Aging Accelerates Endotoxin-Induced Thrombosis

Increased Responses of Plasminogen Activator Inhibitor-1 and Lipopolysaccharide Signaling with Aging

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Although older subjects are susceptible to thrombosis under septic conditions, the underlying molecular mechanisms have not been fully elucidated. Since elevated plasminogen activator inhibitor-1 (PAI-1) primarily contributes to endotoxin-induced thrombosis, we first compared the induction of PAI-1 by lipopolysaccharide (LPS) between young and aged mice. The higher induction of PAI-1 antigen and mRNA with increased renal glomerular fibrin deposition was observed in LPS-treated aged mice compared to young mice. *In situ* hybridization analysis showed that the aging-associated induction of PAI-1 mRNA by LPS was pronounced in hepatocytes and in renal glomerular cells. The increased magnitude of the response of aged mice to lower doses of LPS was observed in terms of renal glomerular fibrin deposition and PAI-1 mRNA induction in the tissues. Furthermore, older PAI-1 deficient mice treated with LPS developed much less fibrin deposition in kidneys. Importantly, a larger induction of receptor molecules for LPS (eg, CD14 and Toll-like receptor 4) was demonstrated in LPS-treated aged mice as compared with young mice. The enhanced LPS signaling in aged mice was also demonstrated by the marked induction of nuclear factor- κ B in the tissues after endotoxin treatment. As a consequence, increases in an inflammatory cytokine, tumor necrosis factor- α , were pronounced in plasma and tissues of LPS-treated aged mice. These results emphasize the key role played by PAI-1 in aging-associated deterioration in this thrombosis model, and suggest that the hyperresponse of PAI-1 gene to LPS results from the enhanced LPS signaling

and the subsequent inflammatory response in aged mice. (*Am J Pathol* 2002, 161:1805–1814)

The increased incidence of thromboembolism in the elderly suggests that age-related changes occur in the vascular and hemostatic systems, including platelets, coagulation and fibrinolytic proteins, and endothelium. For example, the plasma levels of some major risk factors for thrombotic tendency (ie, fibrinogen and factor VII) have been shown to increase in subjects 60 years of age and older.^{1,2} Recently, the molecular mechanism of the age-associated increase in factor IX was investigated, and the age-responsive regulatory elements were identified in this gene.³ The fibrinolytic system is impaired by aging since a progressive prolongation of the euglobulin lysis time⁴ and an increase in plasminogen activator inhibitor-1 (PAI-1), a principal regulator of fibrinolysis,⁵ have been observed with aging.^{2,6} Clinically, elevated levels of plasma PAI-1 are observed in a variety of thrombotic conditions,⁷ including myocardial infarction,⁸ deep vein thrombosis,⁹ and disseminated intravascular coagulation.¹⁰ The inappropriate expression of PAI-1 in the tissues may underlie the occurrence of local thrombotic events (eg, cerebral infarction and cardiovascular disease), which are frequently observed in the elderly.

Sepsis caused by gram-negative bacteria is frequently associated with thrombotic complications and is characterized histologically by microvascular fibrin deposition in several organs,¹¹ tissue necrosis, and vascular disruption. Endotoxin (lipopolysaccharide; LPS) profoundly alters the fibrinolytic system of both humans¹² and experimental animals,¹³ frequently leading to a procoagulant state. Elderly individuals are susceptible to endotoxin-induced effects than the young,¹⁴ and aged rats demonstrate increased susceptibility to hemorrhage and intravascular hypercoagulation following endotoxin administration.¹⁵ These LPS-

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mediated changes result in an increased mortality of aged rats as compared to young rats.¹⁵ In these studies, a larger increase in PAI-1 activity and a more significant decrease in the total PA activity were demonstrated in plasma of aged rats treated with endotoxin in comparison with young rats.¹⁶ These observations suggest that aged animals may tend to develop thrombosis due to the high anti-fibrinolytic potential in endotoxemia and in inflammatory processes.

We previously reported that fibrin deposition in the tissues of LPS-treated mice correlated with changes in the local expression level of key procoagulant and fibrinolytic genes, including PAI-1.¹⁷ In the current study, we treated young (8 weeks old) and aged (24 months old) mice with LPS, and analyzed renal fibrin deposition in association with the expression of PAI-1 gene. Renal glomerular fibrin deposition and renal PAI-1 gene expression were markedly induced and sustained in LPS-treated aged mice compared with young mice. This increased response of the aged mice to LPS in the PAI-1 induction, together with the observation that little fibrin was detected in LPS-treated PAI-1 deficient mice, suggests that PAI-1 contributes to the observed thrombotic tendency in aged mice of endotoxemia. Finally, we investigated the expression of CD14 and Toll-like receptor 4 (TLR4), two major receptor molecules for LPS, and of transcription factor, nuclear factor- κ B (NF- κ B), in LPS-treated young and aged mice since the response of PAI-1 gene to LPS may be dependent on the LPS recognition and signaling via these molecules. The expression of these molecules were also markedly induced by LPS in aged mice as compared with young mice, suggesting that a larger induction of PAI-1 and subsequent increased fibrin deposition results from the enhanced LPS signaling in these mice.

Materials and Methods

Animals and Experimental Protocols

Male C57BL/6J mice, 8 weeks and 24 months of age, were obtained from SLC Japan and through the National Institute of Aging. Mice were injected intraperitoneally (i.p.) either with 5 μ g of LPS (0.2 mg/kg) (*Escherichia coli* serotype O111:B4; Sigma Chemical Co., St. Louis, MO) in saline (Baxter, Deerfield, IL), or with saline alone. At 2, 4, 8, 16, 24 hours after LPS injection, the mice were sacrificed by overdose inhalation anesthesia with methoxyflurane (Pitman-Moore, Mundelein, MD). The blood was collected into 20 mmol/L EDTA (final concentration), centrifuged at 3000 $\times$ g for 5 minutes, and then the plasma was removed and stored at -70°C . Tissues were rapidly removed by standard dissection techniques, and either minced and immediately frozen in liquid nitrogen for preparation of total RNA and protein extracts, or fixed in chilled (4°C) 4% paraformaldehyde and embedded in paraffin for *in situ* hybridization. In the latter experiments, tissues were rapidly perfused with cold PBS (15 minutes) through the left ventricle into the opened vena cava at an approximate rate of 600 μ l/min using a peristaltic pump

to wash out fibrinogen. The tissues were then perfused with chilled 4% paraformaldehyde for 15 minutes, and then removed surgically, fixed overnight in fresh 4% paraformaldehyde, and embedded in paraffin for fibrin immunohistochemistry. Kidneys and livers also were harvested at 2 and 4 hours after LPS injection and prepared for analysis by quantitative RT-PCR and fibrin immunohistochemistry using the same methods as above. To investigate the dose dependency of the response to LPS in young and aged mice, we injected i.p. 2-month-old and 24-month-old mice with increasing doses of LPS (ie, 0.05, 0.25, 1, and 5 μ g), and analyzed them as above.

In separate experiments, male or female PAI-1 deficient¹⁸ and wild-type mice were obtained from Scripps Clinic Rodent Breeding Colony (these animals were originally provided by Drs. P. Carmeliet and D. Collen, University of Leuven, Belgium). They were injected i.p. with 5 μ g of LPS as above, and 4 hours later, they were sacrificed for subsequent immunohistochemical analysis for fibrin as described below.

Determination of Active PAI and Tumor Necrosis Factor- α Antigen in Mouse Plasma

Active PAI-1 antigen in plasma was determined by using the t-PA binding assay as previously described.¹⁹ Active PAI-1 levels (ng/ml) were calculated from a standard curve constructed by using recombinant mouse PAI-1. Total tumor necrosis factor (TNF)- α antigen in plasma (pg/ml) was measured by ELISA-view kit (BioSource International, Inc., Camarillo, CA).

Quantitative Reverse Transcription-PCR

We have developed a quantitative reverse transcription (RT)-PCR assay to determine the concentration of PAI-1 mRNA in murine tissues, as described previously.¹⁷ Briefly, total tissue RNA was prepared using the Ultraspec RNA Isolation System (Biotech Laboratories, Inc., Houston, TX), and the integrity of the 18S/28S ribosomal RNA was monitored by electrophoresing 10 μ g of total RNA through a 1.2% agarose/formaldehyde gel. The control RNA (cRNA), which contains a pair of primer sequences for PAI-1, was *in vitro* transcribed using the Riboprobe Gemini II (Promega, Madison, WI). Thereafter, 1 μ g of total tissue RNA and a fixed amount of the cRNA, which function as the competitor for PAI-1 mRNA, were combined and reverse transcribed using a Gene Amp RNA PCR kit (Perkin-Elmer/Cetus, Norwalk, CT). The RT mixtures were then amplified using specific primers for PAI-1 in the presence of ^{32}P -end-labeled sense primer (5×10^5 cpm). After PCR amplification of 30 to 32 cycles (95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute), 20- μ l aliquots of the PCR products were electrophoresed on a 1.5–2.5% agarose gel. The appropriate bands corresponding to the cRNA product (438 bp) and PAI-1 mRNA product (540 bp) were excised from the gel and the incorporated radioactivity in each was determined using a scintillation counter. The amounts of PAI-1 mRNA were determined by extrapolation using the cRNA

standard curve. Variations in sample loading were assessed by measuring β -actin mRNA.

A similar method using the cRNA competitor was used for the quantitation of CD14 mRNA levels in murine tissues. The sequences of primers for quantitation of mouse CD14 mRNA were as follows: sense, 5'-CCACCTTAGACCTGTCTGACAATCC-3'; antisense, 5'-CAGCCTGTG-TAACTGAGATCCAGC-3'.²⁰ The band for the cRNA was 132 bp and that for CD14 mRNA was 331 bp. The expression of TLR4 mRNA in murine tissues was similarly analyzed by semiquantitative RT-PCR using primers as follows: sense, 5'-GGTCAAGGAACAGAAGCAGTTCTTG-3'; antisense, 5'-TCAAGGACAAATGAAGATGACGCCAG-3'.²¹ The size of target band for mouse TLR4 mRNA was 540 bp. Each density of the RT-PCR band was measured by densitometry and the relative contents were calculated based on the density of control (0 hour) young liver as 1.0.

Statistical Analysis

All statistical analyses were performed with STATA ver.7 software (STATA Corp., College Station, TX). Comparison of all quantitative RT-PCR results between two age groups (8 weeks vs. 24 months) was performed with the two-sample *t*-test. Welch's method was applied when variance between two-group was unequal. One-way analysis of variance (analysis of variance) was applied to examine the difference among four LPS dosage groups (0.05, 0.25, 1.0, and 5.0 μ g) within same age or sample groups. Considering the issue for multiple comparison, corrected *P* value, the obtained *P* value multiplied by the numbers of testing in the same analysis which may give a conservative estimation, was applied to define the statistical significance in *t*-test. Bonferroni's correction of *P* value for multiple comparison was applied for one-way analysis of variance. *P* < 0.05 was considered statistically significant.

In Situ Hybridization

In situ hybridizations for PAI-1 mRNA were performed using riboprobes as described previously.²² After hybridization, the slides were dehydrated by immersion in a graded alcohol series containing 0.3 mol/L NH_4Ac , and dried. The slides were then coated with NTB2 emulsion (Kodak; 1:2 in water), and exposed in the dark at 4°C for 4 to 8 weeks. The slides were developed for 2 minutes in D19 developer (Kodak), fixed, washed in water, and counterstained with hematoxylin and eosin. No specific hybridization signal could be detected in parallel sections using ³⁵S-labeled sense probes for nonspecific hybridization in each experiment (data not shown).

Fibrin Immunohistochemistry

Immunohistochemical staining was performed using the Histostain-SP Kit (Zymed Laboratories, South San Francisco, CA), as described previously.²² Briefly, the tissue sections were deparaffinized, treated with 2% hydrogen peroxide, and incubated with 10% normal goat serum for

30 minutes. The slides were then incubated with 10 μ g/ml of rabbit anti-mouse fibrinogen/fibrin antibody (a kind gift of Dr. E. Plow, Cleveland Clinic), containing 0.1% goat serum at 4°C overnight, followed by incubation for 1 hour at 25°C. In control experiments, tissues were incubated with preimmune (normal) rabbit IgG instead of the primary antibody. The slides were then washed and treated sequentially with biotinylated goat anti-rabbit IgG (Zymed), streptavidin-peroxidase conjugate (Zymed) and aminoethylcarbazole chromogen containing 0.03% hydrogen peroxide (Zymed). After rinsing in distilled water, the slides were counterstained with Gill's modified hematoxylin, rinsed well with tap water, and mounted in GVA-mount (Zymed). The specificity of the antibody for fibrin in the extensively perfused tissues was indicated by the absence of staining in all tissues from control mice (not shown; 17). Quantitative evaluation of fibrin was achieved by counting the number of fibrin-positive glomeruli in each kidney section (magnification, $\times 400$) in a blinded fashion. The data are shown as an average of three mice in each group.

Immunoblot Analysis for TLR4 and NF- κ B

Resident peritoneal exudative cells of LPS-treated young and aged mice were collected by peritoneal lavage with cold PBS(-). After washing with RPMI (ICN Biomedicals, Aurora, OH)-HEPES (10 mmol/L, pH 7.4), cells were plated and incubated for 2 hours at 37°C in 5% CO_2 . Non-adherent cells were removed by washing the plates with RPMI-HEPES twice and lysed in buffer consisting of 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 50 mmol/L iodoacetamide, 1 mmol/L PMSF, 10 μ g/ml soybean trypsin inhibitor, 2 mmol/L MgCl_2 , and 2 mmol/L CaCl_2 . Thus, whole cell lysates of peritoneal macrophages were collected, and then, TLR4 antigen in the lysates was analyzed by SDS-PAGE and immunoblotting. Briefly, each 2 μ g of the lysates were electrophoresed under non-reduced conditions on a 10% SDS-PAGE and transferred to PDVF membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were soaked in TBS (20 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl) containing 5% nonfat milk and 0.1% Tween-20 for 2 hours at room temperature to block additional protein binding sites, and washed three times (15 minutes/wash) in TBS containing 0.1% Tween-20 (TBS-T). The membranes were then incubated with an MTS510 mAb,²³ a rat antiserum specific for mouse TLR4 associated with MD-2 (1.6 mg/ml, 1:1000 dilution in TBS-T; kindly provided by Drs. S. Akashi and K. Miyake, The University of Tokyo, The Institute of Medical Science, Division of Infectious Genetics, Tokyo, Japan), washed 4 times in TBS-T, and incubated for 2 hours with biotin-labeled goat anti-rat antibody (KPL, Gaithersburg, MD). After three washes in TBS-T, the membranes were incubated for 1 hour at 37°C with peroxidase-labeled streptavidin (0.2 μ g/ml; KPL) and developed with the enhanced chemiluminescent (ECL) detection system (Amersham International, Buckinghamshire, UK) according to manufacturers' instructions.

In separate experiments, the nuclear extracts were prepared from the livers of LPS-treated (2 hours, 4 hours)

Figure 1. PAI-1 antigen in plasma and PAI-1 mRNA in tissues of young and aged mice treated with LPS. Eight-week-old (○) and 24-month-old (●) mice were injected i.p. with either 5 μ g of LPS or with saline alone (0 time point) and plasma and livers were collected at the indicated times. Active PAI-1 antigen levels in plasma (**left panel**) were measured by the t-PA binding assay as described in Materials and Methods. The data are expressed as the mean and SD ($n = 5$) in each category. PAI-1 mRNA levels in livers (**center panel**) and kidneys (**right panel**) were determined by quantitative RT-PCR assay as described in Materials and Methods. The data represent the average and SD ($n = 5$). The corrected P value for comparison between 8-week-old and 24-month-old mice was <0.05 in all time points, except for 0 hours for kidney and 0 and 24 hours for liver.

young and aged mice, as follows. The frozen liver tissues were homogenized in chilled hypotonic buffer (10 mmol/L HEPES pH 7.9, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.5 mmol/L PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin), and pelleted by centrifugation at 12000 rpm for 10 minutes at 4°C. The pellets were resuspended in high-salt buffer (20 mmol/L HEPES pH 7.9, 25% glycerol, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 0.42 mol/L NaCl, 0.5 mmol/L PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin) and the nuclear proteins were extracted by incubation for 30 minutes on ice. The nuclear debris was pelleted by centrifugation at 12,000 rpm for 20 minutes at 4°C. Each 20 μ g of the nuclear proteins was electrophoresed on a 10% SDS-PAGE, and then, the blot was prepared in the same way as above. The expression of NF- κ B in the nuclear proteins was analyzed by using rabbit anti-NF- κ B antibody (Cell Signaling Technology, Inc., Beverly, MA; 1:2000 dilution in TBS-T), which detects p50 and p65 of the mouse NF- κ B. The blot was incubated with this specific antibody, followed by the incubation with biotin-labeled goat anti-rabbit antibody (KPL) and by the detection with ECL as described above.

Results

PAI-1 Antigen and mRNA Levels in LPS-Treated Young and Aged Mice

Figure 1 shows the time course changes in plasma PAI-1 antigen and PAI-1 mRNA in livers and kidneys of young and aged mice after i.p. administration of 5 μ g of LPS. The aged mice had significantly higher active PAI-1 antigen in their plasma in the basal state. For example, 8-week-old mice had 3.66 ± 0.88 ng/ml of PAI-1 antigen in plasma, while 24-month-old mice contained 11.9 ± 3.72 ng/ml, respectively. On LPS treatment, the magnitude of the induction of plasma PAI-1 was much greater in aged mice than young mice (Figure 1, left). Active PAI-1 antigen in plasma of 24-month-old mice continued increasing for at least 8 hours after LPS treatment, and reached levels that were threefold higher than those in

8-week-old mice. A similar difference was observed at the mRNA level in livers, with a four- to fivefold greater induction in PAI-1 mRNA at 8 hours in the 24-month-old mice compared with 8-week-old mice (Figure 1, center). Again, the maximal induction of PAI-1 mRNA in the liver of 24-month-old mice occurred at later time point (ie, at 8 hours after LPS). In kidneys of both groups, the maximal induction of PAI-1 mRNA was observed at 2 hours after LPS, but again, the induction was dramatic in aged mice compared with young mice (Figure 1, right). The differences of PAI-1 expression levels in each time point according to each tissue remained significant even when corrected P values were applied.

Although PAI-1 mRNA was induced severalfold in the heart and lung, the level of induction did not significantly differ in the young versus aged mice (data not shown). Meanwhile, endotoxin caused a two- to threefold increase in t-PA mRNA and a 50 to 60% decrease in u-PA mRNA in kidneys (not shown), in agreement with previous studies.¹⁷ However, the magnitude of these changes in t-PA and u-PA mRNA expression did not significantly differ between young and aged mice. Thus, the clearance of endotoxin-induced glomerular fibrin may be impaired in aged mice due to larger induction of systemic and regional PAI-1 expression.

In Situ Hybridization Analysis of PAI-1 mRNA in Tissues of LPS-Treated Young and Aged Mice

High resolution *in situ* hybridization analysis for PAI-1 mRNA demonstrated that the same cell populations in livers and kidneys responded to endotoxin between young and aged mice, even though the magnitude of induction is much greater in the older animals than in young mice. For example, PAI-1 mRNA was induced in hepatocytes and in sinusoidal endothelial cells in livers of young (Figure 2A) and aged (Figure 2B) mice, but the magnitude of induction was considerably higher in the older animals. Similar results were obtained in the kidneys where hybridization signals for PAI-1 mRNA were markedly increased in glomerular

Figure 2. *In situ* hybridization analysis of PAI-1 mRNA in tissues of LPS-treated young and aged mice. Liver and kidney sections from 8-week-old (**A, C**) and 24-month-old (**B, D**) mice treated with 5 μ g of LPS for 3 hours were analyzed by *in situ* hybridization using 35 S-labeled cRNA probes for PAI-1 mRNA. **A** and **B**: Livers. **Arrowheads** indicate hepatocytes and **arrows** denote endothelial cells. **C** and **D**: Kidneys. G, glomeruli; T, tubules. Magnification, $\times 400$. All slides were exposed for six weeks at 4°C.

cells in aged mice (Figure 2D) compared with those in young mice (Figure 2C).

Renal Glomerular Fibrin Deposition in LPS-Treated Young and Aged Mice

Young (8 weeks) and aged (24 months) mice were injected i.p. with 5 μ g of LPS, and then sacrificed at various times for subsequent analysis. In these experiments, 2 out of 11 of the older mice died within 8 hours after LPS injection. In contrast, none of young mice (0 out of 9) had died at this time, and in fact, they were all still alive 24 hours after endotoxin treatment. This significant difference ($P < 0.05$) in survival suggests that aged mice are more susceptible to endotoxin than young mice. Quantitative evaluation of renal glomerular fibrin deposition in the young and aged mice after LPS administration was achieved by performing fibrin immunohistochemistry and then counting positive glomeruli as described in Materials and Methods (Figure 3). Fibrin deposition at 2 and 4 hours after LPS was observed in 15 to 18% of the glomeruli in kidneys of young mice. In contrast, fibrin deposits were detected in approximately 30% of the glomeruli of aged mice at 2 hours, and by 4 hours, fibrin was detected in more than 50% of their glomeruli. More than 40% of the glomeruli from aged mice remained positive for fibrin at 8 hours after LPS compared to less than 5% in

young mice. The inset shows an example of the fibrin staining. At 8 hours, fibrin was readily demonstrated in glomerular capillaries in the kidneys of aged mice (Figure 3, inset, right). However, almost all of glomerular fibrin present at 2 to 4 hours in young mice had disappeared at this time (Figure 3, inset, left). These results demonstrate that aged mice are more vulnerable to renal thrombosis than young mice in endotoxemia.

LPS Dose Dependency of Glomerular Fibrin Deposition and PAI-1 mRNA Induction

Experiments were performed to investigate the dose-dependency of the response to LPS in terms of renal glomerular fibrin deposition and PAI-1 mRNA induction in livers and kidneys of both young and aged mice. Mice were injected i.p. with increasing doses of LPS, and 4 hours later, the kidneys and livers were removed and analyzed for PAI-1 mRNA by quantitative RT-PCR and for fibrin by immunohistochemistry. In all cases, the older mice showed larger responses to endotoxin than the younger animals in terms of glomerular fibrin deposition and PAI-1 mRNA induction (Figure 4). For example, fibrin deposition was induced at lower doses of LPS in the older mice and in more glomeruli (ie, 50 to 60% in aged mice treated with 1–5 μ g of LPS versus less than 20% in young mice) (Figure 4, left). The

Figure 3. Renal glomerular fibrin deposition in young and aged mice treated with LPS. Quantitative evaluation of glomerular fibrin deposition in young ($\square$, 8 weeks old) and aged ($\bullet$, 24 months old) mice injected with LPS. Mice were injected i.p. with $5 \mu\text{g}$ of LPS and sacrificed at the indicated times. Mice injected with saline alone were sacrificed at 4 hours after injection and served as controls (0 time point) in each group. The kidneys were removed and rapidly perfused, fixed, and then, serial sections were analyzed by immunohistochemistry for fibrin as described in Materials and Methods. Quantitation was achieved by counting the number of glomeruli in each tissue section that were positive for fibrin. The figure shows the average percentage of positive glomeruli and SD ($n = 3$) at each time point. **Inset**, fibrin immunohistochemistry in the kidney of young (**left panel**) and aged (**right panel**) mice at 8 hours after LPS administration (magnification, $\times 100$). Fibrin deposits were detected as red spots (**arrows**).

magnitude of the induction of PAI-1 mRNA in livers (Figure 4, center) and kidneys (Figure 4, right) in each LPS dosage was greater in aged mice than young mice. The differences were statistically significant even when multiple comparisons were considered (not shown).

Glomerular Fibrin Deposition in PAI-1 Deficient Mice Treated with LPS

Young (8 weeks old) and older (32 weeks old) wild-type and PAI-1 deficient mice were injected i.p. with $5 \mu\text{g}$ of LPS and sacrificed 4 hours later for subsequent analysis. Quantitative evaluation of renal glomerular fibrin deposition was achieved by performing fibrin immunohistochemistry, and then counting the number of positive glomeruli as described in Materials and Methods. In young mice, fibrin deposition was observed in 10 to 20% (mean: 15%) of the glomeruli of wild-type mice, but was only detected in 1 to 2% glomeruli from PAI-1 deficient mice (Figure 5, left). In 32-week-old wild-type mice, the number of positive glomeruli increased to 19 to 32% (mean: 25%), but again, only 1 to 2% of the glomeruli from PAI-1 deficient mice were positive, and no fibrin was detected in two of them (Figure 5, right). These results indicate that LPS-induced glomerular fibrin deposition may be dependent on the PAI-1 induction, especially in older mice. Moreover, they suggest that this aging-associated induction in PAI-1 may be a primary contributor to the increased thrombosis observed in aged mice treated with endotoxin.

LPS-Mediated Induction of Molecules for LPS Binding and Signaling

Since the induction of PAI-1 gene may result from the LPS signaling and subsequent inflammatory responses, we then investigated the gene expression of major receptors for LPS (eg, CD14 and TLR4) in the tissues of LPS-treated young and aged mice (Figure 6). Much larger induction of CD14 mRNA was demonstrated in livers and kidneys of aged mice than young mice within 24 hours after LPS administration by using quantitative RT-PCR assay (Figure 6, A and B). Similarly, we revealed that the expression level of TLR4 mRNA in livers and kidneys of LPS (2 hours,

Figure 4. LPS Dose dependency of glomerular fibrin deposition and PAI-1 mRNA induction in young and aged mice. Eight-week-old (**hatched bar**) and 24-month-old (**solid bar**) mice were injected i.p. with the indicated doses of LPS. Livers and kidneys were removed 4 hours later and analyzed for fibrin by immunohistochemistry and for PAI-1 mRNA by quantitative RT-PCR. The percentage of glomerular fibrin deposits were determined (**left panel**), and PAI-1 mRNA in the liver (**center panel**) and kidney (**right panel**) was quantitated as described in Materials and Methods. The data were represented as the mean and SD ($n = 5$) in each category. The comparison with Bonferroni's correction showed that differences in PAI-1 mRNA induction were statistically significant between different LPS dosages and between young and aged in each LPS dose ($P < 0.05$).

Figure 5. Renal glomerular fibrin deposition in wild-type and PAI-1-deficient mice treated with LPS. Quantitative evaluation of LPS-induced glomerular fibrin deposition in young (8 weeks old) wild-type (●) and PAI-1-deficient (○) mice ($n = 8$), or in older (32 weeks old) wild-type (●) and PAI-1-deficient (○) mice ($n = 6$). Mice were injected i.p. with 5 μ g of LPS and sacrificed at 4 hours after injection. The kidneys were removed and rapidly perfused, fixed, and then, serial sections were analyzed by immunohistochemistry for fibrin as described in Materials and Methods. Quantitation was achieved by counting the positive glomeruli for fibrin out of 200 glomeruli in each kidney section. The figure shows the percentage of positive glomeruli for fibrin in each mouse. The **horizontal bars** represent the mean of each group.

4 hours)-treated aged mice was higher than young mice (except kidney, 2 hours) by performing semiquantitative RT-PCR analysis (Figure 6C). Furthermore, the expression of TLR4 antigen with the accessory component MD-2, which associates with TLR4 on the cell surface and may be a link between LPS and TLR4 itself,^{23,24} in peritoneal macrophages obtained from LPS-treated young and aged mice was analyzed by immunoblotting. The expression of TLR4 antigen (~120 kd) associated with MD-2 (~30 kd) were increased by LPS in the lysates of peritoneal macrophages obtained from aged mice, but not in young mice (Figure 6D). Thus, the LPS-mediated induction of molecules for LPS binding and signaling was more pronounced in aged mice as compared with young mice.

As another mechanistic analysis on the LPS signaling *in vivo*, we investigated the expression of transcription factor, NF- κ B, which induces a variety of genes encoding inflammatory mediators, in the tissue obtained from LPS (2 hours, 4 hours)-treated young and aged mice (Figure 7). The expression of NF- κ B, detected as bands of 50 and 65 kd, was more increased by LPS in the liver from aged mice compared with young mice.

TNF- α Antigen and mRNA Levels in LPS-Treated Young and Aged Mice

TNF- α , a principal inflammatory cytokine, is one of the known mediators that regulate coagulant and fibrinolytic gene expression *in vivo*.^{25,26} To investigate whether inflammatory responses are more activated in aged versus

young animals under septic condition, we compared TNF- α antigen levels in plasma and TNF- α mRNA levels in the tissues of young and aged mice treated with endotoxin (Figure 8). The maximal induction of TNF- α antigen in plasma was observed at 2 hours after LPS injection and this induction was considerably higher in aged mice (60-fold) compared with young mice (20-fold) (Figure 8, left). Quantitative RT-PCR analysis revealed that TNF- α mRNA in the liver (Figure 8, center) and kidney (Figure 8, right) was induced maximally at 1 to 2 hours after LPS injection, and again, the level of induction was higher in aged mice than young mice. These results suggest that the LPS signaling and subsequent inflammatory responses are more enhanced in aged mice than young mice.

Discussion

To investigate the possibility that aged animals may tend to develop thrombosis due to the high anti-fibrinolytic potential in endotoxemia, we treated young and aged mice with LPS, and analyzed the expression of PAI-1 in association with renal fibrin deposition. We observed that the expression of PAI-1 was dramatically induced by LPS in plasma and in the tissues of aged mice as compared with young mice (Figure 1). The actual cells induced by LPS to express PAI-1 mRNA in the liver and kidney were similar to those identified in our previous studies (eg, hepatocytes and sinusoidal endothelial cells in the liver,²⁷ glomerular and peritubular endothelial cells in the kidney^{17,22}), and this distribution did not appear to differ between young and aged mice (Figure 2). These results suggest that the greater induction of PAI-1 mRNA may reflect increased sensitivity of the PAI-1 producing cells to endotoxin in aged mice. This hypothesis is supported by the observation that the PAI-1 mRNA response of aged mice to relatively low doses of LPS (0.25 and 1 μ g) in the liver and kidney was strikingly higher than in young mice (Figure 4). We demonstrated increased susceptibility of aged mice to renal glomerular fibrin deposition as well after LPS treatment compared with young mice (Figures 3 and 4). Moreover, the kinetics of glomerular fibrin deposition (max. 2–4 hours) were similar to those for the induction of PAI-1 mRNA in tissues, and for PAI-1 antigen in the plasma of aged mice (Figures 1 and 3). These observations suggest that there is a predisposition to tissue microthrombosis in aged mice under pathological conditions such as sepsis, and that the rapid and sustained induction of PAI-1 gene expression may directly contribute to this phenomenon.

These results raise the possibility that the systemic and local induction of PAI-1 by LPS may promote renal fibrin deposition in sepsis (Figure 4). This hypothesis is supported by a number of studies. For example, Carmeliet et al showed that PAI-1 deficient mice were relatively resistant to LPS-induced thrombosis in the foot pad,¹⁸ and Biemond et al²⁸ and Levi et al²⁹ demonstrated that neutralizing antibodies to PAI-1 prevented thrombosis, thrombus extension, and reocclusion in models of experimental thrombosis. Monoclonal antibodies against PAI-1

Figure 6. CD14 and TLR4 expression in the tissues and/or macrophages from LPS-treated young and aged mice. Eight-week-old (○) and 24-month-old (●) mice were injected i.p. with either 5 μ g of LPS or with saline alone (0 time point), and then the livers and kidneys were removed at the indicated time points. The expression level of CD14 mRNA in livers (A) and kidneys (B) was determined by quantitative RT-PCR assay as described in Materials and Methods. The data are represented as the mean and SD ($n = 5$) in each category. * $P < 0.01$ in 24-month vs. 8-week; ** $P < 0.005$ in 24-month vs. 8-week. The expression of TLR4 mRNA in livers and kidneys of LPS-treated (0 hours, 2 hours, 4 hours) mice was also examined by semiquantitative RT-PCR assay and the results obtained by densitometric analysis were shown in C. Each density of the RT-PCR band was measured by densitometry and the relative contents were calculated based on the density of control (0 hours) young liver as 1.0. Y or hatched bars, young mice (8 weeks old); O or closed bars, old mice (24 months old). The expression of TLR4 antigen associated with MD-2 in the lysates of peritoneal macrophages obtained from LPS-treated (0 hours, 2 hours, 4 hours) young and aged mice, was analyzed by SDS-PAGE and immunoblotting (D). 8w, 8-week-old mice; 24m, 24-month-old mice.

also prevented endotoxin-induced DIC in rabbits.³⁰ Figure 5 shows that there is much less renal fibrin deposition in PAI-1 deficient mice compared with wild-type mice, and that this phenomenon appears to be age-dependent. Thus, it seems likely that the enhanced fibrinolytic potential in PAI-1 deficient mice protects these animals against

LPS-induced fibrin deposition. Based on these observations, we suggest that the age-related elevation in PAI-1 contributes directly to their increased thrombosis in LPS-treated mice.

LPS binding to its major receptor on the cell surface, CD14, triggers a cascade of signaling events ultimately leading to cytokine production and to septic shock.³¹ Expression of human CD14 in transfected murine cells³² and in transgenic mice³³ has been shown to increase sensitivity to LPS. CD14 gene expression itself was dramatically induced in a variety of tissues, including the liver and kidney, by LPS.³⁴ The expression of CD14 in rat cardiac tissues was more increased in aged animals after LPS treatment, suggesting that innate immune response would be augmented with aging.³⁵ Another molecule, TLR4, constitutively expressed by lymphocytes and macrophages, is now identified as a signaling receptor for LPS.^{36,37} The TLR4 gene is mutated in the LPS low responder mouse strain C3H/HeJ²¹ and the LPS-mediated PAI-1 induction was extremely attenuated in this mouse strain.³⁸ Thus, the LPS recognition and signaling via

Figure 7. NF- κ B expression in the livers of LPS-treated young and aged mice. Eight-week-old and 24-month-old mice were injected i.p. with 5 μ g of LPS, and the livers were removed at 2 hours and 4 hours after LPS. The nuclear extracts of livers obtained from non-treated and LPS-treated (2 hours, 4 hours) young and aged mice were prepared, and then, the expression of NF- κ B was analyzed by SDS-PAGE and immunoblotting using anti-NF- κ B antibody. Lanes 1, 3, 5: 8-week-old mice (1, no LPS; 3, LPS 2 hours; 5, LPS 4 hours); lanes 2, 4, 6: 24-month-old mice (2, no LPS; 4, LPS 2 hours; 6, LPS 4 hours). Arrows denote specific bands for NF- κ B antigen (p50 and p65).

Figure 8. TNF- α expression in plasma and tissues of LPS-treated young and aged mice. Eight-week-old (○) and 24-month-old (●) mice were injected i.p. with either 5 μ g of LPS or with saline alone (0 time point), and then at various time points, plasma was collected, and the livers and kidneys were removed. TNF- α antigen levels in plasma (**left panel**) were measured by ELISA assay as described in Materials and Methods. The expression level of TNF- α mRNA in livers (**center panel**) and kidneys (**right panel**) was determined by quantitative RT-PCR assay as described in Materials and Methods. The data are expressed as the mean and SD ($n = 5$) in each category. * $P < 0.05$ in 24-month vs. 8-week.

these molecules would conclusively influence the response of PAI-1 gene to endotoxin. We observed that the magnitude of induction of CD14 and TLR4 mRNA in livers and kidneys was greater in LPS-treated aged mice than young mice (Figure 6). Furthermore, peritoneal macrophages from aged mice expressed large amounts of TLR4 antigen associated with MD-2, which greatly enhances the LPS signaling,²⁴ in response to LPS, but those from young mice did not (Figure 6). The marked increase in the expression of CD14 and TLR4 gene in LPS-treated aged mice could augment the LPS binding and its signaling inside cells. The expression of transcription factor, NF- κ B, was also more induced in LPS-treated aged mice (Figure 7), supporting that older mice have enhanced LPS signaling. As NF- κ B mediates the expression of a number of rapid responsive genes involved in the whole body inflammatory response to injury,³⁹ these changes may contribute to the enhanced activation of the inflammatory cascade in aged mice. In this regard, we detected higher levels of TNF- α in plasma of LPS-treated aged mice versus young mice (Figure 8), suggesting that the LPS signaling was actually enhanced in aged mice. This response of TNF- α may result in the dramatic induction of PAI-1 in aged mice because PAI-1 mRNA was previously shown to be markedly induced by TNF- α *in vivo*.³⁸ Taken together, the greater magnitude of the induction of CD14 and TLR4 gene in LPS-treated aged mice may cause a larger increase in the PAI-1 expression, leading to the enhanced tissue microthrombosis.

In conclusion, we demonstrate increased fibrin deposition in renal glomeruli of aged mice treated with endotoxin as compared with young mice. This thrombotic tendency in aged animals may result from dramatic increases in the expression of PAI-1 gene, and this response of PAI-1 may be attributed to the enhanced LPS signaling by the induction of LPS receptor molecules (eg, CD14 and TLR4) and NF- κ B. The increased response of aged mice to endotoxin, with the subsequent induction of various genes related to inflammation and thrombosis, could accelerate the progression of tissue damage due to impairment of the microcirculation during endotoxemia.

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