ORIGINAL ARTICLE

Roles of fibrin deposition and protease activated receptor-1 in renal cytokine/chemokine production and inflammatory cell infiltration in rats of different ages

Shupeng Lin, Xuefeng Sun, Suozhu Shi, Chunsheng Xi, Quan Hong, Yang Lu, Xiangmei Chen

State Key Lab Kidney Diseases, Department of Nephrology, Chinese PLA General Hospital & Military Medical Postgraduate College, Beijing, China

Received 8th May 2011. Revised 14th June 2011. Published online 7th July 2011.

Summary

The present study aimed to investigate the roles of fibrin deposition and protease activated receptor-1 (PAR-1) in renal cytokine/chemokine production and inflammatory cell infiltration in rats of different ages. Acute inflammation was induced by lipopolysaccharide (LPS) in rats which were then treated with tranexamic acid (TA), TA+urokinase (UK) or TA+low-molecular-weight heparin (HP). Fibrin deposition, inflammatory cells and expressions of PAR-1, monocyte chemoattractant protein-1 (MCP-1) and intercellular adhesion molecule 1 (ICAM-1) were detected. A reduction in fibrin deposition and PAR-1 expression in the LPS+TA+HP group was associated with decreased infiltration of inflammatory cells and down-regulated expressions of MCP-1 and ICAM-1. In the LPS+TA+UK group, the fibrin deposition, but not the PAR-1 expression, was reduced, However, the infiltration of inflammatory cells decreased and the expressions of inflammatory cells and expressions of PAR-1, MCP-1 and ICAM-1 between young and old rats undergoing the same treatment. These findings demonstrated that fibrin deposition plays more important roles than PAR-1 dose in cytokine/chemokine production and inflammatory cell infiltration of inflammatory cells than PAR-1 dose in cytokine/chemokine production of cytokines/chemokines and infiltration of inflammatory cells.

Key words: coagulation; fibrin deposition; protease activated receptor-1; ageing; kidney

INTRODUCTION

Studies of acute and chronic inflammatory diseases in humans have shown that a local increase in

- Xiangmei Chen, State Key Lab Kidney Diseases, Chinese PLA General Hospital & Military Medical Postgraduate College, 28 Fuxing Road, Beijing 100853, China
- □ xmchen301@126.com
- ***** +8610-6693-5462
- +8610-6813-0297

© Journal of Applied Biomedicine

procoagulants and fibrin deposition is important in the pathogenesis of inflammatory injury (Furie and Furie 1988, Neale et al. 1988, Levi and van der Poll 2010). This has also been confirmed in a variety of glomerulonephritis (Neale et al. 1988, Cunningham et al. 2004, Hertig and Rondeau 2004). Recent studies reveal a close association between the coagulation system and inflammation and immune responses. Interestingly, it has been shown that blocking the coagulation in the experimental glomerulitis can attenuate the glomerular injury and local inflammation, which indicates a pivotal cross-talk between the coagulation and local inflammation (Welty-Wolf et al. 2001, Miller et al. 2002).

Epidemiology shows an association between the hypercoagulation state and the increased incidence of thromboembolism in the elderly, which suggests age-related changes in the vascular and hemostatic systems. For example, some risk factors for thromboembolism including fibrinogen, factor VII and factor VIII were increased in the plasma of subjects who are ≥ 60 years (Balleisen et al. 1985, Aillaud et al. 1986). The fibrin deposition in the glomerule was reported to increase in lipopolysaccharide (LPS)-treated aged mice (Yamamoto et al. 2002). In addition, elderly individuals are more susceptible to inflammatory stimuli than the young, and aged rats demonstrate more serious inflammatory injury and an increased mortality following endotoxin administration as compared to young rats (Carthew et al. 1991). However, whether an ageing-related hypercoagulation state enhances the susceptibility to inflammatory stimuli in elderly animals is as yet unclear.

Recent studies suggest that thrombin is a physiological mediator of inflammatory events. Administration of recombinant hirudin, a highly specific thrombin antagonist, reduces the pathology and leukocyte infiltration in a mouse glomerulonephritis model (Cunningham et al. 2000). Hirudin and its analogs also prevent the occurrence of inflammation and ameliorate the inflammation in the carrageenin induced inflammation model (Cirino et al. 1996) and in mouse arthritis models (Varisco et al. 2000, Marty et al. 2001). These findings strongly suggest that thrombin plays an important role in the immunity and inflammation. Thrombin is a serine protease that cleaves fibrinogen to form fibrin monomers and uniquely cleaves cell surface receptors, known as PARs. Evidence has demonstrated that thrombin plays critical roles in the regulation of inflammation in two ways: the biological activities of fibrin and thrombin induced activation of the protease activated receptor-1 (PAR-1).

PAR-1 is constitutively expressed in the glomerular endothelial cells, mesangial cells and epithelial cells and in the endothelial cells of the interstitial renal vasculature. The *in vitro* PAR-1 activation by thrombin or TRAP (a thrombin receptor activating peptide) results in the production of pro-inflammatory mediators, including IL-8 (Ueno et al. 1996), E-selectin, platelet-derived growth factor (Shankar et al. 1994), intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) (Grandaliano et al. 1994).

Thrombin may also influence inflammation through stimulating the fibrin deposition. Fibrin is a ligand for ICAM-1 (Languino et al. 1993), CD11b/CD18 (CR3, Mac-1) (Diamond and Springer 1993) and CD11c/CD18 (CR4, p150/95) (Nham 1999).

Thus, extravascular fibrin may act as a provisional adhesion matrix for leukocyte accumulation at sites of inflammation. Moreover, fibrin can disrupt the organization of endothelial cells and increase the vascular permeability (Dang et al. 1985). *In vitro* studies confirm that fibrin directly up-regulates the expressions of ICAM-1 and MCP-1 in the endothelial cells, and tumour necrosis factor alpha (TNF- α) and IL-1 β expressions in the macrophages (Perez and Roman 1995).

Until recently, few studies have evaluated the effects of fibrin and PAR-1 on inflammation in the elderly. In the present study, LPS was used to induce renal acute inflammation in young and aged rats which were then treated with tranexamic acid (TA) alone, TA+urokinase (UK) or TA+ low-molecular-weight heparin (HP). The expressions of PAR-1 and fibrin and the inflammatory cell infiltration were detected and the changes in the mRNA and protein expressions of MCP-1 and ICAM-1 investigated.

MATERIALS AND METHODS

Animals and Study Design

Young (3-month-old) and aged (28-month-old) female Wistar rats, weighting 220±20 g and 420 ± 30 g, respectively, were purchased from Beijing Experimental Animal Centre and acclimatized in our laboratory vivarium for 7 days before drug administration. All animal procedures were performed according to the Proper Care and Use of Laboratory Animals. The animals were given *ad libitum* access to food and water. Both young and aged rats were randomly divided into five groups: normal control group (NC group, n=8): rats were treated with saline alone (vehicle for LPS); LPS group (n=8): rats were intraperitoneally treated with LPS (14 mg/kg); LPS+TA group (n=8): rats were treated with TA (75 mg/kg) intraperitoneally at 30 min after LPS administration; LPS+TA+HP group (n=8): rats were subcutaneously treated with low-molecular-weight heparin (200 U/kg) at 30 min before LPS administration, and then intraperitoneally with TA (75 mg/kg) at 30 min after LPS administration, and the LPS+TA+UK group (n=8): rats were intraperitoneally treated with TA (75 mg/kg) at 30 min after LPS administration and then intravenously with UK (25,000 U/kg) 30 min later. At 4 h after LPS injection, the rats were sacrificed by an overdose of inhalation anesthesia with ether. Kidney tissues were collected. A fraction of the tissues was immediately frozen in liquid nitrogen for isolation of total RNA and protein extraction, and the remaining tissues were embedded immediately in Optimal Cutting Temperature (OCT) Compound (Miles Scientific, Naperville, USA) and then snap-frozen in liquid nitrogen for immunofluorenscence staining as described below.

Reagents

LPS was purchased from Sigma Chemical Co. (St Louis, USA) and dissolved in 0.9% saline before use. TA Injection and UK Injection were purchased from Dongting Pharmaceuticals Co., Ltd. (Hunan Province, China) and Fengyuan Pharmaceutical Factory (Anhui Province, China), respectively.

The following monoclonal and polyclonal antibodies were used in this study: mouse anti-rat CD11b (Mac-1 α-chain) and WT.5 (BD Pharmigen) which label neutrophils and some myeloid cells; FITC-conjugated fibrin polyclonal antibody (Dako Ltd., Glostrup, Denmark); mouse anti-rat ICAM-1 and PAR-1 monoclonal antibody and goat anti-rat MCP-1 polyclonal antibody were purchased from Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz, USA). Peroxidase-conjugated anti-mouse/goat IgG was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd. (Beijing, China).

Immunofluorescence staining for fibrin and CD11b

Tissue sections frozen in OCT (Miles Laboratories, Elkhart, USA) were cut into serial sections (4 µm in thickness) on a cryostat, and fixed in acetone for 5 min at room temperature. The detection of fibrin was performed using a direct method, with a FITC-conjugated rabbit anti-fibrin antibody. Fluorescent images were obtained with a confocal laser scanning microscope (Bio-Rad MRC1024ES, Bio-Rad Laboratories Inc., Hercules, USA). Fibrin deposition at a minimum of 30 glomeruli per rat was quantitatively evaluated by measuring the intensity of fluorescence in the glomerular areas with LaserPix 4.0 software (Bio-Rad Laboratories Inc., Hercules, USA). For the evaluation of infiltrating neutrophils, CD11b immunofluorescence staining was performed with an indirect method. A minimum of 20 glomeruli was assessed per animal. and results were expressed as cell number per glomerular cross section (c/gcs).

Northern blot for MCP-1, ICAM-1 and PAR-1

Total RNA was extracted from the kidney tissues using TRIzol (GIBCO BRL, Grand Island, USA) according to the manufacturer's directions. RNA (20 μ g) was denatured and electrophoresed through a 1% agarose gel containing formaldehyde and transferred to Hybond[®] N⁺ nylon membranes (Amersham Biosciences, UK) by capillary action. The transferred RNAs were cross-linked to the nylon membrane with an ultraviolet light cross linker. The quality of RNA was assessed by ethidium bromide staining. After transferring, the blots were pre-hybridized at 42 °C for 3 h. Then, membranes were hybridized with each cDNA probe labelled by the random primer method (Boehringer Mannheim Biochemica, Germany) with $\left[\alpha^{-32}P\right]$ -dCTP at 42 °C for 20 h. After hybridization, the blots were washed twice with $2\times$ standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), and then once with $0.1 \times SSC/0.1\%$ SDS at 42 °C for 15 min. The hybridized membranes were exposed at -70 °C for 72 h. Autoradiography films (Kodak, Rochester, USA) were scanned using the UVP-2000 system. For quantitative densitometric measurements of Northern blots, all the signals were normalized by comparison with the signals of 28S RNA.

The primers for MCP-1 were as follows: sense, 5'-ATG CAG GTC TCT GTC ACG-3', antisense, 5'-CTA GTT CTC TGT CAT ACT-3'(94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, 28 cycles, product size: 448 bp). The primers for ICAM-1 were: sense, 5'-GAT GCT GAC CCT GGA GAG CA-3', antisense, 5'-CAG GGA CTT CCC ATC CAC CT-3' (94 °C for 45 s, 55 °C for 30 s, 72 °C for 90 s, 35 cycles, product size: 409 bp). The primers for PAR-1 were: sense, 5'-CCGCAGCGTGTTATT-3', antisense, 5'-CGCAGAGGAGGTAAG-3' (94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, 35 cycles, product size: 389 bp).

Western blot for MCP-1, PAR-1 and ICAM-1

Tissues were homogenized in 1 ml of lysis buffer (20 mM HEPES-KOH, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, digitonin at 500 µl/ml, 0.1 mM phenylmethylsulphonylfluoride, aprotinin at 2 mg/ml, leupeptin at 10 mg/ml, and pepstatin at 5 mg/ml), with a handheld homogenizer. All samples were centrifuged at 10,000 g for 30 min at 4 °C, and the protein concentration in each lysate was determined spectrophotometrically. The proteins extracted were solubilized by boiling in SDS loading buffer. Then, 50 µg of total protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a 0.45 µm-pore nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by the semidry method (Bio-Rad Laboratories, Hercules, USA). The nitrocellulose membranes were blocked in 10 ml of Tris-buffered saline (TBS) buffer (10 mM Tris-HCl, 0.15 M NaCl, 8 mM sodium azide, 0.05% Tween-20) containing 3% bovine serum albumin (BSA) overnight at 4 °C. The membranes were then incubated with the primary antibody, goat

anti-rat MCP-1 polyclonal antibody, mouse anti-rat ICAM-1 and PAR-1 monoclonal antibody (1:200) for 2 h at room temperature. Thereafter, the nitrocellulose membrane was washed three times with TBS containing 3% BSA, and incubated for 90 min at room temperature with peroxidase-conjugated AffiniPure goat anti-mouse IgG (1:2000). After washing with TBS, blots were developed with enhanced chemiluminescent (ECL) reagents. Rabbit polyclonal anti- β -actin antibody (1:100, Santa Cruz Biotechnology) was used as the control for each sample.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Inc., Chicago, USA). Quantitative data were expressed as mean±standard deviation (SD) and analysed by one-way analysis of variance (ANOVA), followed by the LSD post hoc test, for comparisons of the difference among the rats in five groups of the same age group. Student's t-test or analysis of covariance (ANCOVA) was used to compare the difference between two age groups with same treatment. We used the significance level 2α =0.05.

RESULTS

Induction and regulation of glomerular fibrin deposition

Fibrin immunofluorenscence staining was evaluated quantitatively by measuring the intensity of fluorescence in the glomerular areas (Fig. 1). There was almost no fibrin deposition in the glomeruli of young and aged rats in the NC group. LPS could induce the fibrin deposition in the young and aged rats in the LPS group. In addition, LPS+TA increased the fibrin deposition, while LPS+TA+HP and LPS+TA+UK decreased it. The fibrin fluorescence intensity in the aged rats was higher than that in the young ones with the same treatment (statistically significant). There was no significant difference in the fibrin fluorescence intensity between LPS+TA+HP group and LPS+TA+UK group.

Changes of PAR-1 protein and mRNA expressions

PAR-1 protein expressions and quantitative image analysis are shown in Fig. 2A and B, respectively. There was little PAR-1 expression in the glomeruli of normal young rats. In the NC group, the protein expression of PAR-1 in the aged rats was higher than that in the young ones $(0.52\pm0.11 \text{ vs}. 0.25\pm0.03,$ statistically significant). In the LPS group, the protein expression of PAR-1 in the aged rats was higher than that in the young animals $(1.61\pm0.31 \text{ vs}. 0.33\pm0.07,$ statistically significant). In the LPS+TA group, the protein expression of PAR-1 in young (0.42 ± 0.10) and aged (1.62 ± 0.22) rats were slightly higher than that in the LPS group, but without significant difference. When compared with the LPS+TA group, PAR-1 expression in young (0.20 ± 0.06) and aged (1.0 ± 0.21) rats of LPS+TA+HP groups was markedly decreased (statistically significant). However, PAR-1 expression in the young (0.38 ± 0.13) and aged (1.54 ± 0.22) rats of LPS+TA+UK groups was not markedly decreased when compared with that of LPS+TA groups (statistically not significant).

The results from Northern blot are shown in Fig. 2C and D. Densitometric analysis revealed that the changes in the PAR-1 mRNA expression were similar to that of protein expression.

Infiltration of CD11b positive cells in glomeruli is PAR-1 independent, but fibrin dependent

Most of the infiltrating cells were neutrophils in the acute phase of LPS induced inflammation (Naruse et al. 1985). The immunofluorescence staining of CD11b, a marker of neutrophils, can reveal the source of infiltrating cells, and was evaluated by positive cell count (Fig. 3). There were few CD11b positive cells in the glomeruli of young and aged rats of the NC groups. The number of CD11b positive cells of the aged rats in the LPS group was higher than that of the young rats (13.5±3.4 vs. 7.9±2.1 c/gcs, statistically significant). The number of CD11b positive cells was significantly augmented in the glomeruli of the young and the aged rats in the LPS+TA group when compared with that in the LPS group, while the number of CD11b positive cells of aged rats in the LPS+TA group was significantly higher than that of the young ones (21.5±5.3 vs. 14.8±3.6, statistically significant). Compared with the LPS+TA group, the number of CD11b positive cells was significantly reduced in the glomeruli of young and aged animals in the LPS+TA+HP group (7.2±0.8, vs. 17.5±3.9, statistically significant). In the young and aged rats of LPS+TA+UK group, the CD11b positive cells were markedly reduced when compared with those in the LPS+TA group (7.5±2.9, vs. 16.5±3.1, statistically significant). The changes in the number of CD11b positive cells were similar to those in the fibrin deposition in the glomeruli but not in PAR-1 expression. In LPS+TA groups, the fibrin deposition increased, while the expression of PAR-1 was not changed as compared to LPS groups, and the CD11b positive cells still increased. Similarly, in the LPS+TA+UK groups, the fibrin deposition decreased, but PAR-1 expression was similar to that in the LPS+TA groups, and the CD11b positive cells also



Fig. 1. Fibrin immunofluorescence staining. (A) Representative photomicrographs from confocal laser scanning. (\times 400). (B) Immunofluorescence density in the immunofluorescence staining of fibrin. Data were expressed as mean \pm SD.



decreased. The findings indicated that CD11b positive cell infiltration in the glomeruli is PAR-1 independent, but dependent on fibrin.

Changes in the in vivo productions of MCP-1 and ICAM-1 were fibrin dependent, but PAR-1 independent

MCP-1 protein expressions and quantitative image analysis are shown in Fig. 4A and B, respectively. MCP-1 was hardly detectable in the normal glomeruli of the young and the aged rats. The MCP-1 expression in the glomeruli of the aged rats was higher than that of the young rats in the LPS group (statistically significant). Moreover, the MCP-1 expression in the young and aged rats in the LPS+TA group was markedly higher than that in the LPS group, while in the aged rats of the LPS+TA group it was significantly higher than that of the young ones (statistically significant). When compared with the LPS+TA group, the MCP-1 expression of both young and aged rats in the LPS+TA+HP group was markedly decreased, and higher in the aged rats of the LPS+TA+HP group than in the young animals (statistically significant). In the young and aged rats of the LPS+TA+UK group, the MCP-1 expression was significantly reduced as compared to the LPS+TA group (statistically significant). Northern blot for MCP-1 (Fig. 4C and D) revealed that the changes in the MCP-1 mRNA expression were similar to the protein expression of MCP-1.



Fig. 2. **Protein and mRNA expressions of PAR-1.** (*A*) Western blot assay of PAR-1. (*B*) Densitometric analysis of bands in Western blot. (*C*) Northern blot assay of PAR-1. (*D*) Densitometric analysis of bands in Northern blots. Data were expressed as mean±SD. Line 1, 2, 3, 4 and 5 represent young NC group, LPS group, LPS+TA group , LPS+TA+HP group and LPS+TA+UK group, respectively. Line 6, 7, 8, 9 and 10 represent aged NC group, LPS group, LPS+TA group, LPS+TA+HP group, LPS+TA+HP group and LPS+TA+HP group and LPS+TA+HP group and LPS+TA+HP group and LPS+TA+UK group respectively.

The ICAM-1 protein expressions and quantitative image analysis are shown in Fig. 5A and B, respectively. The protein expression of ICAM-1 in the aged animals was higher than that in young animals in the LPS group $(1.03\pm0.31 \text{ vs}.0.21\pm0.08\%,$ statistically significant). Moreover, the ICAM-1 protein expression in young and aged rats in the LPS+TA group was markedly higher than that in the LPS group, and in the aged rats of the LPS+TA group it was dramatically higher than in the young rats $(1.22\pm0.34 \text{ vs. } 1.01\pm0.29\%$, statistically significant). When compared with the LPS+TA group, the ICAM-1 protein expression in the LPS+TA+HP and LPS+TA+UK groups was markedly decreased, and in the aged animals of the LPS+TA+HP and LPS+TA+UK groups it was also higher than in the young ones $(0.77\pm0.24 \text{ vs. } 1.21\pm0.31, 0.81\pm0.23 \text{ vs.} 1.31\pm0.34$, respectively, statistically significant).





Fig. 3. **CD11b immunofluorescence staining.** (*A*) Representative photomicrographs from confocal laser scanning. (\times 400). (*B*) CD11b positive cells in the immunofluorescence staining. Data were expressed as mean \pm SD.

Results of Northern blot are shown in Fig. 5C and D. The changes in the ICAM-1 mRNA expression were similar to those in the protein expression.

In the LPS+TA groups, the fibrin deposition increased, while the PAR-1 expression was not changed as compared to LPS groups, and the expressions of MCP-1 and ICAM-1 still increased. Similarly, in the LPS+TA+UK groups, the fibrin deposition decreased, but the PAR-1 expression remained relatively stable as compared to the LPS+TA groups, and the expressions of MCP-1 and ICAM-1 also decreased. The expressions of MCP-1 and ICAM-1 had a closer relationship with fibrin deposition than PAR-1 did. These findings indicated that fibrin but not PAR-1 may regulate the MCP-1 and ICAM-1 production *in vivo*.

DISCUSSION

Recent studies suggest that thrombin is an important physiological mediator in inflammatory events in two ways: the biological activities of fibrin, and the thrombin induced activation of PAR-1. But the contributions of PAR-1 and fibrin to the inflammation in older subjects are still unknown.

In the present study, LPS was used to induce inflammation in young and old rats which were then treated with TA, HP and UK, with the aim of investigating the *in vivo* pro-inflammatory effects of fibrin and PAR-1. TA increased the fibrin deposition through its antifibrinolytic effects, HP reduced the fibrin deposition by binding anti-thrombin III, and UK reduced the fibrin deposition through its



Fig. 4. **Protein and mRNA expressions of MCP-1.** (*A*) Western blot assay of MCP-1. (*B*) Densitometric analysis of bands in Western blot. (*C*) Northern blot assay of MCP-1. (*D*) Densitometric analysis of bands in Northern blot. Data were expressed as mean±SD. Line 1, 2, 3, 4 and 5 represent young NC group, LPS group, LPS+TA group, LPS+TA+HP group and LPS+TA+UK group, respectively. Line 6, 7, 8, 9 and 10 represent aged NC group, LPS group, LPS+TA group, LPS+TA+HP group and LPS+TA+HP group and LPS+TA+UK group respectively.

profibrinolytic effects. Our data showed there were significant changes in the fibrin deposition and expression of PAR-1 among five different treatment groups, which demonstrated that TA, HP and UK could interfere with the level of glomerular fibrin deposition and expression of PAR-1. The animal model and methods used in this study facilitated the investigation of the pro-inflammatory effects of fibrin and PAR-1 *in vivo*.

A critical characteristic of inflammatory disease is the migration of leukocytes from the circulation across the endothelium, and into the affected tissues. Leukocyte extravasation from the blood into the tissues is a multistep process involving a series of coordinated interactions between leukocytes and endothelial cells. MCP-1 and ICAM-1 are important molecules involved in leukocyte extravasation. In the glomerulus, MCP-1 is mainly detectable in the



Fig. 5. **Protein and mRNA expressions of ICAM-1.** (A) Western blot assay of ICAM-1. (*B*) Densitometric analysis of bands in Western blot. (*C*) Northern blot assay of ICAM-1. (*D*) Densitometric analysis of bands in Northern blot. Data were expressed as mean±SD. Line 1, 2, 3, 4 and 5 represent young NC group, LPS group, LPS+TA group, LPS+TA+HP group and LPS+TA+UK group, respectively. Line 6, 7, 8, 9 and 10 represent aged NC group, LPS group, LPS+TA group, LPS+TA+HP group and LPS+TA+HP group and LPS+TA+UK group respectively.

vascular endothelial cells. MCP-1 is predominantly expressed on the surface of endothelial cells and can interact with their cognate receptors on the specific leukocytes, which triggers the activation of adhesion molecules resulting in tight adhesion (Wada et al. 2001). ICAM-1 mediates the tight adhesion between leukocytes and endothelial cells. It has been reported that ICAM-1 can recognize the bridging ligand fibrin(ogen) (Altieri 1999). To further explore the pro-inflammatory effects of fibrin deposition and PAR-1, we determined the changes in the protein and mRNA expressions of MCP-1 and ICAM-1. Our results showed that, in the LPS+TA groups, the fibrin deposition increased, while the PAR-1 expression was not changed as compared to the LPS groups, and the expressions of MCP-1 and ICAM-1 still increased. Similarly, in the LPS+TA+UK groups, the fibrin deposition decreased, but the PAR-1 expressions remained relatively stable as compared to the LPS+TA groups, and the expressions of MCP-1 and ICAM-1 and ICAM-1 correspondingly decreased. The findings imply that it is fibrin but not PAR-1 that activates the

in vivo productions of chemokine/cytokines including MCP-1 and ICAM-1, which then stimulate the macrophage adhesion.

Szaba and Smiley (2002) investigated roles for thrombin, PAR-1, and fibrinogen in PAR-1 deficient and fibrinogen-deficient mice with peritonitis. Their results demonstrated that thrombin played an important role in stimulating the *in vivo* adhesion of inflammatory peritoneal macrophages, which is PAR-1 independent, but dependent on fibrinogen. They also revealed that thrombin could stimulate the peritoneal accumulation of cytokines and chemokines in a fibrinogen-dependent manner. These findings were consistent with ours.

Of course, there is controversy on the pro-inflammatory effects of fibrin and PAR-1. In the study by Cunningham et al (2000), PAR-1 deficient mice were used to establish a crescentic glomerulonephritis model and results showed significant protection from crescentic glomerulonephritis when compared with wild-type mice: crescent formation, inflammatory cell infiltration and serum creatinine significantly reduced in the PAR-1 deficient mice. The findings suggest that receptor-mediated effects of thrombin rather than its coagulant effects are responsible for the majority of the contributions of thrombin to the renal injury in this model. Of note is the fact that, although the systemic coagulation, platelet count and function were normal in PAR-1 deficient mice, these mice had significantly less fibrin deposition in the glomeruli during development of glomerulonephritis. For this reason, the pro-inflammatory effects of fibrin deposition can not be excluded.

Interestingly, in the young and aged rats receiving the same treatment, there was a significant difference in fibrin deposition between them, suggesting that there was a difference in the response to the same stimuli between young and aged rats. Taken together, the aged rats were more susceptible to the development of fibrin deposition, indicating that ageing accelerates the glomerular fibrin deposition, which was consistent with results in the study of Yamamoto et al (2002). Our data on age differences in rats suggest a similar picture in human subjects. Clinical studies have shown that elderly individuals are susceptible to endotoxin-induced effects than the young, and with increased susceptibility to haemorrhage and intravascular hypercoagulation following endotoxin administration (Horan and Pendleton 1995). In other studies on haematological disease, aged mammals which are healthy are also susceptible to experimental drug-induced marrow hypoplasia or anaemia (Berger 1987a, b). In addition, there was also a significant difference in the

expressions of MCP-1 and ICAM-1 between young and aged rats, suggesting that more fibrin deposition in aged rats facilitates the glomerular inflammatory cell infiltration by up-regulating MCP-1 and ICAM-1 expressions.

In summary, our findings provide substantial evidence that fibrin deposition plays more important roles than PAR-1 dose in the cytokine/chemokine production and inflammatory cell infiltration *in vivo*, and that ageing may promote glomerular fibrin deposition and subsequent inflammatory response. Our results provide novel insights into the associations among coagulation, inflammation and ageing.

ACKNOWLEDGEMENTS

This work was supported by the Major State Basic Research Development Program of China (2007CB507400).

REFERENCES

- Aillaud MF, Pignol F, Alessi MC, Harle JR, Escande M, Mongin M, Juhan-Vague I. Increase in plasma concentration of plasminogen activator inhibitor, fibrinogen, von Willebrand factor, factor VIII: C and in erythrocyte sedimentation rate with age. Thromb Haemost. 55: 330–332, 1986.
- Altieri DC. Regulation of leukocyte-endothelium interaction by fibrinogen. Thromb Haemost. 82: 781–786, 1999.
- Balleisen L, Bailey J, Epping PH, Schulte H, van de Loo J. Epidemiological study on factor VII, factor VIII and fibrinogen in an industrial population. I. Baseline data on the relation to age, gender, bodyweight, smoking, alcohol, pill-using, and menopause. Thromb Haemost. 54: 475–479, 1985.
- Berger J. Age-related sensitivity of rats to induction of anaemia. Fol Haematol. 114: 408–413, 1987a.
- Berger J. Age-associated sensitivity to experimental drug-induced marrow hypoplasia in laboratory rats. Haematologia. 20: 171–178, 1987b.
- Carthew P, Dorman BM, Edwards RE. Increased susceptibility of aged rats to haemorrhage and intravascular hypercoagulation following endotoxin administered in a generalized Shwartzman regime. J Comp Pathol. 105: 323–330, 1991.
- Cirino G, Cicala C, Bucci MR, Sorrentino L, Maraganore JM, Stone SR. Thrombin functions as

an inflammatory mediator through activation of its receptor. J Exp Med. 183: 821–827, 1996.

- Cunningham MA, Rondeau E, Chen X, Coughlin SR, Holdsworth SR, Tipping PG. Protease-activated receptor 1 mediates thrombin-dependent, cell-mediated renal inflammation in crescentic glomerulonephritis. J Exp Med. 191: 455–462, 2000.
- Cunningham MA, Kitching AR, Tipping PG, Holdsworth SR. Fibrin independent proinflammatory effects of tissue factor in experimental crescentic glomerulonephritis. Kidney Int. 66: 647–654, 2004.
- Dang CV, Bell WR, Kaiser D, Wong A. Disorganization of cultured vascular endothelial cell monolayers by fibrinogen fragment D. Science. 227: 1487–1490, 1985.
- Diamond MS, Springer TA. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. J Cell Biol. 120: 545–556, 1993.
- Furie B, Furie BC. The molecular basis of blood coagulation. Cell. 53: 505–518, 1988.
- Grandaliano G, Valente AJ, Abboud HE. A novel biologic activity of thrombin: stimulation of monocyte chemotactic protein production. J Exp Med. 179: 1737–1741, 1994.
- Hertig A, Rondeau E. Role of the coagulation/fibrinolysis system in fibrin- associated glomerular injury. J Am Soc Nephrol. 15: 844–853, 2004.
- Horan MA, Pendleton N. The relationship between aging and disease. Clin Geron. 5: 125–141, 1995.
- Languino LR, Plescia J, Duperray A, Brian AA, Plow EF, Geltosky JE, Altieri DC. Fibrinogen mediates leukocyte adhesion to vascular endothelium through an ICAM-1-dependent pathway. Cell. 73: 1423–1434, 1993.
- Levi M, van der Poll T. Inflammation and coagulation. Crit Care Med. 38 (Suppl. 2): S26–34, 2010.
- Marty I, Peclat V, Kirdaite G, Salvi R, So A, Busso N. Amelioration of collagen-induced arthritis by thrombin inhibition. J Clin Invest. 107: 631–640, 2001.
- Miller DL, Welty-Wolf K, Carraway MS, Ezban M, Ghio A, Suliman H, Piantadosi CA. Extrinsic coagulation blockade attenuates lung injury and proinflammatory cytokine release after intratracheal lipopolysaccharide. Am J Respir Cell Mol Biol. 26: 650–658, 2002.

- Naruse T, Tsuchida A, Ogawa S, Yano S, Maekawa T. Selective glomerular thrombosis in rats induced by combined injections of nephrotoxic antiserum and lipopolysaccharide. J Lab Clin Med. 105: 146–156, 1985.
- Neale TJ, Tipping PG, Carson SG, Holdsworth SR. Participation of cell-mediated immunity in deposition of fibrin in glomerulonephritis. Lancet. 2: 421–424, 1988.
- Nham SU. Characteristics of fibrinogen binding to the domain of CD11c, an alpha subunit of p150,95. Biochem Biophys Res Commun. 264: 630–634, 1999.
- Perez RL, Roman J. Fibrin enhances the expression of IL-1β by human peripheral blood mononuclear cells: implications in pulmonary inflammation. J Immunol. 154: 1879–1887, 1995.
- Shankar R, de la Motte CA, Poptic EJ, DiCorleto PE. Thrombin receptor-activating peptides differentially stimulate platelet-derived growth factor production, monocytic cell adhesion, and E-selectin expression in human umbilical vein endothelial cells. J Biol Chem. 269: 13936–13941, 1994.
- Szaba FM, Smiley ST. Roles for thrombin and fibrin(ogen) in cytokine/chemokine production and macrophage adhesion *in vivo*. Blood. 99: 1053–1059, 2002.
- Ueno A, Murakami K, Yamanouchi K, Watanabe M, Kondo T. Thrombin stimulates production of interleukin-8 in human umbilical vein endothelial cells. Immunology. 88: 76–81, 1996.
- Varisco PA, Peclat V, van Ness K, Bischof-Delaloye A, So A, Busso N. Effect of thrombin inhibition on synovial inflammation in antigen induced arthritis. Ann Rheum Dis. 59: 781–787, 2000.
- Wada T, Yokoyama H, Matsushima K, Kobayashi K. Chemokines in renal diseases. Int Immunopharmacol. 1: 637–645, 2001.
- Welty-Wolf KE, Carraway MS, Miller DL, Ortel TL, Ezban M, Ghio AJ, Idell S, Piantadosi CA. Coagulation blockade prevents sepsis induced respiratory and renal failure in baboons. Am J Respir Crit Care Med. 164: 1988–1996, 2001.
- Yamamoto K, Shimokawa T, Yi H, Isobe K, Kojima T, Loskutoff DJ, Saito H. Aging accelerates endotoxin-induced thrombosis increased responses of plasminogen activator inhibitor-1 and lipopolysaccharide signaling with aging. Am J Pathol. 161: 1805–1814, 2002.