhibitor, and 300 U of superscript II RNase H (Invitrogen, Carlsbad, CA). PCR was performed by using 0.9 µl of the reverse transcription reaction mixture as a template, 0.4 µM of gene-specific primers, 1× PCR buffer, 0.25 mM dNTPs and 1.5 U of KlenTaq DNA polymerase (Ab Peptides Inc., St. Louis, MO). The amplification was carried out at 94°C for 2 min, then for 25 cycles at 94°C for 45 s, 55°C for 1 min, and 72°C for 45 s, followed by a final extension at 72°C for 10 min. GAPDH and each target gene primer were added to the same reaction tube. The PCR products were electrophoretically separated on a 2% agarose gel and stained with ethidium bromide. The densitometry analysis was performed using a gel documentation system (Bio-CAPT; Marne-la-Vallée, France). The values were expressed as ratios of the target gene to the internal control GAPDH. The primers used for the PCRs were given in Table 1.

Real-time PCRs were using 10 µl of cDNA, 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems) and 1.25 µl of the specific probe/primer mixed in a total volume of 25 µl. The thermal cycle conditions were as follows: 1× 2 min 50°C, 1× 10 min 95°C, 40 cycles denaturation (15 s, 95°C) and combined annealing/extension (1 min, 60°C). Amplifications were normalized to GAPDH using the 2−ΔΔCT method (Applied Biosystems).

Western blot analysis

Each urine sample was run on a 10% SDS-polyacrylamide gel. The gel was electroblotted onto a nitrocellulose membrane, incubated for 2 h in 20 ml of blocking buffer (5% skim milk), and incubated with goat anti-Sparc (R&D), goat anti-S100α6 (Santa Cruz), goat anti-Anxa2 (Santa Cruz), rabbit anti-OPN (Assay Designs Inc.) or rabbit anti-Lcn2 (a generous gift from Professor Marit Nilsen-Hamilton, Iowa State University, Iowa City, IA) antibodies at 4°C overnight. Blots were washed three times and incubated with horseradish peroxidase-conjugated rabbit anti-goat or goat anti-rabbit antibodies (Pierce) for 1 h at room temperature. Membranes were washed three times, and the membrane-bound antibody detected was incubated with chemiluminescent reagent plus (PerkinElmer Life Sciences, Boston, MA) and captured on X-ray film. Data were presented as the ratio of the density of each target protein to the creatinine concentration of urine.

Data analysis

Values were presented as the mean±SE. Individual experimental group means of data were compared with controls using the Student t-test. A P-value of <0.05 was considered statistically significant.

Results

Clinical and pathological evaluation of the CLN model

The mice developed an increasing proteinuria (Figure 1a) that was detectable at week 3 after the induction of the CLN model, and it progressively increased, reaching a plateau (~60 mg/ml) by week 6, and remained at this high level until week 9, when the animals were sacrificed. Haematuria was identified in a few of the mice at 6 and 9 weeks, respectively. Concurrently, progressive impairment of renal function was observed by elevation of serum creatinine levels (Figure 1b).

Pathologically, although there were no discernible changes (Figure 2a and b) until week 6 when the animals showed thickening of glomerular capillary walls (Figure 2c), the glomerular lesion further transformed to extensive crescent formation (Figure 2d) in most of the glomeruli examined at week 9. Up to 80% of the crescents were circumferential and the remainder were segmental. The major histological type of the crescent was cellular, and only a few of the crescents were fibrocellular. Glomerulonephrosis and scattered fibrin thrombi in some of the affected glomeruli were also identified.

spreadsheets for analysis. The average of median ratios from replicates was calculated for each spot. Spots representing housekeeping genes were used to normalize the entire slide so that all slides could be compared directly. Finally, the ratios were taken as log2 transformation, and the SD of the mean was then calculated from these log2 ratios. We chose 1.5 SD as our cut-off for the determination of expression outliers.
Importantly, although only a small number of the glomeruli (7.0±3.1%) did show crescent formation at week 6, dramatically increased crescents were observed at week 9 (80.4±11.0%).

As illustrated in Figure 2e–l, IF showed IgG and C3 deposition in a granular pattern along the glomerular capillary wall, starting early at week 3 (Figure 2f and j), with greatly increased fluorescence at week 6 (Figure 2g and k) and week 9 (Figure 2h and l), after the induction of the CLN model. Glomerular IgA and IgM deposits were also identified in a similar pattern, with a much lower fluorescence intensity.

**Gene expression in the renal cortex**

To determine the profile of altered gene expression associated with CLN, cDNA microarray chip analysis was performed on cortical renal tissue of the CLN model at week 9 and the F1 normal control (data not shown). In comparison with normal or experimental groups showing glomerular lesions at 9 weeks, when florid crescent formation was noted, enhanced expression of all 11 genes that are related to injury, inflammation and cell–matrix interaction including Sparc, Tmsb10, S100a6, Anxa2, OPN, Lcn2, Col3a1, Mglap, C3, B2m and Lyzs was identified (Figure 3).
Glomerular gene expression

To determine whether the upregulated gene profile in the cortical tissue was associated with crescent formation, time course gene expression analysis by quantitative real-time PCR was performed on glomeruli isolated by LMD. As shown in Figure 4, there was a significant increase in the expression of six genes in the glomeruli of the CLN model, compared with normal controls ($P < 0.05$). The genes affected were *Sparc*, *Tmsb10*, *S100a6*, *Anxa2*, *OPN* and *Lcn2*. Expression of *Sparc* was upregulated very early at week 3, followed by that of *Tmsb10*, *S100a6* and *Anxa2* at week 6, and then that of *OPN* and *Lcn2* at week 9 after the induction of the CLN. It is of note that all these genes had the highest mRNA expressions at week 9, when florid crescent formation was fully developed.

Cellular localization of gene expression

We utilized ISH and IHC to identify the cellular source of gene expression in the renal tissues from the time course studies. As shown in Figures 5 and 6, ISH showed a time-dependent increase in mRNA expression for *Sparc*, *Tmsb10*, *S100a6*, *Anxa2*, *OPN* or *Lcn2* in the CLN model. The gene expression was localized mainly in the glomeruli, although it was also observed focally in the epithelial cells of some renal tubules. It was at week 9, when florid crescent formation was present, that the experimental mice showed the most extensive and intensive mRNA expression *in situ* for all these genes, compared with those of F1 normal controls. Examination of glomerular resident cells revealed that the expression of individual genes was limited mainly to the epithelial cells of the crescents, with only focal and minor intensity in podocytes or mesangial cells. The *Sparc* gene was the only exception, showing significant early expression (week 3) predominantly in the podocytes (at this point, no glomerular crescent had developed). During the late phase (week 9) when florid crescent formation was present, *Sparc* expression was diffuse throughout the epithelial cells of the crescents.

IHC was used to confirm the glomerular expression of proteins encoded by *Sparc*, *S100a6*, *Anxa2*, *OPN* and *Lcn2*. As shown in Figures 7 and 8, the protein patterns were similar in both distribution and intensity of the staining to those identified by ISH. Compared with F1 normal controls, CLN mice with florid crescent formation at 9 weeks showed the most widespread and enhanced expression of all these proteins in their glomeruli. Because no specific antibody is available for mouse Tmsb10, we tried to use an anti-human Tmsb10 for the IHC, which again showed the expression of this protein mainly in the crescent of the glomerulus of the CLN model at week 9 (data not shown).
Detection of Sparc, Lcn2 and OPN in urine

A major objective of our studies was to identify whether gene products associated with crescent formation are excreted in the urine. As illustrated in Figure 9, western blot analysis of the urine levels of Sparc, OPN and Lcn2 were significantly increased in the CLN model in a time-dependent manner, compared with F1 normal controls. Meanwhile, although both OPN and Lcn2 were undetectable, Sparc showed a trace amount in the urine early in week 3. Although proteinuria was significant at week 3 after disease induction, it was at week 9, when florid crescent formation was present, that the urine samples showed the highest levels of the proteins, compared with those from F1 normal controls. There were only traces or no detectable amounts of S100a6 and Anxa2 in urine for both the CLN model and F1 normal controls, and no significant difference between them was noted.

Discussion

In this study, we used a cursory approach of global gene expression to identify differential gene expression associated with the development of glomerular crescents in a lupus nephritis model. Capitalizing on the approach of Eikmans et al. [4] of real-time mRNA analysis of kidney tissues in a time course study, we observed that Sparc, Tmsb10, S100a6, Anxa2, OPN and Lcn2 were differentially upregulated in the affected glomeruli. Monitoring the urine levels of Sparc, OPN and Lcn2 proteins proved to be important in identifying these proteins as biomarkers associated with the development of the glomerular crescents in the CLN model. To our knowledge, this is the first demonstration of altered co-expression of Sparc, Tmsb10, S100a6, Anxa2, OPN and Lcn2, and the development of the lupus nephritis model.

Although our findings are constrained by the lack of an evident interconnecting mechanism among the identified genes that might contribute to pathogenesis, the pathophysiological potential of each gene needs to be addressed. Accordingly, the Sparc gene might play an important role in both the development and progression of glomerular injury as represented by crescent formation and its potential progression to sclerosis. In the CLN model, Sparc early expression was limited to the podocytes, and in the later phase of crescent formation was localized in the epithelial cells of the crescent. Active proliferation of parietal epithelial cells has been considered as a major feature of crescent formation. Most crescents progress to sclerosis with time [2]. Sparc exerts its ability to modulate the interactions between cells and extracellular matrix proteins [10]. It is known to inhibit endothelial cell adhesion [11], and has been shown to inhibit proliferation in a variety of cell types, including
fibroblasts [12]. In our data, it is uncertain whether the glomerular crescent itself expressed a high level of Sparc to control the excessive proliferation of the parietal cells or it may represent an interaction between the epithelial cells and extracellular matrix that transforms the crescent into a progressive sclerosis in the CLN model.

McCready et al. [13] isolated a cDNA clone encoding Tmsb10 from a human kidney. Although Abiko and Sekino [14] showed a close relationship between the
altered T-cell function in patients with lupus nephritis and β-thymosins (i.e. Tmsb4, Tmsb8 and Tmsb9), our data are the first to demonstrate the expression of Tmsb10 in the glomerular crescents of the CLN model.

\[\text{Lcn2} \] and \[\text{OPN} \] [16], injury and inflammation-related genes, were overexpressed in the CLN model. \[\text{Lcn2} \] was identified in primary cultures of mouse kidneys that were induced to proliferate [17], and its encoded protein was markedly expressed in the proximal tubules of early ischaemic mouse kidney [15]. On the other hand, OPN protein was detected in the glomerulus of the remnant kidney model [16], and in the glomerular crescents of a crescentic glomerulonephritis model in rats [18] and human crescentic glomerulonephritis [19]. Although enhanced expression of OPN by renal tubule epithelial cells was observed in a lupus nephritis model of MRL/\textit{pr} mice [20] and in patients with lupus nephritis [21], our findings are the first to demonstrate the expression of OPN in the epithelial cells of the glomerular crescents of experimental CLN. It is well known that glomerular crescents are a composite of proliferative epithelial cells, monocytes/macrophages and lymphocytes [22]. Although both OPN and Lcn2 appeared in the late stage of the CLN model, the expression of both proteins might reflect the influence of influx of mediators of macrophages and lymphocytes on the epithelial cells in the crescent.

\[\text{S100a6} \] is reported to play a role in the regulation of renal tubule cell proliferation and regeneration in the recovery process after acute ischaemic injury [23]. The S100a6 product was originally identified as a target protein for \text{Anxa2}, annexin VI and annexin XI [24,25]. As a result, the two proteins S100a6 and Anxa2 could interact cooperatively and contribute to the formation of the glomerular crescent in this CLN model.

It is well known that platelet-derived growth factor (PDGF)-B and PDGF-B receptor have been implicated in the pathogenesis of crescents. In the present study, there was no significant increase of mRNA expression of experimental CLN. It is well known that glomerular crescents are a composite of proliferative epithelial cells, monocytes/macrophages and lymphocytes [22]. Although both OPN and Lcn2 appeared in the late stage of the CLN model, the expression of both proteins might reflect the influence of influx of mediators of macrophages and lymphocytes on the epithelial cells in the crescent.

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of PDGF-B receptor, compared with the normal control, by the cDNA microarray analysis. Because we used whole cortical renal tissue in the microarray analysis, this may have masked the glomerular PDGF-B receptor expression. Besides, since no PDGF-B was originally included in the microchip that we used to perform the microarray analysis, it is uncertain whether this growth factor also plays a role in the CLN model.

In conclusion, our findings from the present study suggest that upregulated expression of Sparc, Tmsb10, S100a6, Anxa2, OPN and Lcn2 is closely associated with the development of glomerular lesions, especially crescent formation, in the CLN model, although the role of tubular cells in chronic renal failure progression in lupus nephritis could be noted. Sparc may play a critical role in both the initiation and progression of glomerular injury. More importantly, Sparc, Lcn2

Fig. 7. Distribution and cellular localization of gene products by IHC during the course of glomerular lesion development. Kidney sections from F1 normal control (week 0) (a–e), week 3 (f–j), week 6 (k–o) and week 9 (p–t) of the CLN model for Sparc (a, f, k and p), S100a6 (b, g, l and q), Anxa2 (c, h, m and r), OPN (d, i, p, n and s) and Lcn2 (e, j, o and t). Positive cells are stained in red. Arrowheads in (f) and (k) indicate podocytes. Arrows in (p), (q), (r), (s) and (t) indicate glomerular crescents. All at 400×.
and OPN in urine might serve as important biomarkers for the non-invasive diagnosis of CLN.

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Conflict of interest statement. None declared.

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