Degradation of the extracellular matrix leads to the release of fragments, which elicit biological responses distinct from intact molecules. We have reported that \(\alpha_1:Se^{2091-Arg^{2108}}\) a peptide derived from the \(\alpha_1\)-chain of laminin-1, triggers protein kinase C-dependent activation of MAPK\(\text{erk}^1/2\), leading to the up-regulation of macrophage urokinase type plasminogen activator and matrix metalloproteinase (MMP)-9 expression. Since intact laminin-1 failed to trigger these events, we hypothesized that \(\alpha_1:Se^{2091-Arg^{2108}}\) is cryptic or assumes a conformation not recognized by macrophages. Here we demonstrate that elastase cleavage of laminin-1 generates fragments, which stimulate proteinase expression by RAW264.7 macrophages and peritoneal macrophages. In contrast, fragments generated by MMP-2, MMP-7, or plasmin had no effect on macrophage proteinase expression. Elastase-generated laminin-1 fragments were fractionated by heparin-Sepharose chromatography. Heparin-binding fragments stimulated macrophages’ proteinase expression severalfold greater than nonbinding fragments. The heparin binding fragments reacted with antibodies directed against regions of the \(\alpha_1\)-chain including \(\alpha_1:Se^{2091-Arg^{2108}}\) and the globular domain. A peptide from the first loop of the globular domain \(\alpha_1:Se^{2179-Arg^{2189}}\) triggered the phosphorylation of MAPK\(\text{erk}^1/2\) and stimulated the expression of macrophage urokinase type plasminogen activator and MMP-9. Moreover, a heparin-binding fraction isolated from an aortic aneurysm contained fragments of \(\alpha_1\)-chain and stimulated macrophages’ proteinase expression. Based on these data, we conclude that cryptic domains in the COOH-terminal portion of the \(\alpha_1\)-chain of laminin are exposed by proteolysis and stimulate macrophages’ proteinase expression.

The synthesis and activation of serine and matrix metalloproteinases (MMP)\(^1\) by monocytes and macrophages play an important role in their migration through extracellular matrix (ECM) and clearance of extravascular fibrin and necrotic debris (1–7). In earlier studies, we tested the hypothesis that the ECM regulates macrophage proteinase expression by culturing macrophages on ECM purified from Engelbreth Holm Swarm (EHS) sarcoma (Matrigel\(^{TM}\)) (8, 9). Results demonstrated that the expression of urokinase type plasminogen activator (uPA) and MMP-9 by murine RAW264.7 macrophages, human THP-1 monocytes, and human bone marrow-derived macrophages were strongly up-regulated. This was the first demonstration that the engagement of ECM by macrophages stimulates their expression of both uPA and MMP-9. Since the uPA/plasmin system is a physiologic activator of MMPs (10, 11), the ECM emerges as a potent regulator of the macrophage-degradative phenotype.

The ECM component responsible for stimulating macrophage proteinase expression was identified as laminin-1 (8). Laminins are large heterotrimeric molecules (~500–1000 kDa) with multiple domains that mediate their attachment to cells and other ECM components (12). Twelve laminin heterotrimers (assembled from five \(\alpha\), three \(\beta\), and three \(\gamma\) chains) have been identified in mammals. Laminin-1 was the first of this family to be identified (13) and remains the best understood of the laminin isoforms (12, 14). It consists of \(\alpha_1\) (~400 kDa), \(\beta_1\) (~200 kDa), and \(\gamma_1\) (~200 kDa) chains. The NH\(_2\)-terminal portions of the \(\alpha_1\)-, \(\beta_1\)-, and \(\gamma_1\)-chains are free, whereas much of the rest of the chains are twisted in a coiled-coil. The COOH-terminal portion of the \(\alpha_1\)-chain extends past the coiled-coil region and forms a large oblong globule (G-domain) consisting of five homologous repeats. The G-domain is the principle heparin-binding region of laminin-1 (15, 16).

In an effort to identify the domains of laminin-1 responsible for stimulating macrophage proteinase expression, we examined synthetic peptides, which were reported to support cell adhesion and stimulate a variety of biological responses. Incubation of RAW264.7 macrophages and THP-1 monocytes with \(\alpha_1:Se^{2099-Arg^{2104}}\) stimulated their expression of both uPA and MMP-9 (8). Neither a scrambled \(\alpha_1\)-chain peptide nor \(\beta_1\)-chain peptides had any effect on macrophage proteinase expression. Thus, a peptide derived from the \(\alpha_1\)-chain of laminin-1 stimulates both uPA and MMP-9 expression by macrophages.

ECM components contain cryptic domains, which are...
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exposed by proteolysis and elicit biological responses distinct from intact molecules. For example, it was recently reported that a cryptic domain in laminin-5 that stimulates cell motility is exposed following cleavage with MMP-2 or MT1-MMP (17, 18). The synthetic laminin-1 peptide α112099SRARKQASIKVAVSDAD2108 (9) stimulates macrophages uPA and MMP-9 (8) is derived from the region of the α1-chain associated with the coiled-coil and is probably not exposed in the intact molecule (16). Therefore, we compared the ability of intact laminin-1 and α1-chain peptide to regulate macrophage proteinase expression. Results of those experiments demonstrated that intact laminin-1 had no effect on macrophages’ proteinase expression, whereas uPA and MMP-9 expression were stimulated in a dose-dependent manner by α112099SRARKQASIKVAVSDAD2108 (9). Moreover, incubation of macrophages with the α1-chain peptide, but not intact laminin-1, triggers a phosphorylation cascade resulting in the activation of protein kinase C, which in turn leads to the activation of MAPKerk1/2. The observed signaling events were causal to the induction of proteinase expression, since inhibition of tyrosine kinases, protein kinase C, or MEK-1 (MAP kinase) blocked the ability of α112099SRARKQASIKVAVSDAD2108 to induce uPA or MMP-9 expression (9). Taken together, these data suggest that a cryptic domain of laminin-1 induces a signaling pathway distinct from intact laminin-1 and up-regulates macrophage proteinase expression. The unmasking of this cryptic domain may play a role in regulation of macrophage degradative phenotype and tissue remodeling.

In studies reported here, we have sought to identify the proteinases responsible for exposing the domain(s) in laminin-1 that regulate the macrophage-degradative phenotype. Results demonstrate that laminin-1 is susceptible to cleavage by a variety of proteinases including elastase, MMP-2, MMP-3, MMP-7, and plasmin. However, only cleavage by elastase generated fragments that stimulated proteinase expression by RAW264.7 macrophages and thioglycollate-elicited macrophages. Laminin fragments were fractionated by affinity chromatography on heparin-Sepharose. Heparin binding fragments of laminin-1 induce a signaling pathway distinct from intact laminin-1 and up-regulates macrophage proteinase expression. The unmasking of this cryptic domain may play a role in regulation of macrophage degradative phenotype and tissue remodeling.

MATERIALS AND METHODS

Cell Culture—Murine RAW264.7 macrophages (20) were obtained from American Type Culture Collection. Cells were maintained as adherent cultures in Roswell Park Memorial Medium (RPMI; without HEPES) supplemented with 10% Cellc straw bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), and 4% glucose (Invitrogen). Experiments to determine the effect of laminin fragments and peptides on macrophage proteinase expression were carried out in macrophage serum-free medium (MSFM; Invitrogen). Isolation of Peritoneal Macrophages—Thioglycollate-elicited peritoneal macrophages were obtained from Swiss Webster mice by the method of Edelson and Cohn (21) as described previously (2). Mice were injected intraperitoneally (3 ml/mouse) with 3% Brewer thioglycollate (Difco). 4 days later, cells were harvested by lavage with cold DPBS. Peritoneal cells were recovered by centrifugation and resuspended in RPMI-10% FBS and plated into appropriate wells. Cells were allowed to adhere for 2 h and then washed free of nonadherent cells.

Preparation of Laminin—Murine laminin-1 (BD Biosciences) was incubated with bovine pancreatic elastase (Sigma), active recombinant human MMP-2 (Calbiochem), active recombinant human MMP-7 (Calbiochem), or human plasmin (American Diagnostics). Incubation conditions were as follows. 27 nm laminin-1 was incubated with 0.48 nm elastase for 2.5–120 min in 0.05 m ammonium bicarbonate buffer, pH 7.9; 5.8 nm laminin-1 was incubated with 50 μM MMP-2 for 2–24 h in DPBS; 5.8 nm laminin-1 was incubated with 50 μM MMP-7 for 24 h in DPBS; and 22 nm laminin-1 was incubated with 235 nm plasmin in DPBS for 2–24 h. Following incubation, 6% SDS-sample buffer containing 5% β-mercaptoethanol was added to the samples and boiled for 3 min.

Western Blot for Laminin—In experiments to monitor the degradation of murine laminin-1 by selected proteinases, intact laminin-1 and degraded laminin-1 were electrophoresed in 4–15% polyacrylamide gradient gels under reducing conditions. Proteinases were transferred to a PVDF membrane, following which the membrane was blocked in TTBS containing 5% dry defatted milk for 1 h. Following one wash (15 min) in TTBS, the membrane was incubated 1 h with 1.0 μg/ml rabbit anti-murine laminin-1 (Collaborative Biomedical Products) in TTBS containing 5% dry defatted milk. The membrane was washed (twice in TTBS) and reblocked in TTBS containing 5% dry defatted milk for 15 min. The membrane was then incubated 1 h with biotinylated rabbit anti-mouse IgG (1:10,000; Pierce), washed (twice in TTBS) and incubated for 1 h with preformed avidin-biotin-horseradish peroxidase complexes (Pierce) in DPBS plus 0.1% Tween 20. Bound HRP was visualized utilizing enhanced chemiluminescence.

Heparin-Sepharose Chromatography of Elastase-derived Laminin Fragments—Laminin-1 (5 mg) was digested with elastase (2.5 μg) at 4 °C for 1 h and room temperature for 20 h in 50 mM bicarbonate buffer, pH 7.9. Proteolysis was stopped by the addition of excess phenylmethysulfonyl fluoride. The sample was loaded on a heparin-Sepharose column (5 ml), which was previously equilibrated with ammonium bicarbonate buffer. Fractions of 1 ml were collected and monitored for the presence of protein by UV spectrophotometry. Unbound laminin fragments were washed from the column with buffer. Bound fragments were eluted with 0.5 mM NaCl in ammonium bicarbonate buffer. Peak fractions were concentrated by ultrafiltration and dialyzed against DPBS at 4 °C.

Domain Mapping of Laminin Fragments—Following elastase digestion, fractionation by heparin-Sepharose chromatography, heparin-Sepharose-bound fragments were electrophoresed in 4–15% polyacrylamide gradient gels under reducing conditions. Proteinases were transferred to a PVDF membrane, following which the membrane was blocked in TTBS containing 5% dry defatted milk for 1 h. Following one wash (15 min) in TTBS, the membrane was incubated with either a rabbit antibody directed against a recombinant protein corresponding to amino acids 1656–2099 of the α1-chain of laminin-1 (Santa Cruz Biotechnology) or a rabbit antibody directed against the COOH-terminal 50-kDa portion of the G-domain (16) (RG50; kindly provided by Peter Yurchenco, UMDNJ) in TTBS containing 3% dry defatted milk. The membrane was washed (twice in TTBS) and reblocked in TTBS containing 5% dry defatted milk for 15 min. The membrane was then incubated 1 h with biotinylated goat anti-rabbit IgG (1:10,000; Pierce), washed (twice in TTBS), and incubated for 1 h with preformed avidin-biotin-HRP complexes (Pierce) in DPBS plus 0.1% Tween 20. Bound HRP was visualized utilizing enhanced chemiluminescence.

Preparation of Cell Lysates—RAW264.7 macrophages were lysed in Tris buffer, pH 7.5, containing 20 mM Tris-HCl, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM sodium vanadate, 2 mM sodium pyrophosphate, 1 mM phenylmethysulfonyl fluoride, and 10 μg/ml aprotinin. Lysates were centrifuged (14,000 × g) for 20 min at 4 °C. The supernatants were recovered, normalized for protein, and mixed with SDS sample buffer with β-mercaptoethanol and boiled for 5 min. Equal amounts of cell lysates were applied to gels based on protein content.

Western Blot Identification of Phosphorylated MAPK (p38/ERK)—Cell lysates were electrophoresed in 4–15% polyacrylamide gradient gels. Proteinases were transferred to a PVDF membrane, following which the membrane was placed in blocking buffer for 1 h. Following one wash in PBS, the membrane was incubated 1 h in blocking buffer containing 0.1 μg/ml rabbit anti-phospho-p38/ERK (p44/p42 MAP kinase IgG (New England Biolabs). The membrane was washed (twice in TBSB) and incubated for 1 h in blocking buffer containing 0.3 μg/ml goat anti-rabbit.
IgG conjugated to HRP (Transduction Laboratories). The membrane was washed in TBPS (three times) followed by PBS (once). Bound HRP was visualized utilizing enhanced chemiluminescence.

**Determination of Plasminogen Activator Activity—**Plasminogen activator activity was quantitated utilizing a sensitive functional assay for plasmin (22). Aliquots of conditioned media were added to microtiter wells containing 32 μl of DPBS plus 0.05% Tween 20, 1 μg of the plasmin substrate b-Val-Leu-Lys-aminio methyl coumarin (Enzyme Systems Products), and 0.5 μg of bovine plasminogen (American Diagnostica). 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).

**Determination of Metalloproteinase Activity—**The presence of metalloproteinase activity in cellular conditioned media was determined utilizing enzyme zymography as previously described (8). Conditioned media were mixed with SDS sample buffer (without mercaptoethanol) and incubated 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 (twice) in 2.5% Triton X-100 to remove SDS. The gel was incubated at 37 °C for 4 h in 200 mM NaCl containing 40 mM Tris–HCl and 10 mM CaCl₂, pH 7.5, and stained with Coomassie Blue. The presence of gelatinolytic activity was identified as clear bands on a uniform blue background following destaining.

**Northern Blot for MMP-9 mRNA Levels—**Total RNA was isolated from RAW264.7 macrophage as previously described (23). The poly(A) mRNA fraction was isolated utilizing the Poly(A)tract® mRNA isolation system (Promega, Madison, WI) according to the manufacturer’s instructions. Samples were electrophoresed in agarose, transferred to nylon membrane (Schleicher and Schuell), and hybridized with a 32P-labeled murine cDNA for MMP-9 (24) (kindly provided by Dr. G. Opdenakker, Rega Institute for Medical Research, University of Leuven, Belgium).

**Isolation and Identification of Laminin Fragments from Human Aortic Aneurysm—**A specimen of surgically removed abdominal aneurysm (¼ g, wet weight) was dissected free of fat and thrombus, minced, and extracted with 4 ml of 5M urea in 50 mM Tris, pH 7.12, containing 10 mM EDTA for 6 h at room temperature and overnight at 4 °C. Degradation of laminin-1 stimulated their expression of both uPA and MMP-9 (24). Samples were mixed and incubated for 1 h with end over end mixing. Protein A-Sepharose was removed from the suspension by brief centrifugation. The supernatants were collected and diluted 1:3 with RPMI medium.

**RESULTS**

**Elastase-generated Fragments of Laminin-1 Stimulate Macrophage Proteinase Expression—**Laminin family members are susceptible to cleavage by a variety of proteinases including elastase (15, 25), MMP-2 (17, 26), MMP-7 (27), and plasmin (28, 29). To directly test whether cleavage of laminin-1 exposes cryptic domains, which subsequently stimulate macrophage protease expression, we incubated intact murine laminin-1 with elastase. Degradation of laminin-1 was monitored by SDS-PAGE followed by Coomassie Blue staining and/or Western blot with polyclonal anti-laminin-1 antibody.

We next determined whether elastase-generated fragments of laminin-1 would stimulate macrophage protease expression. uPA and MMP levels in macrophage conditioned media were quantitated utilizing a fluorescent bioassay and zymography, respectively. As seen in Fig. 2, an overnight incubation of RAW264.7 macrophages with elastase-generated fragments of laminin-1 stimulated their expression of both uPA and MMP-9 to levels achieved utilizing the previously reported stimulatory peptide α1:Ser2091–Arg2108. In contrast to either elastase-degraded laminin-1 or the α1-chain peptide, intact laminin-1 has no effect on macrophages’ protease expression. These data are proof of the principle that proteolysis of laminin-1 generates fragments that regulate macrophage protease expression.

We next determined whether the exposure of stimulatory domains in laminin-1 by elastase was specific. For this purpose, the effect of fragments of laminin-1 generated by MMP-2, MMP-7, or plasmin on macrophage proteinase expression was determined. As seen in Fig. 3, the α1-chain of laminin-1 was degraded by active recombinant MMP-2, MMP-7, and plasmin. However, fragments generated by these proteinases failed to regulate macrophage uPA or MMP-9 expression (data not shown).

These data demonstrate that selective digestion of laminin-1...
produces fragments that stimulate macrophage proteinase expression. However, in the inflammatory setting, laminin-1 would be subjected to a variety of proteinases, which may modify the elastase-generated fragments to produce biologically inactive fragments. To determine whether the stimulatory activity of elastase-generated laminin-1 fragments is sensitive to other proteinases, we incubated elastase-generated fragments with either 50 nM recombinant MMP-2 or MMP-7 for 4 h and tested their ability to up-regulate uPA expression. Conditioned media recovered from control RAW264.7 macrophages contained 77 ± 3 milliunits of uPA/10⁵ cells (n = 3; means ± S.E.). uPA expression by macrophages incubated (18 h) with elastase-generated fragments increased to 285 ± 25 milliunits/10⁵ cells. Elastase-generated fragments incubated with either MMP-2 or MMP-7 stimulated macrophage uPA expression to 221 ± 21 and 263 ± 24 milliunits/10⁵ cells, respectively. Thus, incubation of elastase-generated fragments with either MMP-2 or MMP-7 had little effect on their ability to stimulate macrophage uPA expression.

Heparin-binding Fragments of Laminin-1 Exhibit Enhanced Proteinase-inducing Activity—The principal heparin-binding region of laminin-1 has been mapped to the COOH terminus of the α1-chain (amino acids 1856–2099) that overlapped the peptide sequence previously reported to stimulate proteinase expression (Fig. 4, bottom). Predictably, the intact α1-chain of undigested laminin was strongly immunoreactive reactive with the anti-α1:1856–2099 antibody. The anti-peptide antibody failed to react with the laminin fragment(s) that did not bind heparin (i.e. peak 1). In contrast, a single immunoreactive band was observed at 120 kDa in fragments that bound to and were eluted from the heparin column (peak 2). Similarly, proteins in peaks 1 and 2 were examined for reactivity with anti-RG50, a polyclonal antibody directed against the terminal 50-kDa portion of the globular domain of laminin-1. As expected, the intact α1-chain of undigested laminin was strongly immunoreactive with anti-RG50. Anti-RG50 failed to react with peak 1 laminin fragments but did react with a heparin binding fragment of ~50 kDa in peak 2. Based on these data, we conclude that peak 2 proteins are derived from the tail region of laminin. Moreover, one of these fragments (120 kDa) contains the sequence previously reported to stimulate macrophages' proteinase expression.

To determine whether unbound and bound laminin fractions differentially affected uPA expression, peaks 1 and 2 were dialyzed with DPBS and incubated with RAW264.7 macrophages (50 μg/ml) overnight. As seen in Fig. 5, incubation of macrophages with α1:Ser2089–Arg2108 stimulated their expression of uPA, whereas intact laminin-1 had no effect. Following digestion with elastase, both unbound and bound laminin fractions stimulated macrophage uPA expression relative to control cells. The heparin-binding laminin fragments (peak 2) stimulated uPA expression 3-fold more than fragments that did not bind heparin.

A Peptide from the G-domain of Laminin-1 (SN Peptide; α1:Ser2179–Ser2198) Stimulates Macrophages' Proteinase Expression—In addition to mediating the binding of laminin-1 to heparin, the G-domain contains the epitopes that support cell adhesion. In this regard, α1:Ser2179–Ser2198 (SN peptide),
which is located in the first loop of the G-domain, is responsible for the binding of a variety of cells to laminin-1 (19, 32, 33). Another G-domain peptide (\(\text{H}9251\)1:Asn2183–Gly2194; AG-10), which comprises the central portion of the SN peptide, stimulated the invasion of cross-linked gelatin films by melanoma cells (34). Therefore, we determined the effect of \(\text{H}9251\)1:Ser2179–Ser2198 on macrophages’ proteinase expression. As seen in Fig. 6, macrophage uPA expression was increased 8-fold following overnight incubation with the SN peptide (100 \(\mu\)g/ml), whereas intact laminin-1 had no effect. Importantly, uPA expression induced by elastase-generated laminin-1 fragments and the SN peptide were similar to that achieved with monocyte colony stimulating factor (75 ng/ml). Likewise, levels of MMP-9 activity in conditioned media derived from RAW264.7 macrophages incubated with elastase generated fragments or the SN peptide were similarly increased (Fig. 7). The observed increase in MMP-9 activity was further examined by determining the effect of elastase-generated fragments and SN peptide on steady state levels of MMP-9 mRNA. Following a 24-h incubation with
Serum-starved cells were incubated with SN peptide (100–μg/ml) for 0–20 min, following which cell lysates were prepared. Phosphospecific Erk-1 (p44) and Erk-2 (p42) were identified by Western blot utilizing polyclonal anti-phosphospecific p44/42 MAPK as described under "Materials and Methods." For purposes of comparison, mRNA levels for constitutively expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are presented.

We previously reported that the induction of macrophages protease expression by α1:Ser2091–Arg2108 was dependent on a signaling pathway that resulted in the activation of MAPKv1/2 (9). Treatment of cells with the MEK-1 inhibitor U0126 blocked the ability of α1:Ser2091–Arg2108 to stimulate the phosphorylation/activation of MAPKv1/2 and up-regulation of macrophage protease expression (9). Therefore, we determined whether the induction of proteinase expression by SN peptide was dependent on the activation of MAPKv1/2.

Serum-starved cells were incubated with SN peptide (100 μg/ml) for 0–20 min, and levels of phosphorylated MAPKv1/2 were determined by Western blot. Incubation of cells with SN peptide resulted in a clear increase in levels of phosphorylated MAPKv1/2 (Fig. 8A). Preincubation of macrophages with the MEK-1 inhibitor U0126 blocked SN peptide-induced uPA (Fig. 8B) and MMP-9 expression (Fig. 8C). Thus, these data demonstrate that two α1-chain peptides from the tail region of laminin-1 trigger MAPK-dependent up-regulation of macrophage protease expression.

The stimulatory effect of elastase-generated laminin fragments or peptide, the levels of MMP-9 mRNA were markedly elevated over controls and cells incubated with intact laminin-1 (Fig. 7). Thus, the stimulation of MMP-9 activity was mirrored by an increase in MMP-9 gene activity.

Fig. 7. SN peptide up-regulates macrophage MMP-9 activity and mRNA levels. RAW264.7 cells were suspended in RPMI containing 10% FBS and aliquoted into T25 flasks (5 × 10⁶/flask). Following 4–6-h adherence, cells were washed to remove serum, and media were replaced with MSFM alone or MSFM containing intact laminin (50 μg/ml), elastase-generated laminin fragments (50 μg/ml) or SN peptide (100 μg/ml). The next day, conditioned media were recovered and assayed for MMP-9 activity by zymography. The poly(A) mRNA fractions were isolated from macrophage monolayers, and MMP-9 mRNA levels were determined by Northern blot hybridization utilizing a murine cDNA for MMP-9 as described under "Materials and Methods." For purposes of comparison, mRNA levels for constitutively expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are presented.

Fig. 8. Inhibition of MEK-1 blocks SN peptide induced uPA and MMP-9 expression. A. RAW264.7 macrophages (2 × 10⁶/well) were cultured in RPMI medium without serum for 24 h. Cells were incubated with SN peptide (100 μg/ml) for 0–20 min, following which cell lysates were prepared. Phosphospecific Erk-1 (p44) and Erk-2 (p42) were identified by Western blot utilizing polyclonal anti-phosphospecific p44/42 MAPK as described under "Materials and Methods." B and C, macrophages (0.5 × 10⁶/well) were preincubated for 30 min with 10 μM MEK-1 inhibitor U0126, following which 100 μg/ml SN peptide was added. Conditioned media (24 h) were collected and assayed for uPA and MMP activities as described under "Materials and Methods." The uPA data represent the means ± S.E. of three individual wells.

Fig. 9. SN peptide up-regulates MMP-9 expression by peritoneal macrophages. Thioglycollate-elicited macrophages were cultured in RPMI-10% fetal calf serum overnight. Cells were washed to remove serum, and media were replaced with MSFM alone or MSFM containing intact laminin-1 (50 μg/ml), elastase-generated laminin fragments (50 μg/ml), or SN peptide (100 μg/ml). Following 24-h incubation at 37 °C, MMP activity in conditioned media was assessed by zymography as described under "Materials and Methods." uPA expression by thioglycollate-elicited peritoneal macrophages was markedly elevated (2), and the exposure of these cells to laminin fragments or α1-chain peptides did not further stimulate their expression of uPA (data not shown). Thus, our observation that elastase-generated laminin fragments and the SN peptide stimulate proteinase expression by RAW264.7 macrophages is confirmed in primary macrophages.

Laminin-1 Fragments Recovered from Abdominal Aortic Aneurysm Stimulate Proteinase Expression by Macrophages—The
that were reactive with the anti-

unfractionated extract, peak 1, and peak 2 contained proteins

against T

extract (fraction 5) and peak 2 (fractions 24 and 25) were analyzed by Western blot utilizing polyclonal antibodies directed

P2

RPMI containing 10% FBS and aliquoted into 96-well plates (105/well). Following 4

Materials and Methods.

Unbound laminin fragments were washed from the column with wash buffer (peak 1). Bound fragments were eluted with wash buffer containing 0.5 M NaCl (peak 2). Fraction 5 of peak 1 and fractions 24 and 25 of peak 2 were collected. B, standard laminin-1 (Std Lmn), total extract (T), peak 1 (P1; fraction 5) and peak 2 (P2; fractions 24 and 25) were analyzed by Western blot utilizing polyclonal antibodies directed against α1-chain amino acids 1856–2099 (anti-α1:1856–2099) as described under “Materials and Methods.” C, RAW264.7 cells were suspended in RPMI containing 10% FBS and aliquoted into 96-well plates (105/well). Following 4–6 h adherence, cells were washed to remove serum, and media were replaced with MSFM alone (Ctrl) or MSFM containing peak 1 (P1; 50 μg/ml), peak 2 (P2; 50 μg/ml), or MCSF (75 ng/ml). The next day, media were recovered and assayed for uPA activities as described under “Materials and Methods.” The uPA data represent the means ± S.E. of three individual wells. D, peak 1 (P1) and peak 2 (P2) were incubated with anti-α1:1856–2099 followed by Protein A-Sepharose as described under “Materials and Methods.” Following centrifugation, the supernatants were recovered and tested for their ability to stimulate uPA expression.

Fig. 10. Enhanced stimulation of macrophage uPA expression by heparin-binding laminin-1 fragments derived from aortic aneurysm. A, a tissue extract prepared from an aortic aneurysm was fractionated on a heparin-Sepharose column as described under “Materials and Methods.” Unbound laminin fragments were washed from the column with wash buffer (peak 1). Bound fragments were eluted with wash buffer containing 0.5 M NaCl (peak 2). Fraction 5 of peak 1 and fractions 24 and 25 of peak 2 were collected. B, standard laminin-1 (Std Lmn), total extract (T), peak 1 (P1; fraction 5) and peak 2 (P2; fractions 24 and 25) were analyzed by Western blot utilizing polyclonal antibodies directed against α1-chain amino acids 1856–2099 (anti-α1:1856–2099) as described under “Materials and Methods.” C, RAW264.7 cells were suspended in RPMI containing 10% FBS and aliquoted into 96-well plates (105/well). Following 4–6 h adherence, cells were washed to remove serum, and media were replaced with MSFM alone (Ctrl) or MSFM containing peak 1 (P1; 50 μg/ml), peak 2 (P2; 50 μg/ml), or MCSF (75 ng/ml). The next day, media were recovered and assayed for uPA activities as described under “Materials and Methods.” The uPA data represent the means ± S.E. of three individual wells. D, peak 1 (P1) and peak 2 (P2) were incubated with anti-α1:1856–2099 followed by Protein A-Sepharose as described under “Materials and Methods.” Following centrifugation, the supernatants were recovered and tested for their ability to stimulate uPA expression.

regulatory role of laminin-1 on macrophage proteinase expression in vivo is unclear, since laminin-1 is rare in normal adult tissues (35). However, the expression of laminin-1 may increase under pathological conditions (36–41). Therefore, we determined whether laminin-1 fragments were present in an extract prepared from an abdominal aortic aneurysm and whether these fragments trigger macrophage proteinase expression. Aneurysms are inflammatory lesions characterized by elevated MMP and serine proteinase expression, which result in the degradation of vascular ECM and loss of structural integrity (10, 42). For this purpose, a tissue extract was prepared and fractionated on a column of heparin-Sepharose, as described for elastase-generated fragments of laminin-1. Unbound proteins (peak 1) were washed from the column and bound proteins (peak 2) were eluted with 0.5 M NaCl (Fig. 10A). The proteins in peaks 1 and 2 were analyzed by Western blot utilizing a polyclonal directed against a sequence in the tail of the α1-chain that overlaps the peptide sequence previously reported to stimulate proteinase expression. As seen in Fig. 10B, anti-α1:1856–2099 reacted strongly with the α1-chain of intact laminin. The unfractionated extract, peak 1, and peak 2 contained proteins that were reactive with the anti-α1-chain antibody. Peak 2 appears to contain intact and fragmented α1-chain. The two prominent bands observed between 50 and 75 kDa in the tissue extract and peak 1 are nonspecific, since they appeared in blots probed with secondary antibody only (data not shown). Peaks 1 and 2 were dialyzed with DPBS and incubated with RAW264.7 macrophages overnight. uPA expression was stimulated severalfold by the peak 2 (heparin-binding) fraction, whereas incubation with the peak 1 fraction had no effect (Fig. 10C). Moreover, following immunodepletion with anti-α1:1856–2099, peak 2 was unable to stimulate macrophages’ uPA expression (Fig. 10D). In contrast, uPA expression by cells incubated with immunodepleted peak 1 was relatively unaffected. Likewise, immunoprecipitation with normal rabbit IgG had no effect on P2 induction of uPA expression (data not shown). Thus, these data support the hypothesis that fragments of α1-chain, capable of stimulating macrophage proteinase expression, are generated in vivo. However, further studies are required to determine whether laminin fragments are present in normal abdominal aorta and if the observed fragmentation occurred ex vivo.

DISCUSSION

The ECM is a complex association of fibrillar proteins and adhesive glycoproteins, which provide structural stability to tissues and a substrate upon which cells adhere, move, and differentiate. The proteolysis of ECM, which occurs during development and under many pathologic conditions, can weaken the structural integrity of tissues, stimulate cellular
invasion, trigger apoptosis or proliferation, and release matrix-bound growth factors (43). In addition, several lines of evidence suggest that ECM components contain cryptic domains, which are exposed by proteolysis and elicit biological responses distinct from intact molecules (17, 18, 43–49). Results of our previous studies, which determined whether the ECM regulates the macrophage-degradative phenotype, demonstrated that macrophages uPA and MMP-9 expression were stimulated in a dose-dependent manner by a synthetic peptide from the \( \alpha_1 \)-chain of laminin-1, whereas intact laminin-1 had no effect on proteinase expression (9). These data lead us to hypothesize that the domains of laminin-1, which stimulate macrophages’ proteinase expression, are cryptic or assume a conformation that is not recognized by macrophages in the intact laminin molecule. Supporting this hypothesis was the observation that an extract of EHS-ECM, which stimulates macrophage proteinase expression, contains both intact laminin-1 and several laminin-1 fragments (9). When depleted of laminin and its fragments by immunoprecipitation with polyclonal anti-laminin-1 IgG, the EHS-ECM extract no longer stimulated macrophage proteinase activity (9). Results of experiments reported here demonstrate that selective cleavage of laminin-1 by elastase generates fragments that stimulate macrophage uPA and MMP-9 expression. These data are proof of the principle that proteolysis of laminin-1 generates fragments with new biologic activities.

Based on their affinity to heparin and reactivity with domain-specific antibodies, we conclude that the stimulatory domains exposed by elastase cleavage of laminin-1 are derived from the tail region of laminin-1. Utilizing synthetic peptides, we have identified two synthetic \( \alpha_1 \)-chain peptides from this region that stimulate macrophage expression of uPA and MMP-9: \( \alpha_1:2091\text{SRARKQASIKVAVSADR}^{2108} \) (or the hexapeptide \( \alpha_1:2099\text{SIKVAV}^{2104} \)) and \( \alpha_1:2179\text{SIINNNRWHSIYITRFNGMGS}^{2198} \). Both peptides are located in E8, an elastase-generated fragment of laminin-1 (14), which is the region of laminin primarily responsible for cell binding (50–52). The E8 fragment is derived from the long arm and consists of a coiled-coil (rodlike) region and G1–G3 of the COOH-terminal G-domain.

Despite the early observations that E8 fragments (50, 51) or anti-E8 (16) can block adhesion of cells to intact laminin-1, the peptide sequences in E8 responsible for cell binding remain controversial. In this regard, the \( \alpha_1:2091\text{SRARKQASIKVAVSADR}^{2108} \) (or the hexapeptide \( \alpha_1:2099\text{SIKVAV}^{2104} \)) supports cell adhesion and stimulates a variety of biological responses including macrophage proteinase expression (8, 9, 53–56). Notwithstanding the number of diverse activities attributed to it, evidence suggests that \( \alpha_1:2091\text{SRARKQASIKVAVSADR}^{2108} \) is not exposed in intact laminin-1 (16). First, the \( \alpha_1 \)-chain peptide is derived from a portion of the \( \alpha_1 \)-chain associated with the coiled-coil portion of the E8 fragment. Second, anti-\( \alpha_1:2095\text{SIKVAV}^{2104} \) has no effect on cell adhesion to the E8 fragment (19). Third, the binding of cells to intact laminin-1 and the E8 fragment was blocked by recombinant G-domain, which does not contain \( \alpha_1:2091\text{SRARKQASIKVAVSADR}^{2108} \) (16). Taken together, these data are consistent with our hypothesis that the domains in laminin that regulate macrophages’ proteinase expression are cryptic in the intact molecule.

As discussed above, the binding of cells to laminin-1 appears to be mediated by the COOH-terminal portion of the \( \alpha_1 \)-chain, which extends past the coiled-coil region and forms the large oblong G-domain. It was previously demonstrated that \( \alpha_1:2179\text{SIINNNRWHSIYITRFNGMGS}^{2198} \) (SN peptide), located in the first loop of the G-domain, supports the adhesion of a variety of cells, and anti-SN peptide antibodies blocked the binding of cells to the E8 fragment (19). These data indicate that the SN peptide, unlike the proximal \( \alpha_1:2095\text{SRARKQASIKVAVSADR}^{2108} \), is exposed in intact laminin-1 and supports cell binding. However, in contrast to the SN peptide, intact laminin-1 fails to trigger MAPK activation and the up-regulation of uPA and MMP-9 expression. The divergent response of RAW264.7 and peritoneal macrophages to SN peptide versus intact laminin-1 indicates differential recognition mechanisms for these ligands. For example, intact laminin engages multiple receptors (12), which may act to suppress the signal triggered by the engagement of a single receptor by the SN peptide. Alternatively, the cell surface receptor(s) that recognize the SN peptide and initiate a signaling pathway that triggers proteinase may not recognize the SN peptide when its conformation is constrained as part of the intact molecule.

Results of experiments reported here provide clear evidence that selective proteolysis of laminin-1 generates fragments that up-regulate macrophages’ proteinase expression. However, the regulatory role of laminin-1 fragments in macrophage-dependent tissue remodeling remains unclear, since laminin-1 expression is rare in normal adult tissues (35). In this regard, the expression of laminin-1 may increase under pathological conditions. For example, laminin-1 is one of several ECM proteins, which are elevated in following vascular injury and in atherosclerotic plaques (36–40). Likewise, laminin-1 is present in the adult kidney (57–59) and increases in immune complex glomerulonephritis (41). Supporting the hypothesis that stimulatory fragments of laminin-1 are present in pathologic specimens, we have demonstrated that an extract prepared from an abdominal aortic aneurysm contains immunoreactive \( \alpha_1 \)-chain fragments and up-regulates uPA expression by macrophages. The stimulatory activity in the tissue extract bound to heparin and was removed by immunoprecipitation with antibody directed against the \( \alpha_1 \)-chain.

In conclusion, the results of experiments reported here support the hypothesis that selective degradation of laminin-1 exposes cryptic domains that alter the macrophage-degradative phenotype through the up-regulation of uPA and MMP-9 expression. Based on heparin-Sepharose chromatography and mapping with antibodies, the stimulatory fragments are derived from the tail portion of laminin-1 involved in cell adhesion and binding to heparin sulfate proteoglycan. Two stimulatory \( \alpha_1 \)-chain peptides from this region have been identified that initiate a phosphorylation cascade resulting in the activation of MAPK\(^{148,234}\) and the up-regulation of proteinase expression. The exposure of cryptic domains in laminin-1 may play a role in regulation of macrophage proteinase expression and tissue remodeling at sites of injury and repair.

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REFERENCES

Macrophages’ Proteinase Expression Is Regulated by Laminin-1

Exposure of Cryptic Domains in the α1-chain of Laminin-1 by Elastase Stimulates Macrophages Urokinase and Matrix Metalloproteinase-9 Expression

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