Prevention of Renal Fibrin Deposition in Endotoxin-induced DIC through Inhibition of PAI-1

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Key words

PAI-1, sepsis, endotoxemia, monoclonal antibodies, kidney

Summary

Plasminogen activator inhibitor-1 (PAI-1) increases in endotoxemia thus possibly cooperating in altering the hemostatic balance in a prothrombotic direction. The effect of the inhibition of PAI-1 with the monoclonal antibody MA-33B8 was studied systemically and in kidneys in a lapine model of endotoxin-induced disseminated intravascular coagulation (DIC). The increase in plasmatric PAI activity in the control group (n = 9) was inhibited in the MA-33B8 treated rabbits (n = 5). Control rabbits showed renal fibrin deposits, whereas only one of the MA-33B8 rabbits did so. These results were confirmed immunohistochemically in kidneys as PAI-1 immunostaining was seen inside the glomeruli and larger vessels in the control group, whereas MA-33B8 rabbits showed a remarkable decrease, demonstrating that MA-33B8 successfully inhibited PAI-1 in the kidneys as well. Therefore evidence for the important role of PAI-1 in fibrin generation in endotoxin-induced DIC is presented, suggesting that strategies aiming at its reduction can be useful in this pathology.

Introduction

Fibrin clots are lysed into soluble fragments by plasmin, a serin-protease which is generated from the activation of plasminogen by another serin-protease called tissue-plasminogen activator (t-PA) (1). Plasminogen activator inhibitor-1 (PAI-1), from the superfamily of serpins, is the main inhibitor of t-PA (2). Therefore, abnormally raised PAI-1 levels could break the hemostatic balance in a prothrombotic direction (3, 4). The significance of high levels of PAI-1 as a prothrombotic factor has been pointed out in several animal models of venous and arterial thrombosis in which the arrest of the inhibitor activity improved the endogenous thrombolysis and decreased the thrombus extension in the case of venous thrombosis (5–7), and facilitated the reperfusion and reduction of reocclusion in the case of arterial thrombosis (6–8). Although some models have dealt with endotoxemia

(9), there have been no attempts so far to study the effect of the inhibition of PAI activity, which is dramatically increased after a continuous infusion of gram negative endotoxin [lipopolysaccharide (LPS)] at a dose sufficient to induce disseminated intravascular coagulation (DIC) (10–16). MA-33B8 is a PAI-1-neutralizing monoclonal antibody (Mo Ab) raised against human PAI-1 and crossreacting with, as well as exerting a strong neutralizing activity against, rabbit PAI-1 (17). The PAI-1 neutralizing effect of MA-33B8 was shown to be associated with an accelerated conversion to the latent conformation (18, 19). In this study we investigated the effect of MA-33B8 in a well defined model of LPS-induced DIC (14–16), in which the Mo Ab is administered to rabbits receiving a continuous intravenous infusion of *E. coli* LPS. As kidneys are a target organ for the formation of fibrin polymers in the microvasculature in DIC and previous studies have postulated a role for PAI-1 in the renal pathology and also in the generation of fibrin deposits (20, 21), special attention is paid to the effect of treatment with MA-33B8 on PAI-1 activity in the renal microcirculation and its relationship with the formation of renal fibrin deposits.

Materials and Methods

Experimental Model

Male New Zealand white rabbits (weight 2.8–3 kg) were used. Animals were anesthetized by an intramuscular injection of 30 mg/kg ketamin hydrochloride and 0.002 mg/kg xylazine hydrochloride followed by intramuscular boosts of ketamin hydrochloride throughout the experiment. DIC was induced in rabbits by intravenous (i.v.) infusion of 100 µg/kg/h LPS for 6 h in 60 ml (10 ml/h) saline through the marginal ear vein. Four groups were established:

- Saline control group (n = 9): administration of saline through both marginal ear veins.
- LPS control group (n = 9): administration of LPS as described above and administration of phosphate buffered saline (PBS, pH = 7.4) through the opposite marginal ear vein (i.v.).
- MA-33B8 group (n = 5): administration of LPS as described above and administration of MA-33B8 (in PBS) through the opposite marginal ear vein (i.v.) at 10 mg/kg in bolus immediately before the start of the experiment, followed by continuous administration at 3 mg/kg/h for the six hours of the LPS infusion.
- Mo Ab P3-X63Ag8 (n = 2): administration of LPS as described above and administration of the non-specific Mo Ab P3-X63Ag8 (22) through the opposite marginal ear vein (i.v.) following the same pattern of administration as the MA-33B8 group.

All the rabbits included in the experiment were administered a total volume of 145 ml.

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Surviving rabbits were sacrificed 24 h after the start by intravenous injection of 60 mg/kg Nembutal (Abbot Laboratories, USA). Kidneys were extracted from all animals (survivors and non-survivors) for subsequent histological and immunohistochemical studies.

Blood Sampling

Blood samples were withdrawn through a catheter inserted into a femoral artery immediately before LPS infusion, and 2, 4 and 6 h after the start. Blood for platelet counts was collected in tubes with K₃-EDTA. Blood collected in 3.2% citrate was used to determine fibrinogen, factor XII, AT III, protein C, and PAI activity. Blood was kept on ice for no longer than 2 h and platelet-poor plasma obtained by centrifugation at 1600 g for 20 min at 4° C and stored at –70° C until assay. The plasma sample withdrawn at 6 h from one of the LPS control group rabbits was not available.

Laboratory Methods

Platelets were counted in a Counter STKS automatic analyzer (Coulter Corp., Hialeah, FL, USA). The Clauss method (23) was used to measure fibrinogen levels. Factor XII was assayed by a one-stage method using factor XII deficient plasma (Organon Teknika, The Netherlands). AT III (24), protein C (25) and PAI activity (26) were determined by commercially available assays based on chromogenic substrates (Coamatic AT III, Coamatic Protein C, and Coatest PAI, Chromogenix, Stockholm, Sweden). Plasma samples with PAI activity levels exceeding the calibration curve were diluted appropriately in PAI-depleted plasma (included in the kit). An additional experiment was planned to exclude the possibility of a quenching of PAI activity by MA-33B8 *in vitro* after blood withdrawal: an amount of MA-33B8 which was similar to that reached in the rabbit blood was added to a sample of PAI-1-rich plasma. The mixture was kept on ice for a period of time similar to that spent from the blood withdrawal until the centrifugation and freezing of the rabbit samples, and subsequently tested for PAI activity by the same method described above.

Histological Examination

Kidney sections were fixed in formalin, embedded in paraffin, stained with Masson's trichrome and examined for the presence of fibrin microthrombi by a pathologist unaware of the experimental design. Tissue sections were scored according to a score from 0 to 4 as previously described (11). Briefly: 0, no fibrin; 1, partial fibrin deposits in some glomeruli; 2, partial deposits in all glomeruli; 3, large quantities of fibrin in all glomeruli; 4, fibrin thrombi in glomerular capillaries and in non-capillary vessels. Ten fields were examined for each tissue section and the average value was calculated.

Immunohistochemistry

Sections were deparaffinized, rehydrated, and treated with 3% H₂O₂ solution in deionized H₂O for 10 min to inhibit endogenous peroxidase. In order to block the Fc region of the Mo Ab MA-33B8 injected in the MA-33B8-treated group, the samples were treated with the Zymed Histomouse kit (Carlton Court, San Francisco, CA, USA) in order to make sure that only the specific antibody used in the immunohistochemistry is detected. Tissues were blocked with normal rabbit serum [1:20 in tris buffered saline (TBS)] and incubated overnight at 4° C with an anti-PAI-1 Mo Ab (murine Mo Ab against human PAI-1 subclass IgG1a, American Diagnostica, Greenwich, CT, USA) which recognizes the inhibitor both in its free and complexed (to plasminogen activators) forms, at a concentration of 100 µg/ml in TBS. After washing with TBS, sections were incubated for 1 h in 1:200 (in TBS) biotinylated rabbit anti-mouse Fc (Dako, Denmark). Slides were treated with the avidin-biotin-peroxidase complex (ABC, Dako, Denmark) diluted 1:100 in TBS, for 1 h. Peroxidase activity was detected with 3-3'-diaminobenzidine hydrochloride (Sigma, St. Louis, MO, USA) and H₂O₂, with nickel enhancement (27). The time of development was the same for all the samples. Slides were counterstained with Harris' hematoxylin, dehydrated and mounted in DPX (BDH,

Poole, England). Negative controls consisted of the omission of the primary antibody and yielded no immunohistochemical reaction. Biochemical experiments suggested that both antibodies cannot bind simultaneously. This implies that consequent to the *in vivo* interaction between MA-33B8 and PAI-1 a reduced signal in the immunostaining of sections from rabbits treated with MA-33B8, might be observed. Even though a reduced signal in the immunostaining could also be due to an enhanced clearance of PAI-1, this seems unlikely in view of the fast clearance of PAI-1 under normal conditions [half life about seven minutes (10)]. Kidney samples were also submitted to immunohistochemistry using an anti-murine IgG antibody, in order to discard the possibility that the stain was due to MA-33B8.

Data Analysis

Within groups: the Wilcoxon test for paired data was used to compare baseline values of the hemostatic parameters with values at two and six hours.

Between groups: results of the hemostatic parameters at 2 and 6 h were converted to percentages assuming a value of 100% for baseline data, and were expressed as mean ± standard error of the mean (SEM). The Mann-Whitney U test was applied for purposes of group comparison. Differences in mortality rate at 24 h were assessed by Fisher's exact test. Differences in kidney fibrin deposits were determined by the Mann-Whitney U test.

The correlation between PAI levels and renal fibrin deposits was studied by the Spearman rank test.

Results

Hemostatic Parameters

No changes in the saline control group were observed along the experiment (data not shown). Table 1 shows the plasma levels of hemostatic parameters along the experiment in the LPS control group. As expected, there is a significant consumption of platelets, fibrinogen, factor XII and protein C with respect to the baseline values. There is also consumption of AT III although it does not reach statistical significance, and a dramatic and continuous increase in PAI activity is also seen. When comparing the hemostatic parameters between the MA-33B8 group and LPS control group, the factor XII consumption significantly improved at 6 h in the rabbits treated with the specific Mo Ab (103 ± 4 vs 70 ± 7, *p* < 0.01, data expressed as mean ± SEM of the percentage with respect to each group's baseline value), as shown in Fig. 1A. The dramatic increase in plasmatic PAI activity observed in the LPS control group was strongly inhibited in the MA-33B8 group, reaching statistically significant differences in PAI values between the two groups at all time points analyzed (163 ± 11 vs 353 ± 46, *p* < 0.05, 179 ± 16 vs 527 ± 33, *p* < 0.01 and 277 ± 52 vs

Table 1 Values given as mean ± SEM. * *p* < 0.05, ** *p* < 0.01 vs baseline values (Wilcoxon test for paired data)

	Baseline	2 hours	4 hours	6 hours
Platelets (x 10⁹/l)	462 ± 154	239 ± 175 **	144 ± 97 **	84 ± 63 **
Fibrinogen (mg/dl)	334 ± 22	309 ± 16 *	272 ± 20 *	239 ± 30 *
Factor XII (%)	125 ± 17	112 ± 9	89 ± 7 *	79 ± 4 *
AT III (%)	101 ± 5	97 ± 7	97 ± 7	91 ± 10
Protein C (%)	77 ± 7	58 ± 7 *	34 ± 4 *	18 ± 4 *
PAI-1 (U/ml)	29 ± 4	103 ± 28 *	136 ± 49 *	339 ± 42 *

Fig. 1 Factor XII consumption (panel A) in the LPS control and MA-33B8 groups and PAI activity (panel B) in the LPS control, MA-33B8 and P3-X63Ag8 groups 2, 4 and 6 h after the start of the LPS infusion. Results are expressed as the mean $\pm$ SEM of the percentage with respect to each group's baseline value which is assumed to be 100%. * $p < 0.05$, ** $p < 0.01$ with respect to the LPS control group (Mann-Whitney U test)

1146 ± 77 , $p < 0.01$ at 2, 4 and 6 h respectively, data expressed as described above), as shown in Fig. 1B. No quenching of PAI activity *in vitro* could be observed after adding MA-33B8 to a PAI-1 rich plasma sample (not shown), thus confirming that the decrease of PAI activity in the MA-33B8-treated rabbits had happened *in vivo*. There were no differences in platelets, fibrinogen and AT III between the two groups. The results obtained with the rabbits given the Mo Ab P3-X63Ag8 fully correspond to the results of the LPS control group, with a dramatic increase in PAI activity (Fig. 1B) and a similar pattern of consumption of platelets and fibrinogen.

Kidney Fibrin Deposits

No fibrin deposits were observed in the saline control group. Eight out of nine LPS control rabbits showed kidney fibrin deposits (1.9 ± 0.4 , mean $\pm$ SEM of the score). Remarkably, four out of five rabbits given MA-33B8 did not show renal fibrin deposits (0.4 ± 0.4 , data given as above) yielding a statistically significant ($p < 0.05$) difference in scores between both groups (Fig. 2A). The two rabbits given P3-X63Ag8 showed strong renal fibrin deposits (mean 2.7), comparable to those obtained in the LPS control group. In addition, when taking all the LPS administered rabbits included in the experiment, a positive correlation at marginal statistical significance levels (Fig. 2B) was found between the kidney fibrin deposits and the PAI activity at 6 h ($r = 0.51$, $p = 0.051$). Five out of nine rabbits from the LPS control group and all the MA33B8 and P3-X63Ag8 treated

Fig. 2 Kidney fibrin deposits. Panel A: Values of the scores in the LPS control and MA-33B8 groups. The mean $\pm$ SEM of each group is represented. * $p < 0.05$ between groups (Mann-Whitney U test). Panel B: Correlation between kidney fibrin deposits and PAI activity at 6 h (Spearman rank test)

rabbits had died by 24 h. These differences were not statistically significant.

Immunohistochemistry of PAI-1 in Kidneys

In saline control rabbits (Fig. 3A), immunostaining for PAI-1 was found in the epithelial cells of the medullary ducts (arrow), as well as in the muscular wall of arteries and in some endothelial cells. A very remarkable increase in PAI-1 labeling was observed in the medulla of the kidneys from the LPS control rabbits (Fig. 3B), where a very strong labeling could be seen inside blood vessels (arrowheads) and tubules (arrows) as well as in the epithelial cells of the ducts, most of them desquamated. Interestingly, MA-33B8 treated rabbits showed a decrease in the medullar PAI-1 immunostaining with respect to the LPS control group (Fig. 3C), as no stained deposits were seen inside the blood vessels (arrowheads), although some epithelial cells were labeled (arrows). The glomeruli of the saline control rabbits showed a very weak or no PAI-1 immunolabeling (Fig. 4A) in contrast to the strong signal observed in the glomeruli of the LPS control animals (Fig. 4B). Again the MA-33B8 treated group showed a decrease in the glomerular PAI-1 immunostaining (Fig. 4C). Negative controls yielded no immunohistochemical reaction in any case (data not shown).

A

B

C

Fig. 3 PAI-1 immunostaining in the renal medulla ($\times 600$). Panel A: collector ducts in a control rabbit, where a weak signal in the epithelium can be seen (arrow). Panel B: medulla of a LPS control rabbit, where very strongly immunostained deposits inside some blood vessels (arrowheads) and tubules (arrows) can be seen. Panel C: medulla of a MA-33B8 treated rabbit, where, although some epithelial cells are stained (arrow), no labeling can be seen inside the blood vessels (arrowheads) or ducts

Discussion

The effect of an efficient Mo Ab able to inhibit rabbit PAI-1 was studied in a well defined model of LPS-induced DIC. The infusion of MA-33B8, whose capacity of inhibiting rabbit PAI activity had been previously described in *in vitro* experiments (17), drastically attenuated

A

B

C

Fig. 4 PAI-1 immunostaining in the renal cortex ($\times 600$). Panel A: cortex of a control rabbit, where glomeruli show very weak or no immunolabeling. Panel B: cortex of a LPS control rabbit, where many glomeruli show strong immunostaining. Panel C: cortex of a MA-33B8 treated rabbit, where a marked decrease in the glomerular immunostaining can be observed with respect to the LPS control group

the dramatic and sustained increase in the plasmatic PAI activity in LPS-treated animals. Importantly, the observed decrease was specific, as demonstrated by the fact that rabbits given the control Mo Ab P3-X63Ag8 at the same dose did not show a decrease in the PAI pattern when compared with the LPS control rabbits, allowing us to exclude the possibility that the action of MA-33B8 is due to an effect not related

to its antigen-specificity. Some studies had previously described a successful *in vivo* plasmatic PAI activity inhibition by Mo Abs in rabbit (5), canine (6), and rat (7–9, 28) models, although none of them dealt with the amount of LPS used in the present experiment. These studies described an increase in endogenous thrombolysis and inhibition of thrombus growth in venous thrombosis models (5–7), a decrease in thrombus size, a partial restoration of blood flow (8) as well as an improved reperfusion and reduction of reocclusion (6, 7) in arterial thrombosis models and finally a decrease in lung fibrin deposition in an endotoxemia model (9). In the present study, four out of five MA-33B8-treated rabbits did not show renal fibrin deposits whereas eight out of nine LPS control rabbits did. Remarkably, the plasmatic PAI levels in the only rabbit from the MA-33B8-treated group which had kidney fibrin clots were higher than the mean value observed in the others (240% vs 164% at 4 h and 477% vs 227% at 6 h) further substantiating the idea that the beneficial effects seen with MA-33B8 are a consequence of the suppression of PAI activity levels. These observations together with our data of correlation between PAI activity and renal fibrin deposits are also compatible with previous studies in which the plasmatic PAI activity appeared to correlate with the fibrin deposition in the renal microvasculature in models of DIC (11), endotoxemia (21) and lupus nephritis (29) and in lungs of endotoxemic rats (9). Our current data also provide additional evidence to support this theory at a local level as well: when compared with the LPS control rabbits, PAI-1 immunostaining is weaker in the renal microcirculation of the MA-33B8-treated rabbits not showing renal fibrin deposits. PAI-1 immunolabeling appears to be more moderate in renal non-capillary vessels in these rabbits as well, showing that MA-33B8 successfully inhibited PAI-1 not only in the major circulation but also at a local level, in an organ which has been described as a target for LPS to stimulate PAI-1 gene expression (20, 30). These immunohistochemical data are in agreement with previous studies which showed that the increase of PAI-1 mRNA in the kidney correlated with the formation of renal microthrombi (20, 21, 29, 31) as well as with the progression of the renal pathology (32). On the other hand, the immunosignal of PAI-1 in renal epithelial tubular cells had been previously described, although such models did not deal with endotoxemia (33–35). One explanation for the fact that not only endothelium but other types of renal cells synthesize PAI-1 could be that the mononuclear cells present in areas of active disease would release a cytokine which induces PAI-1 expression in a wide variety of renal cells (36). Some studies point to TNF- α (37–39) or TGF- β (20, 30, 40–44) as the cytokines which might have such an effect. MA-33B8, although diminishing the PAI-1 signal in epithelial cells, was less successful than in vessels, which could be due to the fact that the Mo Ab would not have been able to reach these cells as easily as the endothelium.

The importance of PAI-1 as a key factor in the formation of fibrin is also supported by the fact that the Mo Ab treated rabbits which did not have renal fibrin deposits did not show any improvement in the other hemostatic parameters measured except factor XII. A possible explanation for the improvement of factor XII in the MA-33B8 treated group could be that PAI-1 at high concentrations in plasma may be able to inhibit factor XII as previously demonstrated *in vitro* (45, 46). MA-33B8 was not able to improve the high mortality rate reached with our experimental protocol. This is most likely due to the fact that LPS not only disrupts the hemostatic balance but also exerts important proinflammatory effects through the induction of the production of cytokines like tumor necrosis factor- α and interleukin-1, which in turn participate not only in thrombotic but also in proinflammatory reactions which could finally lead to fatal shock (47, 48).

In conclusion, PAI-1 has been successfully inhibited by a Mo Ab in an *in vivo* model of LPS-induced DIC in rabbits. This inhibition has been demonstrated in the circulation as well as locally in kidneys; the levels of the inhibitor in both cases seem to correlate with the severity of the disease as reflected by the appearance of renal fibrin clots. Our study clearly demonstrates the usefulness of strategies aiming at a reduction of the dramatic increase in PAI activity observed in gram negative induced sepsis, in order to suppress hemostatic disturbances leading to DIC.

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