Role of Laminin in Matrix Induction of Macrophage Urokinase-type Plasminogen Activator and 92-kDa Metalloproteinase Expression*

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Urokinase-type plasminogen activator (uPA) and 92-kDa matrix metalloproteinase (MMP-9) expression by RAW264.7 macrophages were up-regulated when plated on extracellular matrices. Collagen IV, fibronectin, and tenascin stimulated macrophages' MMP-9 expression. In contrast, laminin stimulated both uPA and MMP-9 expression in a dose- and time-dependent manner. The increase in macrophage uPA activity was preceded by an increase in their steady state levels of uPA mRNA. Laminin-induced uPA expression was most pronounced in RAW264.7 macrophages followed by THP-1 monocytes, J774A.1 macrophages, and bone marrow-derived macrophages. Neither laminin nor matrix induced alterations in THP-1 monocyte expression of plasminogen activator inhibitor (TIMP)-1 or TIMP-2. Synthetic laminin peptides were utilized to identify the laminin domain(s) responsible for induction of uPA expression. Peptides derived from the b1 chain of laminin had no effect on macrophage uPA expression, whereas SIKVAV, derived from a1 chain, stimulated uPA expression 20-fold. Preincubation of THP-1 monocytes with a monoclonal antibody directed against the a6 subunit of the a6b1 laminin receptor blocked matrix induction of uPA without affecting the induction of MMP-9. These results demonstrate that macrophage binding to laminin plays an important role in the regulation of their degradative phenotype via the up-regulation of uPA and MMP-9.

Macrophages utilize serine proteinases, metalloproteinases, and cysteine proteinases to degrade extracellular matrix (1–4). Principal among these proteinases is the serine proteinase uPA1 (5). uPA cleaves the Arg560-Val561 bond of plasminogen, thereby generating plasmin, a serine protease with wide substrate specificities (5). Plasmin binds to and degrades several components of the extracellular matrix including fibronectin, laminin, and proteoglycan core protein (6, 7). Moreover, plasmin activates a family of MMPs (8).

In addition to the cooperative role proteinases have in matrix degradation, they participate in the regulation of several expected biological processes. uPA and plasmin activate hepatocyte growth factor (9) and latent transforming growth factor ß (10), respectively. In addition, plasmin liberates cell- and matrix-bound basic fibroblast growth factor and transforming growth factor ß (11–13). MMPs activate pro-interleukin-1 (14) and release from the cell surface tumor necrosis factor, tumor necrosis factor receptor, colony stimulating factor-1, Fas ligand, and interleukin-6 receptor (15–17). Consequently, cellular regulation of the proteinase cascade has effects beyond matrix degradation.

Macrophages localize and regulate plasminogen activation via their expression of uPA, PAI, uPA receptor, and several binding sites/receptors for plasmin(ogen) (18). The expression of uPA, uPA receptor, and PAI is regulated by a variety of cytokines and growth factors (19–22). Likewise, macrophage MMP expression is highly regulated. Macrophages express interstitial collagenase (MMP-1), 92-kDa gelatinase (MMP-9), 72-kDa gelatinase (MMP-2), and stromelysin (MMP-3) (2, 23). The expression of MMPs and their inhibitor, TIMP, is also regulated by cytokines (24–27). In addition, structural components of the matrix regulate macrophage MMP expression. For example, native and denatured collagen types I and III stimulated MMP-1 expression by alveolar macrophages (28), whereas other matrix components including laminin, fibronectin, and elastin were ineffective (28). In other studies, it was demonstrated that fibronectin, laminin, and entactin stimulate production of 96- and 58-kDa MMPs by murine peritoneal macrophages (29). Finally, the laminin peptide SIKVAV induced human monocyte/macrophage MMP-1 and MMP-9 expression (30). Taken together, these data demonstrate that macrophage-matrix interactions are an important factor in their expression of a degradative phenotype and tissue remodeling.

In experiments reported here, we have determined whether macrophage uPA expression, a pivotal component of the proteinase cascade, is regulated by the extracellular matrix. Results demonstrate that macrophage uPA and MMP-9 expression are up-regulated when cultured on extracellular matrix. The matrix component responsible for the increase in uPA and MMP-9 expression is laminin. Collagen, fibronectin, and tenascin up-regulated MMP-9 expression without affecting uPA expression. The effect of laminin on macrophage uPA expression was mediated by a portion of the long arm of laminin containing the sequence SIKVAV and was blocked by antibody to the a6b1 (VLA-6) integrin.

MATERIALS AND METHODS

Cell Culture—Murine RAW264.7 and J774A.1 macrophages and human THP-1 monocytes were obtained from American Type Tissue Culture (Rockville, MD). RAW264.7 and J774A.1 cells were maintained as adherent cultures in Roswell Park Memorial Medium (RPMI; without HEPES) supplemented with 10% Cellest Gold fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), and 4 mM glutamine (Life Technologies, Inc.). THP-1 monocytes were maintained in suspen-
sion in the same medium. Human bone marrow-derived macrophages were obtained from Dr. Shahin Rafii, Cornell Medical College. When assessed by immunoperoxidase staining, bone marrow-derived macrophages were highly reactive with monoclonal antibodies against the human macrophage markers CD68 (Dako, Carpinteria, CA) (31) and HAM56 (Dako) (32).

Preparation of Matrix-coated Plates—Tissue culture plates (24 well) were coated with Matrigel (Collaborative Biomedical Products, Bedford, MA), a basement membrane matrix derived from Engbreth-Holm-Swarm mouse sarcoma (33). Matrigel was thawed at 4 °C, diluted to 1 mg/ml with macrophase serum-free medium (MSFM) (Life Technologies, Inc.) and aliquoted in 1 ml per well. Plates were incubated at room temperature for 1 h. In other experiments, tissue culture plates were incubated overnight at 4 °C with 10 mg/ml laminin (Life Technologies, Inc.), fibronectin (Life Technologies, Inc.), collagen IV (Calbiochem), or tenascin (Life Technologies, Inc.). The next day, wells were washed with DPBS prior to the addition of cells.

Effect of Matrix and Matrix Components on uPA and MMP Expression—RAW264.7, J774A.1, and bone marrow-derived macrophages were mechanically harvested and collected by centrifugation (400 × g, 10 min). Cells were washed with DPBS (3 ×) and suspended in MSFM supplemented with antibiotics. Cells were aliquoted into wells previously treated with Matrigel or purified matrix components as described above. At the indicated time, media were removed and assayed for uPA and MMP activities.

Effect of Synthetic Laminin Peptides on Macrophage uPA and MMP Expression—Lamin peptides CDPGYIGRS and YIGSR were obtained from Sigma. SIKVAV was purchased from Bio-Synthesis (Lewisville, TX). CDGPYIGR and YIGSR were dissolved in water (1 mg/ml). SIKVAV was suspended in DPBS (5 mg/ml), sonicated, and vortexed until dissolved. Peptides were sterile-filtered and stored at −20 °C. Cells were incubated with MSFM containing 10−50 µg/ml synthetic laminin peptides. The next day media were recovered and assayed for uPA and MMP activity.

Effect of Anti-α6 IgG on Matrix Induction of Macrophage uPA and MMP Expression—Monoclonal anti-α6 (CD49d) IgG was obtained from BioDesign (Kennebunk, ME). Monoclonal anti-FcγRII (CD16) IgG was obtained from Immunotech (Westbrook, ME). Antibodies were dialyzed against DPBS to remove azide and stored at −20 °C. Human THP-1 monocytes were harvested by centrifugation (400 × g, 10 min), washed in DPBS, and resuspended in MSFM containing anti-α6 IgG or anti-FcγRII IgG. Cells were incubated 30 min in a microcentrifuge tube at 37 °C and then transferred to matrix-coated wells. The next day, media were recovered and assayed for uPA and MMP activity.

Determination of Plasminogen Activator Activity—Plasminogen activator activity was quantitated utilizing a previously described modification of a sensitive functional assay for plasmin (34). Aliquots of conditioned media were added to microtiter wells containing 82 µl of DPBS, 0.05% Tween 20 containing 13 µg of the plasmin substrate α-Val-Leu-Lys-ammoniomethylcoumarin (Enzyme Systems Products, Dublin, CA) and 0.5 µg of bovine plasminogen (American Diagnostica, Greenwich, CT). Samples were mixed and incubated at 37 °C for 2.5 h. Cleavage of the substrate was monitored by measuring the increase in fluorescence in a Fluoroscan microplate reader (excitation, 330–380 nm; emission, 430–530 nm). Concentrations of uPA in the test samples were extrapolated from a standard curve utilizing high molecular weight uPA (American Diagnostica). Plasminogen activator activity in macrophage-conditioned media was completely inhibited when preincubated with a polyclonal anti-human uPA IgG (American Diagnostica) as described previously (35).

Northern Blot for uPA, PAI 2, and TIMP-2 mRNA Levels—RNA was isolated from murine RAW264.7 macrophage and human THP-1 monocytes as described previously (35). RNA samples were electrophoresed in agarose, transferred to nylon membrane (Schleicher & Schuell), and hybridized with either α32P-labeled murine cDNA for uPA (36; provided by Dr. J. Degen, Children's Hospital Research, Cincinnati, OH), human cDNA for TIMP-2 (37; ATCC), or human cDNA for PAI2 (38; ATCC). Equal amounts of total RNA were loaded per lane as judged by UV inspection of ethidium bromide-stained agarose gels.

Zymographic Demonstration of Metalloproteinase Activity—Conditioned media were concentrated in an SpeedVac ultracentrifugation chamber with a YM-10 (10-kDa cutoff membrane). SDS sample buffer without mercaptoethanol was added to the media samples and heated for 30 min at 37 °C. Samples and molecular weight markers were electrophoresed in a 10% polyacrylamide gel containing 0.1% gelatin. The gel was then washed (2 ×) in 2.5% Triton X-100 to remove SDS. The gel was incubated at 37 °C for 48 h in 200 µl NaCl containing 40 µl Tris-HCl and 10 µl CaCl2, pH 7.5, and stained with 0.05% Coomassie Blue. The presence of gelatinolytic activity was identified as clear bands on a uniform blue background following destaining.

Western Blot for TIMP-1 Antigens—THP-1 monocyte conditioned media were mixed with sample buffer containing β-mercaptoethanol and immersed in boiling water for 5 min. Recombinant TIMP-1 (Calbiochem) and samples were electrophoresed in 4−15% polyacrylamide gradient gels. Proteins were transferred to nitrocellulose membrane, following which the membrane was blocked in TTBS containing 1% bovine serum albumin for 1 h. Following two washes (TTBS), the membrane was incubated with blocking buffer containing 1 µg/ml monoclonal anti-TIMP-1 IgG (Calbiochem) overnight at 4 °C. The membrane was washed (2 ×, TTBS) and incubated for 1 h with 0.5 µg/ml biotinylated rabbit anti-mouse IgG (Pierce) in blocking buffer at room temperature. The membrane was then washed (2 ×, TTBS) and incubated 1 h with avidin-biotin-horseradish peroxidase complexes (Pierce) in DPBS, 0.5% Tween-20. Bound horseradish peroxidase was detected utilizing enhanced chemiluminescence (Amersham Corp.) as per manufacturer’s instructions.

RESULTS

Macrophase uPA and MMP-9 Expression Is Up-regulated When Plated on Extracellular Matrices—Cellular expression of plasminogen activators and metalloproteinases cooperate in matrix degradation (8, 39). In these experiments, we determined the ability of matrix to regulate macrophase expression of both uPA and metalloproteinases. For these purposes, RAW264.7 macrophages were cultured overnight on either plastic or extracellular matrix-coated (Matrigel) plastic. Conditioned media from cells grown on plastic contained 97 ± 8 milliunits of uPA (Fig. 1). When cultured on matrix, uPA levels in conditioned media increased >6-fold. To determine whether a soluble factor present in the matrix was responsible for the observed increase in uPA activity, matrices were incubated overnight with media alone. The matrix conditioned media were recovered and added to cells grown on plastic. As observed in Fig. 1, uPA expression by cells cultured on plastic was not affected when incubated with matrix-conditioned media. Likewise, matrix induction of macrophase uPA expression was not affected when cells were incubated with matrix-conditioned media. These data demonstrate that an insoluble portion of the matrix was responsible for the observed matrix induction of RAW264.7 macrophase uPA expression.

Metalloproteinase activity in macrophase-conditioned media was determined utilizing SDS-polyacrylamide gel electrophoresis zymography. The metalloproteinases degrade gelatin incor-
porated into a polyacrylamide gel. The degraded areas appear unstained following staining with Coomassie Blue. Conditioned media from cells grown on plastic contained metalloproteinase activity which based on molecular weight appears to be the 92-kDa gelatinase or MMP-9 (Fig. 2). When cultured on matrix-coated plastic, MMP-9 levels in macrophage-conditioned media increased severalfold.

Laminin Up-regulates Macrophage uPA and MMP-9 Expression—A series of experiments were initiated to identify the component(s) of the matrix that were responsible for the observed increase in RAW264.7 macrophage uPA and MMP-9 activities. As seen in Fig. 3, insoluble or soluble collagen IV, fibronectin, and tenascin had little or no effect on uPA expression. In contrast, uPA expression by cells cultured on laminin-coated plastic was increased 5-fold over controls. When laminin was added to cells in the fluid phase, uPA expression was increased >100-fold.

We next compared the effect of laminin on uPA expression by other murine and human monocyte/macrophage cells. Levels of constitutively expressed uPA in conditioned media derived from the control cells varied widely (3–68 milliunits) (Table I). Following an overnight incubation with laminin, uPA levels in conditioned media from all macrophages examined were increased. The increase in uPA activity in conditioned media of macrophages incubated with laminin was most pronounced in murine RAW264.7 macrophages (313-fold) followed by human macrophages incubated with laminin. The next day media were recovered and assayed for uPA activity. The data represent the mean ± S.E. from three separate wells.

![Figure 2](image)

**Figure 2. Extracellular matrix up-regulates macrophage 92-kDa MMP expression.** RAW264.7 cells were suspended in MSFM and aliquoted into untreated 12-well plates (5.0 × 10⁵/well) or matrix-coated plates. The conditioned media were collected 24 h later and concentrated 5.1 by ultrafiltration as described under "Materials and Methods." SDS sample buffer was added to aliquots of concentrated conditioned media, and samples were electrophoresed in composite polyacrylamide-gelatin gels. MMP activity was identified by zymography as described under "Materials and Methods."

![Figure 3](image)

**Figure 3. Effect of individual matrix components on macrophage uPA expression.** RAW264.7 cells were suspended in RPMI, 10% FBS and aliquoted into 12-well plates (5.0 × 10⁵/well). Following 2 h adherence, cells were washed and media changed to MSFM alone or MSFM containing 10 µg/ml collagen type IV, fibronectin, laminin, and tenascin. In addition, cells were cultured in wells previously coated with the individual matrix components as described under "Materials and Methods." Conditioned media were recovered the next day and assayed for uPA activity. The data represent the mean ± S.E. from three separate wells.

| Matrix Component | uPA (milliunits/well) |
|------------------|-----------------------|
| Control          | 23                    |
| Laminin (5 µg/ml) | 7202                  |

**Table I. Effect of laminin on monocyte/macrophage uPA expression**

RAW264.7, J774.1A, and bone marrow-derived macrophages were suspended in RPMI, 10% FBS, aliquoted into 24-well plates (2.5 × 10⁵ cells/well), and allowed to adhere 2 h at 37 °C. Cells were washed with DPBS, and media were replaced with MSFM or MSFM containing laminin. The next day media were recovered and assayed for uPA as described under "Materials and Methods."

| Monocyte/macrophage | Control | Laminin (5 µg/ml) |
|---------------------|---------|-------------------|
| Murine              | 23      | 7202              |
| J774.1A             | 68      | 1202              |
| Human               | 3       | 122               |

RAW264.7 macrophage metalloproteinase expression. RAW264.7 cells were incubated overnight with 0.1–10 µg/ml (0.11–11.1 nM) laminin. As seen in Fig. 6, control cell media expressed small amounts of MMP-9 activity. Following incubation with laminin, the expression of MMP-9 was increased in a dose-dependent manner. Laminin-induced MMP-9 activity was visible at 4 h and increased over the remainder of the experimental period (20 h) (Fig. 6). In contrast to selective effect of laminin on macrophage uPA expression, all matrix components examined stimulated MMP-9 expression by RAW264.7 cells (data not shown). Taken together, these data suggest that when cells were plated on basement membrane-derived extracellular matrix, laminin up-regulates macrophage uPA and MMP-9 expression, whereas several matrix components up-regulate the expression of MMP-9. These findings are significant since the expression of both uPA and MMP-9 have been demonstrated to play a role in cellular migration through matrix (40–43).

**Macrophage Expression of TIMP-1, TIMP-2, and PAI-1 Are Unaffected by Laminin—** The effect of laminin on macrophage expression of the protease inhibitors TIMP-1, TIMP-2, and PAI-1 was determined utilizing Northern and Western blots. Incubation of THP-1 monocytes with laminin for 24 h had no effect on the steady state levels of the 3.5- and 1.0-kilobase TIMP-2 mRNA transcripts (44; data not shown). Likewise, no
Matrix Regulation of Macrophage uPA and MMP Expression

Incubation of RAW264.7 macrophages with 10–50 µg/ml laminin for the indicated periods. Recovered media were assayed for uPA activity. Cells were incubated with 5.0 µg/ml laminin (Lmn) for the indicated periods. Data represent the mean ± S.E. of three separate wells.

Change in TIMP-2 mRNA levels were observed when THP-1 monocytes were incubated on Matrigel for 2, 6, or 24 h. Levels of TIMP-1 protein (28 kDa) expressed by THP-1 monocytes incubated with either laminin or Matrigel for 24 h were unchanged as compared with cells incubated on plastic.

PAI2 is the predominant PAI isoform expressed by macrophages (20). Incubation of human THP-1 monocytes with laminin for 24 h had no effect on the steady state levels of the 2-kilobase PAI2 mRNA transcript (data not shown). Likewise, no change in PAI2 mRNA levels were observed when THP-1 monocytes were incubated on Matrigel for 2, 6, and 24 h. Therefore, it appears that purified laminin as well as intact matrix selectively induce macrophage uPA and MMP expression.

Laminin SIKVAV Peptide Up-regulates Macrophage uPA Expression—We have utilized synthetic laminin peptides to identify the laminin domain(s) responsible for the induction of macrophage uPA expression. CDPGYIGSR and YIGSR are derived from the short arm of the β1 chain of laminin (45) and are reported to promote cell attachment (46, 47). SIKVAV is derived from the long arm of laminin’s α1 chain (45). It is reported to promote adhesion, cell spreading, and stimulate tumor cell and monocyte MMP expression (30, 48). As seen in Table II, incubation of RAW264.7 macrophages with 10–50 µg/ml CDPGYIGSR and YIGSR had no effect on uPA expression. In contrast to peptides derived from the short arm of laminin, incubation of macrophages with SIKVAV stimulated their uPA expression 20-fold. When RAW264.7 macrophages were incubated with a scrambled peptide (IVKVSA) no increase in uPA expression was observed (data not shown).

Anti-laminin Receptor Antibody Inhibits Matrix Induction of uPA but Not MMP-9—The binding of cells to laminin is mediated by several integrins and a nonintegrin receptor (49). E8 is derived from the short arm of laminin (45) and are reported to promote cell attachment (46, 47). SIKVAV (50–52) is a laminin fragment E8 is mediated by α6β1 (VLA-6) integrin (50, 51). Since it was demonstrated that SIKVAV stimulates macrophage uPA expression (Table II), we determined the effect of an antibody to the integrin α6 subunit on SIKVAV and matrix induction of uPA. THP-1 monocytes were preincubated with monoclonal antibody (50 µg/ml) directed against the integrin α6 subunit (CD51) before adding SIKVAV (50 µg/ml). Following an overnight incubation, media derived from control cells contained 10 ± 0.3 milliunits uPA/2.5 × 10^5 cells (mean ± S.E.; n = 4). uPA expression by cells incubated with SIKVAV peptide was increased 5-fold (47.5 ± 0.9 milliunits). When cells were preincubated with monoclonal anti-CD51 IgG (2.5 µg/ml), peptide-induced uPA expression was completely blocked (11 ± 0.5 milliunits), whereas preincubation of cells with monoclonal anti-CD16 IgG (FcRII) had no effect. These data demonstrate that binding of the SIKVAV peptide to the α6β1 integrin mediates laminin induction of uPA expression.

However, the above data do not exclude the possibility that laminin binding to other integrins may also regulate uPA ex-
Macrophage tissue remodeling is regulated by the expression of matrix-degrading proteases and their inhibitors and cellular receptors to direct and localize proteolysis (1, 2, 52–55). The expression of these proteinases is regulated by cytokines and growth factors (22, 24, 25, 27). In addition, structural components of the extracellular matrix have been demonstrated to regulate MMP expression (28–30), bind plasminogen and PAI (56–58), and enhance the kinetics of plasminogen activation (59, 60). In studies reported here, we have demonstrated that macrophage expression of uPA and MMP-9 is up-regulated when cultured on basement membrane extracts. Of the purified matrix components examined, laminin up-regulated macrophage expression of both uPA and MMP-9, whereas collagen IV, fibronectin, and tenasin up-regulated MMP-9 without affecting uPA expression. To our knowledge, this is the first demonstration that macrophage uPA expression, a pivotal component of their tissue remodeling protease cascade, is up-regulated by laminin.

In these studies, macrophage uPA expression was quantitated utilizing a functional assay based on the ability of uPA to generate plasmin from exogenous plasminogen (34). The increase in uPA activity observed in macrophage-conditioned media following incubation with laminin was not due to enhanced kinetics of plasminogen activation. In contrast to tPA-mediated plasminogen activation, intact matrices and purified laminin had little or no effect on uPA-mediated plasminogen activation (59, 60). Furthermore, as demonstrated in these studies, incubation of macrophages with laminin resulted in a rapid and sustained increase in the steady state levels of uPA mRNA which paralleled the appearance of uPA activity in their conditioned media.

Laminin is a large (~900 kDa) multidomain heterotrimer consisting of α, β, and γ chains (45, 49). The best understood laminin isoform is derived from Engelbreth Holm Swarm sarcoma matrices (i.e. Matrigel) and is termed laminin 1 (45, 49). In these studies, we have utilized synthetic peptides and specific anti-integrin antibodies to identify the laminin domain and macrophage receptor that mediate the observed regulation of uPA expression. P1 is a pepsin-generated fragment of laminin containing portions of the α, β, and γ chains of the short arms (45). The P1 fragment promotes cell attachment and MMP-9 expression by tumor cells (51, 61, 62). Cellular binding to the P1 fragment is blocked by antibodies to integrin β1 and β3 subunits and RGD peptides (51). Laminin peptides CDPYIGSR and YIGSR, derived from the β chain in the P1 fragment (45), promote cell attachment and spreading (46, 47). These laminin peptides stimulated MMP-9 expression by tumor cells (48) but block angiogenesis and tumor metastasis (63–65). Incubation of RAW264.7 macrophages with either CDPYIGSR or YIGSR had no effect on their expression of either uPA or MMP-9.

E8 is an elastase-generated fragment of laminin containing portions of the α, β, and γ chains of the long arm (45). The E8 fragment promotes cell attachment (51, 61). Cellular binding to the E8 fragment is blocked by antibodies to either subunit of the α6β1 integrin (50, 51). The laminin peptide SIKVAV is derived from the α chain of the E8 fragment (45). SIKVAV peptide promotes tumor invasion, metastasis, angiogenesis, and MMP expression (30, 48, 66). As reported here, incubation of RAW264.7 macrophages with SIKVAV also strongly up-regulated uPA expression. Taken together, these data suggest that laminin's effect on macrophage uPA expression is mediated through the binding of the long arm of laminin to the α6β1 laminin receptor.

To test the hypothesis that the α6β1 integrin mediates matrix induction of macrophage uPA expression, human THP-1 monocytes were incubated with a monoclonal antibody directed against integrin α6 subunit (CDw49f) prior to plating on intact matrix. As a control, cells were incubated with a monoclonal antibody directed against FcγRIII (CD16). The adherence of THP-1 monocytes to matrix-coated plastic was unaffected by either anti-α6 IgG or anti-CD16 IgG. Matrix induction of THP-1 uPA expression was inhibited by anti-α6 IgG, whereas anti-CD16 IgG had little or no effect at similar concentrations. In contrast to uPA, matrix induction of macrophage MMP-9 expression was unaffected by anti-α6 IgG.

The expression of matrix-degrading proteases regulates a variety of macrophage functions including extravasation, migration, and tissue remodeling (4, 40–42, 67). We and others (3, 4) have demonstrated that macrophage matrix degradation is dramatically enhanced in the presence of plasminogen. Fol-
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