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Fibrin deposition, cell migration, and tissue remodeling are key components in the lesions of inflammatory joint diseases, such as rheumatoid arthritis. The plasminogen activators (PAs), namely, tissue-type PA (t-PA) and urokinase PA, are implicated in these aspects of an inflammatory response, although their precise roles are yet to be defined. We therefore used gene-deficient mice to explore their role in a two-stage arthritis model involving intraarticular methylated BSA injection, followed by systemic IL-1 treatment. We report in this study that both t-PA and urokinase PA are protective for the mild arthritis induced by intraarticular methylated BSA injection alone, since absence of either of them exacerbates the response; following s.c. IL-1 injection, t-PA+/− mice had particularly severe disease. Fibrin deposition appeared to parallel disease severity under the various conditions, suggesting that PA-mediated fibrinolysis may be normally playing a protective role in inflammatory joint disease. The Journal of Immunology, 2001, 167: 1047–1052.

An acute, monoarticular murine arthritis model involves intraarticular injection of methylated BSA (mBSA), followed by s.c. injection of IL-1 into a footpad (10). In addition to its advantages of speed and localization to a specific joint, this model can allow analysis of the possible mode of action of IL-1, an inflammatory mediator implicated in rheumatoid arthritis and other inflammatory conditions (11). However, the mechanism by which IL-1 transforms a mild inflammatory reaction to intraarticular mBSA into an acute destructive arthritis is unclear. We were recently able to use gene-deficient mice and a blocking Ab strategy in this two-stage model to demonstrate that GM-CSF forms an essential part of the proinflammatory action of IL-1 in this model (12).

We have shown before that IL-1 can modulate expression of PAs and PA inhibitors by human cartilage, chondrocytes, and synovial fibroblasts (8, 13–16). We therefore have used t-PA and u-PA gene-deficient mice in the mBSA/IL-1 model to explore further the role of PAs, including in response to inflammatory mediators (8). The availability of PA gene-deficient mice is helping to elucidate whether the PAs have beneficial or deleterious functions in disease (9).

Materials and Methods

Reagents and Abs

mBSA was purchased from Sigma (St. Louis, MO). Human rIL-1β (sp. act., 5 × 10^6 U/mg) was a gift from Amgen (Thousand Oaks, CA). Endotoxin levels were routinely monitored by Limulus lysate tests (CSL, Parkville, Australia), with the minimum detectable level of 0.01 ng/ml.

The rat anti-mouse mAb to Mac-2 (M3/38) was obtained from the American Type Culture Collection (Manassas, VA) and purified on a protein G-Sepharose column. The rabbit anti-mouse fibrinogen serum, which had had nonspecific Ab removed by absorption with plasma from fibrinogen-null mice, was a gift from J. Degen, Division of Development Biology, Children’s Hospital Research Foundation (Cincinnati, OH).

Animals

Mice deficient in t-PA and u-PA had been backcrossed to C57BL/6 for 11 generations and were characterized as previously described (9). Wild-type u-PA+/− and t-PA+/− mice, with the same number of backcrosses, were used as controls. Animals used for all experiments, 8–12 wk old, and weighing between 18 and 25 g, were fed laboratory chow and tap water ad libitum, and housed five to a cage. All experiments were approved by the
Induction of monoarticular arthritis

mBSA/IL-1-induced arthritis in mice was established as described before (10, 12, 17). Briefly, 10 μl mBSA at a concentration of 20 mg/ml in saline was injected into the knee joint. Either 20 μl IL-1β (250 ng) or saline was s.c. administered daily into the left rear footpad for 3 days (days 0 – 2). Contralateral control knee joints received vehicle (saline). As before, mBSA was often administered into both knees, leading to more than one joint being involved (12). Animals were sacrificed at day 7 following mBSA injection.

Histologic assessment of arthritis

Arthritis was assessed by histlogic examination as before (17), but with some modifications (12). Knee joints were exposed by removal of the overlying skin and then excised. They were fixed in periodate-lysine-parafomaldehyde for 4 h and decalcified in 10% EDTA (BDH Chemicals, Victoria, Australia) and 7.5% polyvinylpyrolidone (Sigma) in Tris buffer (pH 6.95) for 7–10 days (18). A transverse cut was made when the bones were fully decalcified and processed to paraffin. Tissue sections were cut at 5 μm onto aminoalkylsilane-coated slides and stained for routine histology with H&E. Five defined pathologic features were graded for severity from 0 (normal) to 5 (severe), according to Staite et al. (10), in a blinded manner as follows and as described before (12, 17).

1. Soft tissue inflammation, assessed in the infrapatellar fat pads, joint capsule, and the area adjacent to the periosteal sheath, was graded according to the extent of cellular infiltrate.

2. Cartilage destruction, assessed in the articular cartilage, was graded according to the extent of cartilage erosion.

3. Synovial hyperplasia, assessed in the synovium, was graded according to the extent of synovial proliferation.

4. Pannus formation, assessed in the periosteal sheath and the area adjacent to the periosteal sheath, was graded according to the extent of fibrovascular tissue.

5. Vascular proliferation, assessed in the synovium, was graded according to the extent of new blood vessel formation.


t-PA$^{-/-}$ or t-PA$^{+/+}$ mice were given an intraarticular injection of mBSA; either saline or IL-1 (250 ng) was given s.c. at day 0 and for the following 2 days. Joint inflammation at day 7 was scored by histologic analysis on a scale of 0 – 5 for the five histopathologic features as described previously (12, 17) (Materials and Methods). Values are presented as mean total scores (± SEM) (maximum total score = 25) obtained from t-PA$^{+/+}$ mice (saline, n = 14 joints of 8 mice; IL-1, n = 19 joints of 12 mice) and from t-PA$^{-/-}$ mice (saline, n = 15 joints of 9 mice; IL-1, n = 17 joints of 10 mice). * p < 0.01, mBSA treatment: t-PA$^{-/-}$ vs t-PA$^{+/+}$. ** p < 0.001, mBSA/IL-1 treatment: t-PA$^{-/-}$ vs t-PA$^{+/+}$. † p < 0.001, mBSA/IL-1 vs mBSA treatment.

FIGURE 1. Enhanced arthritis in response to both mBSA and mBSA/IL-1 in t-PA$^{+/+}$ mice. t-PA$^{+/+}$ or t-PA$^{-/-}$ mice were given an intraarticular injection of mBSA; either saline or IL-1 (250 ng) was given s.c. at day 0 and for the following 2 days. H&E sections of knee joints were obtained at day 7 from animals treated as follows: t-PA$^{+/+}$ (A and B, saline; E and F, IL-1); t-PA$^{-/-}$ (C and D, saline; G and H, IL-1). J, joint space; C, articular cartilage; S, synovium, P, pannus formation. Original magnification, ×100 (left panels) and ×250 (right panels).

FIGURE 2. Enhanced arthritis in response to both mBSA and mBSA/IL-1 in t-PA$^{-/-}$ mice. t-PA$^{+/+}$ or t-PA$^{-/-}$ mice were given an intraarticular injection of mBSA; either saline or IL-1 (250 ng) was given s.c. at day 0 and for the following 2 days. Joint inflammation at day 7 was scored by histologic analysis on a scale of 0 – 5 for the five histopathologic features as described previously (12, 17) (Materials and Methods). Values are presented as mean total scores (± SEM) (maximum total score = 25) obtained from t-PA$^{+/+}$ mice (saline, n = 14 joints of 8 mice; IL-1, n = 19 joints of 12 mice) and from t-PA$^{-/-}$ mice (saline, n = 15 joints of 9 mice; IL-1, n = 17 joints of 10 mice). * p < 0.01, mBSA treatment: t-PA$^{-/-}$ vs t-PA$^{+/+}$. ** p < 0.001, mBSA/IL-1 treatment: t-PA$^{-/-}$ vs t-PA$^{+/+}$. † p < 0.001, mBSA/IL-1 vs mBSA treatment.

FIGURE 3. Enhanced arthritis in response to mBSA in u-PA$^{-/-}$ mice. u-PA$^{+/+}$ or u-PA$^{-/-}$ mice were given an intraarticular injection of mBSA; either saline or IL-1 (250 ng) was given s.c. at day 0 and for the following 2 days. H&E sections of knee joints were obtained at day 7 from animals treated as follows: u-PA$^{+/+}$ (A and B, saline; E and F, IL-1); u-PA$^{-/-}$ (C and D, saline; G and H, IL-1). J, joint space; C, articular cartilage; S, synovium, P, pannus formation. Original magnification, ×100 (left panels) and ×250 (right panels).
Materials and Methods

The mBSA/IL-1 model (10) can be studied both as a host response to an Ag delivered directly to the joint (21) as well as a model for how IL-1 may function as a proinflammatory cytokine in arthritic disease (12). As for C57BL/6 mice (12, 17), t-PA^+/+ mice developed mild arthritis after intraarticular administration of mBSA into the knee joint (Fig. 1, A and B) (total score, 4.3 ± 0.4; n = 14 joints; Fig. 2). This response was characterized by mild inflammation of soft tissue, synovial hyperplasia, and minimal leukocyte infiltration into the joint space (10, 12, 17). This mBSA-induced joint inflammation was more severe in t-PA^−/− mice (Fig. 1, C and D) (total score, 7.2 ± 0.9; n = 15 joints; Fig. 2), involving more extensive synovial lining hyperplasia, exudation of inflammatory
traction and angiogenesis. Joint space exudate was identified as leukocytes scattered discretely or in aggregates in the joint space. Synovitis (synovial hyperplasia) was defined as hyperplasia of the synovium, but did not include pannus formation. Pannus was defined as hypertrophic synovial tissue forming a tight junction with the articular surface. Evaluation of cartilage and bone damage was based on loss of cartilage matrix, disruption and loss of cartilage surface, and the extent and depth of the subchondral bone erosion.

Immunohistochemistry

Fibrinogen deposition was identified in knee joints as in the literature (19, 20). Briefly, paraffin-embedded sections (5 μm) were deparaffinized and incubated with 5% BSA and 20% normal goat serum for 30 min. Slides were overlaid with rabbit anti-mouse fibrinogen serum (diluted 1/1000) for 30 min, and subsequently incubated with biotinylated goat anti-rabbit IgG (Dako, Carpinteria, CA) and peroxidase-conjugated streptavidin (Dako). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide (H₂O₂, 30% w/v; Sigma) in methanol. Peroxidase activity was demonstrated by incubation with 3,3′-diaminobenzidine/tetrahydrochloride (Sigma)-H₂O₂ solution. Slides were counterstained with hematoxylin. Staining specificity was confirmed with nonimmune rabbit serum. Fibrin immunostaining in the knee joints was scored blindly on a scale of 0 (no fibrin deposition at all) to 5 (maximum fibrin staining).

For identification of synovial macrophages, deparaffinized sections were incubated with a specific mAb against Mac-2, and subsequently stained using a peroxidase-conjugated rabbit anti-rat IgG (Dako) for 1 h. Endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol. Peroxidase activity was demonstrated by incubation with 3,3′-diaminobenzidine/tetrahydrochloride (Sigma)-H₂O₂ solution.

Results

t-PA deficiency enhances arthritis induced by both mBSA and mBSA/IL-1

Statistical analysis

Data were analyzed using the Mann-Whitney U two-sample rank test to determine the level of significance between means of groups for histologic scores (12, 17). To evaluate the relationship between variables, the univariate correlation was used. Results are expressed as the mean ± SEM. For each test, p = 0.05 was regarded as statistically significant.

FIGURE 4. Enhanced arthritis in response to mBSA in u-PA^−/− mice. u-PA^+/+ or u-PA^−/− mice were given an intraarticular injection of mBSA; either saline or IL-1 (250 ng) was given s.c. at day 0 and for the following 2 days. Joint inflammation at day 7 was scored by histologic analysis on a scale of 0–5 for the five histopathologic features as described previously (12, 17) (Materials and Methods). Values are presented as mean total scores (±SEM) (maximum total score = 25) obtained from u-PA^−/− mice (saline, n = 14 joints of 8 mice; IL-1, n = 14 joints of 7 mice) and from u-PA^+/+ mice (saline, n = 16 joints; IL-1, n = 17 joints, 9 mice each group). *, p < 0.001, mBSA treatment: u-PA^−/− vs u-PA^+/+. ††, p < 0.005, u-PA^−/−; mBSA/IL-1 vs mBSA treatment. †, p < 0.05, u-PA^−/−; mBSA/IL-1 vs mBSA treatment.

FIGURE 5. Fibrinogen deposition. t-PA^+/+, t-PA^−/−, u-PA^−/−, and u-PA^+/+ mice were given an intraarticular injection of mBSA; either saline or IL-1 (250 ng) was given s.c. at day 0 and for the following 2 days. At day 7, sections were stained with antifibrinogen serum and counterstained with hematoxylin (Materials and Methods), and were taken from animals treated as follows: t-PA^+/+ (A, saline; D, IL-1); t-PA^−/− (B, saline; E, IL-1); u-PA^−/− (C, saline; F, IL-1); u-PA^+/+ (data not shown). J, Joint space; C, articular cartilage; S, synovium; P, pannus formation. Original magnification, ×250.
cells and the presence of basophilic fragments in the joint space (see below), and mild pannus formation.

Subcutaneous IL-1 administration exacerbated the response in t-PA−/− mice (Fig. 1, E and F) (total score, 10.4 ± 0.9; n = 19 joints). By comparison, the arthritis resulting from this protocol was particularly severe in t-PA−/− mice (Fig. 1, G and H) (total score, 16.1 ± 0.9; n = 17 joints; Fig. 2); the histopathologic features were similar to what was noted following mBSA treatment alone in these mice, but more severe again, and included cartilage and subchondral bone damage.

u-PA deficiency enhances arthritis induced by mBSA

Following intraarticular mBSA injection, we observed in u-PA+/+ mice similar histologic features to those noted in t-PA−/− (see above) and C57BL/6 mice (12, 17) (Fig. 3, A and B) (total score, 4.4 ± 0.7; n = 14 joints; Fig. 4). In u-PA-deficient mice, the inflammatory response in the knee joint was significantly increased by comparison (Fig. 3, C and D) (total score, 8 ± 0.6; n = 16 joints; Fig. 4) with features similar to what was seen in t-PA−/− mice treated in like manner (Fig. 1, C and D).

The additional administration of IL-1 again resulted in a significant enhancement of the inflammatory response in the joints injected with mBSA in both wild-type (Fig. 3, E and F) (total score, 9.1 ± 1.1; n = 14 joints; Fig. 4) and u-PA−/− mice (Fig. 3, G and H) (total score, 11.7 ± 0.7; n = 17 joints; Fig. 4). However, in contrast to t-PA−/− and t-PA+/+ mice, the difference in the mean values of the total scores of the joint inflammation induced by mBSA/IL-1 in u-PA−/− mice and their wild-type controls was not statistically significant.

Fibrinogen deposition

Given the potentiation of the joint inflammation in response to both mBSA and IL-1 in t-PA−/− mice, we determined whether there was increased fibrinogen deposition associated with these enhanced responses. Following mBSA injection in t-PA−/− mice, widespread fibrinogen immunoreactivity was observed in the joint space, in the synovial lining and sublining layers, in the inflamed synovial tissue, and on the cartilage surface (Fig. 5B); the corresponding fibrinogen deposition in t-PA+/+ mice was much less (Fig. 5A). The immunoreactivity was scored and the differences were significantly different (Fig. 6A). IL-1 injection enhanced further the intensity of fibrinogen immunoreactivity (Figs. 5, D and E, and 6A). The specificity of the fibrin staining was confirmed by the absence of immunoreactivity when normal rabbit serum was included as a control (19) (data not shown).

Similar observations on fibrinogen immunoreactivity were made in u-PA−/− (Fig. 5, C and F) and u-PA+/+ mice following mBSA and mBSA/IL-1 administration, with the quantitation presented in Fig. 6B.

Analysis of fibrinogen deposition in the joints (pooled data from all of the above groups) according to disease activity, as assessed by histologic score, revealed a strong positive correlation (r = 0.88, p < 0.0001; Fig. 6C).

Macrophage numbers

We previously reported with C57BL/6 mice (12, 17) that increased macrophage numbers in the inflamed joints, as judged by Mac-2 immunohistochemistry, correlated with disease severity in the arthritis model under study. As can be seen in Fig. 7, the extent of Mac-2 immunostaining correlated with the intensity of the joint inflammatory reaction in t-PA−/− (Fig. 7, B and E) and t-PA+/+ (Fig. 7, A and D) mice and also in u-PA−/− (Fig. 7, C and F) and u-PA+/+ mice (data not shown), in response to both mBSA and mBSA/IL-1 treatments.

Discussion

The more severe disease noted above with the t-PA−/− mice in the arthritic response to mBSA is suggestive of a protective role for t-PA. This is the first report providing direct evidence for an involvement of t-PA in the response to an arthritogenic agent. It could be that the particularly severe disease noted in t-PA−/− mice in the added presence of IL-1 is, in fact, due to the loss of a normal protective effect of t-PA in response to the IL-1 administration; however, it should be taken into account when interpreting these findings that before the IL-1 injection, there was already a stronger inflammatory reaction in t-PA−/− mice than in t-PA+/+ mice. This is the first report providing direct evidence for an involvement of t-PA in the response to an arthritogenic agent. It could be that the particularly severe disease noted in t-PA−/− mice in the added presence of IL-1 is, in fact, due to the loss of a normal protective effect of t-PA in response to the IL-1 administration; however, it should be taken into account when interpreting these findings that before the IL-1 injection, there was already a stronger joint reaction to mBSA in the t-PA−/− mice. Recently, using this same model, we were able to show clearly with GM-CSF−/− mice that GM-CSF had a proinflammatory role in the second IL-1-responsive stage, but no obvious one in the first stage, since the inflammatory reaction to intraarticular mBSA was not altered by GM-CSF deficiency (12).

In support of the concept for t-PA being possibly involved in both stages of the model as a fibrinolytic enzyme is the increased fibrinogen deposition in the t-PA−/− mice noted at the two stages. There is evidence that intraarticular fibrin can have deleterious effects (4). Consistent with this notion and our data, it has been shown in two other murine arthritis models that intraarticular fibrin contributes to the maintenance of synovial inflammation and correlates with disease severity (19, 20). However, an analysis of
whether t-PA activity is expressed in the arthritic joints in the mBSA/IL-1 model is needed to confirm this putative local action. Several functions for the deposited fibrin have been proposed, such as impediment of normal synovial nutrition, enhanced vascular permeability and chemotactic activity of fibrinogen degradation products, enhanced cellular adherence and migration on the fibrin matrix, and enhanced fibrin-mediated inflammatory cytokine production by macrophages (Ref. 19 and references therein). In rheumatoid disease, accumulation of extravascular fibrin is a prominent feature in the inflamed joints (4–6). Decreased t-PA activity has been reported in rheumatoid synovia, although the presence of fibrin D-dimers in rheumatoid synovial tissue and fluids is consistent with plasmin activity endeavoring to degrade fibrin clot formation (7). The enhanced exacerbation due to IL-1 in t-PA<sup>−/−</sup> compared with the exacerbation in t-PA<sup>+/+</sup> mice suggests that increased fibrin formation somehow forms part of the response in the inflamed joints to IL-1 in this murine arthritis model; this may also be part of the proinflammatory action of IL-1 in rheumatoid disease. In this connection, IL-1 has been widely shown in vitro to induce procoagulant, i.e., tissue factor, activity including in monocytes/macrophages (22, 23). Very recently, in two different murine arthritis models, increased expression of genes involved in coagulation and fibrinolysis (e.g., those for tissue factor and u-PA) were noted, and it was suggested that the pattern favored procoagulant activity (24). The enhanced arthritis in our model would appear to have some analogy to the enhanced renal injury observed in another monoarticular arthritis model, called Ag-induced model (19), in which the enhanced disease severity in u-PA<sup>−/−</sup> mice correlated with the extent of fibrinogen deposition in the joint. It was concluded in this previous study that u-PA had the major role in fibrin removal; however, this conclusion cannot be drawn from our studies reported above for the response to intraarticular mBSA in t-PA<sup>−/−</sup> and u-PA<sup>−/−</sup> mice since both PAs appear to be implicated. Even though subsequent IL-1 treatment enhanced disease severity in the u-PA<sup>−/−</sup> mice, in contrast to the t-PA<sup>−/−</sup> mice, the severity was not significantly worse than that in the wild-type controls subjected to the same protocol. The interpretation as to whether u-PA has any role in the response to IL-1, protective or otherwise, is difficult to assess on account of the higher preexisting histologic score in the u-PA<sup>−/−</sup> mice following mBSA injection. What can be said is that the disease can still progress to some extent in response to IL-1 even in the absence of u-PA; however, unlike for t-PA, we found no evidence either that its absence contributed to the disease progression due to IL-1.

From the literature, it has been suggested that plasmin-mediated degradation of neighboring tissue can have either beneficial or deleterious consequences depending on the nature of the matrix (2). As in other examples (2, 26), plasmin degradation of fibrin may be advantageous in terms of reducing the inflammatory response. In contrast, excessive plasmin-mediated degradation of other matrix components may be associated with pathologic manifestations (27, 28). In this particular nonsystemic arthritis model, tissue (i.e., cartilage and bone) damage is relatively minor, and a potentially deleterious role for u-PA/plasmin in tissue remodeling (1, 8, 14, 29, 30) may not come into play. It is also possible that the acute nature of the model may have some bearing on the contribution of u-PA.

**FIGURE 7.** Mac-2 expression and arthritis severity. Mice were treated as in Fig. 5 except that sections were stained with mAb to Mac-2 (Materials and Methods); counterstaining was again with hematoxylin. Same nomenclature and magnification as for Fig. 5. t-PA<sup>−/−</sup> (A, saline; D, IL-1); t-PA<sup>+/+</sup> (B, saline; E, IL-1); u-PA<sup>−/−</sup> (C, saline; F, IL-1).
to the pathology. In this connection, u-PA+/− mice showed a relatively defective lung inflammatory response to an infectious insult compared with u-PA++/− control mice only after a delayed period of 14–21 days (31). It would be of interest to explore the role of u-PA in more severe, chronic, and/or systemic arthritis models, such as the collagen-induced model (32).

The above data implicate both t-PA and u-PA as being protective for the mild inflammatory response to intraarticular mBSA and possibly t-PA in the subsequent systemic IL-1 administration. This protection could be through fibrin degradation (see above); however, other mechanisms could be contributing to their effects on the inflammatory response, for example, plasmin-mediated activation or liberation of cytokines, such as latent TGF-β, basic fibroblast growth factor, hepatocyte growth factor, vascular endothelial growth factor, and IL-1β (33, 34). Divergent roles for t-PA and u-PA in fibrinolysis have been postulated (9). The former PA has been implicated in fibrin degradation within the vasculature, whereas u-PA has been assumed to function on cell surfaces to promote lysis of fibrin at extravascular sites (35). However, other observations suggest that endogenous u-PA can contribute to intravascular fibrinolysis (9).

From the data above, there is a suggestive relationship between increased macrophage numbers (Mac-2 expression) and the extent of fibrinogen deposition; in this connection, macrophages are a possible source of tissue factor, which is likely to be a key initiator of the fibrin formation in the model (36). As mentioned, IL-1 induces tissue factor expression in monocytes/macrophages in vitro (23). Additional studies are needed to test whether this mechanism is relevant in the model.

Acknowledgments

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5. Weinberg, J. B., A. M. Pippen, and C. S. Greenberg. 1991. Extravascular fibrinolysis (9). As mentioned, IL-1 induces tissue factor expression in monocytes/macrophages in vitro (23). Additional studies are needed to test whether this mechanism is relevant in the model.

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