Angiostatin is a potent inhibitor of tumor angiogenesis and the growth of metastatic foci. Recent studies have indicated that neoplastic cells can generate angiostatin directly or in cooperation with tumor-associated macrophages. In studies reported here, we determined whether angiostatin is generated in mice under non-neoplastic settings. Utilizing murine RAW264.7 macrophages and thioglycollate-elicited peritoneal macrophages, we demonstrate that angiostatin-like fragments are generated as a byproduct of the proteolytic regulation of membrane-bound plasmin. Plasmin proteolysis and subsequent loss in membrane-bound plasmin activity requires active plasmin but was unaffected by inhibitors of metalloproteinases. Lysine binding fragments of plasmin, isolated from macrophage-conditioned media utilizing affinity chromatography, appeared as a major (48 kDa) and two minor bands (42 and 50 kDa) in SDS-polyacrylamide gel electrophoresis and were immunoreactive with anti-kringle 1–3 IgG. Each peptide begins with Lys77 and contains the entire sequence of angiostatin. The affinity isolated plasmin fragments inhibited bFGF-induced endothelial cell proliferation. Lavage fluid recovered from the peritoneal cavities of mice previously injected with thioglycollate contained angiostatin-like plasmin fragments similar to those generated in vitro. This is the first demonstration that angiostatin-like plasmin fragments are generated in a non-neoplastic inflammatory setting. Thus, in addition to regulating pericellular plasmin activity, proteolysis of plasmin generates inactive kringle-containing fragments expressing angiostatic properties.

Angiostatin, a 38-kDa internal fragment of plasminogen, is a potent inhibitor of tumor angiogenesis and the growth of metastatic foci in mice (1–5). It was first isolated from the serum and urine of tumor-bearing mice (5). The isolated fragment of plasminogen extends from Thr398-Val440, contains the first four of five kringles present in plasminogen, and is not enzymatically active (5). Murine-derived angiostatin inhibits bFGF-induced endothelial cell proliferation in vitro and bFGF-induced angiogenesis in vivo (5). In addition, recombinant angiostatin inhibits endothelial cell proliferation and the growth of several diverse carcinomas in mice (3, 4). The inhibitory effect of angiostatin on growth factor-induced endothelial cell proliferation appears to be expressed by recombinant kringle 1, 3 and, to a lesser extent, 2 (6).

The mechanism of formation of angiostatin in mice with tumor burden is not completely understood at this time. It was recently reported that macrophages recruited to Lewis lung carcinomas were responsible for the formation of angiostatin via their expression of metalloelastase (MMP-12) (7). Macrophage expression of MMP-12 is induced by granulocyte-macrophage colony-stimulating factor secreted by the carcinoma cells (7). These data suggest that the carcinoma cells and host macrophages cooperate in the generation of angiostatin. Supporting the role of metalloproteinases in the generation of angiostatin is the observation that stromelysin-1 (MMP-3), matrixin (MMP-7), and type IV collagenase (MMP-9) proteolyzed plasminogen to generate angiostatin fragments in a cell free system (8, 9). In contrast, it has been reported that reduction of disulfide bonds in plasmin, via a reductase secreted by HT1080 fibrosarcoma cells or Chinese hamster ovary cells, triggered serine proteinase-dependent angiostatin formation (10). Likewise, the conversion of plasminogen to angiostatin by prostate carcinoma cells was dependent on urokinase and free sulfhydryl donors (11, 12). Taken together, these data suggest that angiostatin may form via multiple pathways in animals with malignant neoplasia.

The observation that an angiostatic fragment of plasminogen is generated in mice with Lewis lung carcinomas, raises the hypothesis that these or other angiostatic molecules may be generated under non-neoplastic settings as well. In this regard, we previously reported that autoproteolysis was a mechanism by which plasmin activity on the surface of macrophages is regulated in vitro (13). Following incubation with plasmin, THP-1 macrophage membrane-bound plasmin activity fell 80% over a 24-h period, despite the presence of enzymatically active plasmin in the incubation media (13). Incubation of THP-1 macrophages with either 125I-plasminogen or 125I-plasmin resulted in the accumulation of several degradative fragments in their conditioned media. The smaller plasmin fragments (~36 kDa) were enzymatically active but lacked cell-binding activity. Following their release from the macrophage surface, they were rapidly inactivated in the fluid-phase. The larger plasmin fragment (~48 kDa) appeared to be enzymatically inactive and bound to cells in a lysine-dependent manner.

In studies reported here, we have isolated inactive lysine-binding fragments of plasmin from cultures of murine RAW264.7 and thioglycollate-elicited peritoneal macrophages. These kringle-containing fragments are byproducts of the regulation of membrane-bound plasmin activity. They contain the entire sequence of angiostatin and inhibit bFGF-induced endothelial cell proliferation. The loss of membrane-bound plasmin activity and subsequent formation of angiostatin-like fragments were unaffected by inhibitors of metalloproteinase activity. Similar angiostatin-like plasmin fragments were recovered from the peritoneal cavities of mice following thiogly-

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collate-induced inflammation. This is the first demonstration that angiostatin forms in vivo in the absence of neoplasia. Thus, the activation of plasminogen on macrophage surface initiates plasmin proteolysis, which releases catalytically active fragments from the cell surface and generates inactive fragments with angiostatic properties.

MATERIALS AND METHODS

Cell Culture—Murine RAW264.7 macrophages were obtained from American Type Culture Collection (Rockville, MD). RAW264.7 cells were maintained as adherent cultures in Roswell Park Memorial Medium (RPMI; without HEPES) supplemented with 10% CellLect™ Gold fetal bovine serum (FBS),1 penicillin (100 units/ml), streptomycin (100 µg/ml), and 4 mM glutamine (ICN Pharmaceuticals, Costa Mesa, CA).

Isolation of Peritoneal Macrophages—Thiglycolate-elicited peritoneal macrophages were obtained from female mice by the method of Edelson and Cohn (14) as described previously (15). Mice were injected intraperitoneally (3 ml/mouse) with 3% Brewer thiglycollate medium containing 0.3 mM thiglycollate (Difco, Detroit, MI). Four 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.

Quantitative Plasmin Assay and Membrane-bound Plasmin Activity—Plasmin activity was quantitated by measuring the increase in fluorescence following the cleavage of the plasmin substrate: D-Val-Leu-Lys-aminomethyl-coumarin (Enzyme Systems Products, Dublin, CA) as described previously (13). Aliquots of cellular conditioned media were added to microtiter wells containing DPBS, 0.05% Tween-20 and the plasmin substrate. Samples were mixed and incubated at 37 °C for 2.5 h. Cleavage of the substrate was monitored in a Fluoroscan microplate reader. Plasmin activity in the test samples were extrapolated from a standard curve utilizing 0–40 ng/ml human plasmin (American Diagnostica, Diagnostica, Greenwich, CT).

Membrane-bound plasmin activity was quantitated by incubating macrophages with plasminogen in macrophage serum-free media (MSFM; Life Technologies, Inc.) for 2 h at 37 °C. Unbound plasmin was removed, and cells were washed with cold DPBS. MSFM containing the plasmin substrate was added and allowed to incubate 2.5 h. Fluorescence was monitored in a Fluoroscan microplate reader and extrapolated to the fluorescence generated by 0–40 ng/ml plasmin prepared in MSFM. The amounts of membrane-bound plasmin may be overestimated because it was calculated by extrapolation from a standard curve of fluid-phase plasmin. Since the catalytic activity of membrane-bound plasmin has been reported to be increased relative to fluid-phase plasmin (16). We have not, however, examined this possibility directly because we were interested in relative changes in membrane-bound plasmin over time.

Iodination of Lys-plasminogen—Human Lys-plasminogen (American Diagnostica) was iodinated according to the method of McFarlane (17) as described previously (13). Iodinated protein was separated from unincorporated125I utilizing a PD-10 column (Amersham Pharmacia Biotech) preequilibrated with HEPES buffered saline (137 mM NaCl, 4 mM KCl, 1 mM glucose, and 11 mM HEPES, pH 7.4) containing 0.5% human albumin.

Analysis of 125I-Plasmin Fragments by SDS-PAGE and Autoradiography—RAW246.7 macrophages were incubated with125I-Lys-plasminogen in MSFM for 1 and 24 h. Aliquots of conditioned media were diluted with SDS-sample buffer without β-mercaptoethanol and then boiled 3 min. Samples and molecular weight markers were applied to 4–15% polyacrylamide gradient gels. Proteins were transferred to a PVDF membrane and processed for NH2 terminal sequencing in the Cornell Medical College Microsequencing Facility (Dr. Daniel Wellner, Department of Biochemistry).

Endothelial Cell Proliferation Assay—The effect of plasmin fragments on endothelial cell proliferation was performed as described by Cao et al. (6). Low passage (p3–p4) bovine capillary endothelial cells were cultured in RPMI containing 10% FBS and 3 ng/ml bFGF. Cells were harvested by trypsinization, resuspended in media containing 10% FBS without bFGF, and dispersed into gelatinized wells. Following a 24 h incubation, cells were preincubated (30 min) with plasminogen or the affinity isolated plasmin fragments prior to the addition of 1 ng/ml bFGF. Cells were incubated 72 h and counted.

RESULTS

Proteolysis of Membrane-bound Plasmin Generates Enzymatically Active Fragments without Cell Binding Properties—In the absence of exogenous plasminogen, RAW264.7 macrophages express negligible membrane-bound plasmin activity (Fig. 1). Following a 1 h incubation, 66% of the Lys-plasminogen added to the culture media was converted to plasmin by the murine macrophage cell line. Membrane-bound plasmin activity was ~30 ng/10⁴ cells. Virtually all of the added plasminogen was converted to plasmin by a 24-h incubation with RAW264.7 cells. Nonetheless, membrane-bound plasmin activity had fallen ~50%. Thus, RAW264.7 macrophages exhibit a loss in membrane-bound plasmin activity despite the presence of active plasmin in the media. In a similar experiment, we examined by SDS-PAGE autoradiography alterations in125I-Lys-plasminogen incorporated into RAW264.7 macrophages for 1 and 24 h. Under nonreducing conditions, Lys-plasminogen in the 1-h conditioned media comigrated with intact125I-Lys-plasminogen (Fig. 1). In contrast, in the 24-h-conditioned media, several degradative fragments of125I-Lys-plasminogen were visible.

Plasmin in the fluid-phase is rapidly inactivated by plasminogen. Cells (15–20 × 10⁶) were washed three times with DPBS, and media was replaced with MSFM containing 2 ng of Lys-plasminogen for 24–30 h. Macrophage-conditioned media (15 ml) were slowly applied to a column containing 5 ml of lysine-Sepharose. Fractions of 1 ml were collected. Unbound proteins were washed from the column with DPBS containing 0.02% azide. When the absorbance at 280 nm of the column eluate returned to 0, proteins bound to the lysine-Sepharose were eluted with 50 mM eACA in DPBS. Peak fractions were collected and dialyzed against DPBS to remove eACA and azide. Following dialysis, the affinity isolated plasmin fragments were tested for the presence of endotoxin utilizing the Quantitative Chromogenic Limulus Amebocyte Lysate assay (BioWhittaker,Walkersville, MD).

SDS-PAGE and Western Blot Characterization of Kringle-containing Plasmin Fragments—Aliquots of the peak fractions were diluted with SDS-sample buffer without β-mercaptoethanol. Column fractions, intact Lys-plasminogen, and molecular weight markers were run in 4–15% polyacrylamide gradient gels, fixed 1 h in 20% methanol containing 10% acetic acid, and stained overnight in Coomassie Blue. Lys-plasminogen, eACA eluates, and biotinylated molecular weight markers (Amer sham Pharmacia Biotech) were electrophoresed in 4–15% polyacrylamide gradient gels. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was blocked in fresh 5.0% dry defatted milk in Tris-buffered saline, 0.05% Tween-20 (TTBS) for 1 h (4 °C), washed two times with TTBS for 5 min, and was incubated for 3 h in TTBS containing a monoclonal antibody directed against kringle 1–4 of human plasminogen (2.5 µg/ml; American Diagnostica) for 1 h. The membrane was washed (two times; TTBS), incubated for 1 h in 3% dry defatted milk in TTBS containing biotinylated rabbit anti-mouse IgG (1:10,000; Pierce, Rockford, IL), washed (two times; TTBS), and incubated 1 h with preformed avidin-biotin-horseradish peroxidase complexes (Pierce) in DPBS, 0.1% Tween-20. Bound horseradish peroxidase was visualized utilizing enhanced chemiluminescence (Amer sham Pharmacia Biotech, Burlington, IL).

Amino Terminal Sequence Analysis of Plasmin Peptides—Kringle-containing plasmin fragments were electrophoresed in 4–15% polyacrylamide gradient gels. Proteins were transferred to a PVDF membrane. The membrane was stained with 0.25% Coomassie Blue in 50% methanol without acetic acid. Protein bands were cut from the PVDF membrane and processed for NH2 terminal sequencing in the Cornell Medical College Microsequencing Facility (Dr. Daniel Wellner, Department of Biochemistry).

The abbreviations used are: FBS, fetal bovine serum; DPBS, Dulbecco's phosphate-buffered saline; MSFM, macrophage serum-free media; PAGE, polyacrylamide gel electrophoresis; TGF, transforming growth factor; PVDF, polyvinylidene difluoride; TTBS, Tween 20 with Tris-buffered saline; eACA, ε-amino-n-caproic acid; bFGF, bovine fibroblast growth factor; 1 The abbreviations used are: FBS, fetal bovine serum; DPBS, Dulbecco's phosphate-buffered saline; MSFM, macrophage serum-free media; PAGE, polyacrylamide gel electrophoresis; TGF, transforming growth factor; PVDF, polyvinylidene difluoride; TTBS, Tween 20 with Tris-buffered saline; eACA, ε-amino-n-caproic acid; bFGF, bovine fibroblast growth factor.
followed by autoradiography. The arrow points to a plasmin fragment that was previously determined to be enzymatically active by zymography (13).

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Fig. 1. Loss of membrane-bound plasmin activity is associated with plasmin proteolysis in cultures of RAW264.7 macrophages. Cells were plated in RPMI, 10% FBS and allowed to adhere for 4 h. Serum containing media were removed, the cells were washed (three times) with DPBS, and media were replaced with MSFM alone or MSFM containing 1 µg/ml plasminogen. Following incubation for 1 or 24 h at 37 °C, the conditioned media were recovered, and the cells were washed with DPBS. Plasmin activities in the conditioned media and bound to the cell surfaces were quantitated as described under “Materials and Methods.” Data represent the mean ± S.E. of six separate wells. In other studies, cells were incubated with MSFM containing 125I-Lys-plasminogen (1 µg/ml). Conditioned media were collected at 1 or 24 h and analyzed by SDS-PAGE followed by autoradiography. The arrow points to a plasmin fragment that was previously determined to be enzymatically active by zymography (13).

Fig. 2. Isolation and characterization of kringle-containing plasmin fragments derived from cultures of RAW264.7 macrophages. Cells were primed with TGF-β1 to up-regulate their uPA and uPA receptor expression (18). Cells were washed to remove serum, and media were replaced with MSFM containing 2 mg of Lys-plasminogen. A, following 30 h incubation at 37 °C, conditioned media were applied to a lysine-Sepharose column, and bound plasmin fragments were eluted with the lysine analogue eACA. B, proteins in the eACA eluate were analyzed by SDS-PAGE. C, Kringle-containing plasmin fragments were detected by Western blot.

inhibitors under physiologic conditions (19). Therefore, we determined whether the decline in RAW264.7 membrane-bound plasmin activity observed in Fig. 1 would occur in the absence of fluid-phase plasmin activity. When macrophages were incubated with Lys-plasminogen for 1 h in the presence of 10-fold excess of α2-plasmin inhibitor, no plasmin activity was detected in their conditioned media. Despite the absence of fluid-phase plasmin activity, membrane-bound plasmin activity was 6.6 ± 1.3 ng per 10^5 cells (mean ± S.E.; n = 6). Following 24 h of incubation, plasmin activity in the media remained undetectable, and membrane-bound activity fell to 1.3 ± 0.1 ng per 10^5 cells. Conditioned media from cells incubated with 125I-Lys-plasminogen and α2-plasmin inhibitor for 1 h contained both intact 125I-Lys-plasminogen and α2-plasmin inhibitor complexes. Moreover, despite the absence of detectable plasmin activity in 24-h conditioned media, degradative plasmin fragments were visible (data not shown). Taken together, these data demonstrate that following plasminogen activation by RAW264.7 macrophages, membrane-bound plasmin is proteolyzed, resulting in the formation of enzymatically active and inactive fragments, and a loss of membrane-bound plasmin activity.

Isolation and Characterization of Kringle-containing Plasmin Fragments—RAW264.7 macrophages’ expression of uPA and uPA receptor were up-regulated by incubation with TGF-β1 (18). TGF-β1-primed macrophages were incubated for 24 h in serum-free medium containing Lys-plasminogen. Conditioned media were applied to a column of lysine-Sepharose, and fragments of plasmin exhibiting lysine-binding properties were eluted with eACA (Fig. 2A). The eACA eluate was characterized by SDS-PAGE and Western blot utilizing a monoclonal antibody directed against plasminogen kringle 1–3. Coomassie Blue-stained gels revealed that the eluates contained a small amount of intact plasminogen, a prominent 48-kDa fragment and two minor plasmin fragments (42 and 50 kDa) (Fig. 2B). All three plasmin fragments were immunoreactive with anti-kringle IgG (Fig. 2C). Plasmin enzymatic activity expressed by the affinity isolated plasmin fragments was 1000-fold less than similar amounts of intact plasmin (data not shown).

Amino terminal sequence analysis of the individual plasmin fragments was determined to identify the regions of plasminogen from which the fragments were derived (Table I). The NH2-terminal sequence of the plasmin fragments contained that each peptide began at Lys77, the amino-terminal of Lys-plasminogen. Based on the NH2-terminal sequence and molecular weights determined by SDS-PAGE, the plasmin fragments contained the entire sequence of angiostatin (38 kDa; Thr308-Val314). In summary, these data indicate that plasmin fragments isolated from RAW264.7 conditioned media exhibit lysine-dependent binding, are enzymatically inactive, are immunoreactive with anti-kringle 1–3, and contain angiostatin.

Formation of Kringle-containing Plasmin Fragments by Inflammatory Peritoneal Macrophages in Vitro—We next deter-
mimned whether proteolysis of membrane-bound plasmin by fully differentiated macrophage results in loss of membrane-bound plasmin activity and the formation of enzymatically inactive kringle-containing fragments. Thioglycollate-elicited macrophages were incubated with Lys-plasminogen for 1 and 24 h, following which membrane-bound plasmin activity was determined. As reported for RAW264.7 macrophages (Fig. 1), membrane-bound plasmin activity expressed by inflammatory macrophages decreased over 24 h despite the presence of active plasmin in the media (data not shown). Fragments of plasmin in 24-h conditioned media, exhibiting lysine-binding properties, were affinity purified as described above. The aCA-eluted plasmin fragments isolated from the media of inflammatory macrophages exhibited an identical pattern in SDS-PAGE as those isolated from RAW264.7 media (Fig. 3A). Likewise, intact plasmin, plasmin fragments, and higher molecular weight forms were immunoreactive with anti-kringle IgG (Fig. 3B). NH2-terminal sequence analysis of the 48-kDa fragment indicated that the plasmin fragments began at Lys77 and contained the entire sequence of angiotatin (data not shown).

Recovery of Kringle-containing Plasmin Fragments from Peritoneal Exudates in Vivo—To determine whether plasmin proteolysis occurs under inflammatory conditions in vivo, sterile exudates were elicited in the peritoneal cavities of mice via intraperitoneal injection of 3% Brewer thioglycollate medium containing 0.3 ma thioglycollate. After 4 days, the peritoneum was lavaged with DPBS containing aCA. The recovered macrophages were removed by centrifugation, and the supernatant was concentrated by ultrafiltration. Fragments of plasmin exhibiting lysine-binding property were affinity purified as described above. Western blot utilizing a monoclonal antibody directed against human plasmin kringle 1–3 revealed plasmin fragments (Fig. 4), which were similar in size to those derived from RAW264.7 macrophages and thioglycollate-elicited peritoneal macrophages in vitro (Figs. 2 and 3). These data are the first to demonstrate that angiotatin-like plasmin fragments are generated in vivo under non-neoplastic conditions.

Because thioglycollate is a reducing agent, we determined whether the fragments of plasmin recovered from peritoneal exudates induced by intraperitoneal administration of Brewer thioglycollate medium were derived from simple reduction of plasmin. For this purpose, both plasminogen and plasmin were incubated with 3% Brewer thioglycollate medium or 5% β-mercaptoethanol for 1 and 4 h at 37°C. The samples were analyzed by SDS-PAGE (under non-reducing conditions) and Western blot utilizing monoclonal anti-kringle 1–3 IgG. Incubation with Brewer thioglycollate medium did not effect the appearance of plasminogen or plasmin in Western blots. In contrast, reduction with β-mercaptoethanol resulted in a complete loss of reactivity with the anti-kringle 1–3 IgG (data not shown). Together with the observation that elicited peritoneal macrophages generated plasmin fragments in vitro (Fig. 3), it is highly unlikely that the kringle-containing plasmin fragments identified in the peritoneal exudates were because of simple reduction of plasmin by thioglycollate.

Plasmin Proteolysis Is Not Dependent on Macrophage Metalloproteinase Activity—In mice with Lewis lung carcinoma, the malignant cells and host macrophages appear to cooperate in the generation of angiotatin (7). Tumor cell derived GM-CSF induces macrophage MMP-12 expression, which is responsible for proteolysing plasminogen to form angiotatin (7). Therefore, we determined whether metalloproteinases were responsible for the degradation of plasmin observed in RAW264.7 cultures and their subsequent loss in membrane-bound plasmin activity. For this purpose, cells were incubated with Lys-plasminogen in the presence of either of two inhibitors of metalloproteinases: tissue inhibitor of metalloproteinases 1 (TIMP-1) or EDTA. As seen in Fig. 5 (top left), 1-h plasmin binding to RAW264.7 cells was unaffected by TIMP-1. Following 24-h incubation (Fig. 5; bottom), membrane-bound plasmin activity declined 90% and was unaffected by the presence of TIMP-1. Similar results were observed utilizing EDTA (Fig. 5; bottom).
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FIG. 5. Effect of metalloproteinase inhibitors on the loss of RAW264.7 macrophage membrane-bound plasmin activity. Cells (10^4) were aliquoted into 96-well plates in RPMI, 10% FBS. Following adherence for 4 h, cells were washed to remove serum, and media were replaced with MSFM alone or MSFM containing plasminogen (1 μg/ml). Cells were incubated with plasminogen alone or plasminogen and eACA (25 μM), TIMP-1 (300 ng/ml), or EDTA (200 μg/ml). Cells were incubated for 1 or 24 h, media were removed, and cells were washed. Membrane-bound plasmin activity was determined as described under "Materials and Methods." Data represent the mean ± S.E. of six separate wells.

FIG. 6. Effect of metalloproteinase inhibitors on plasmin proteolysis. Cells (0.5 × 10^5) were aliquoted into 12-well plates in RPMI, 10% FBS. Following adherence for 4 h, cells were washed to remove serum, and media were replaced with MSFM alone or MSFM containing plasminogen (2 μg/ml). Cells were incubated with plasminogen alone or plasminogen and eACA (25 μM), TIMP-1 (300 ng/ml), or EDTA (200 μg/ml). Cells were incubated for 1 or 24 h, and media were removed and analyzed for kringle-containing plasmin fragments by Western blot.

left and right). Moreover, when 24-h conditioned media were examined for the presence of kringle-containing plasmin fragments utilizing Western blots, neither TIMP-1 nor EDTA blocked plasminogen proteolysis (Fig. 6).

Kringe-containing Plasmin Fragments Inhibit Basic Fibroblast Growth Factor-induced Endothelial Cell Proliferation—We determined the ability of kringle-containing plasmin fragments, isolated from macrophage-conditioned media, to inhibit bFGF-induced bovine capillary endothelial cell proliferation utilizing procedures described for angiostatin (5, 6). For these experiments, we utilized affinity purified plasmin fragments that contained small amounts of intact plasmin assessed by Western blot. Two separate experiments were performed in which the initial endothelial cell plating densities varied (Table II). In the first experiment, the numbers of cells grown in media containing FBS increased 6-fold during the 4-day experimental period. When media containing FBS was supplemented with bFGF, cell numbers increased ~18-fold over the initial plating density. The addition of intact plasminogen had no effect on cell numbers. In contrast, the number of endothelial cells was markedly reduced when incubated with media containing FBS, bFGF, and macrophage-derived plasmin fragments. In the second experiment, the ability of macrophage-derived plasmin fragments and an elastase-derived fragment of plasminogen to inhibit bFGF-induced proliferation were compared. Cells grown in media containing FBS increased in number 11-fold over the initial plating density. As was the case in experiment 1, incubation with intact plasminogen had no effect on cell numbers; whereas, the numbers of cells were similarly reduced when incubated with the plasmin fragments.

To test whether contaminating endotoxin was responsible for the observed inhibition of endothelial cell growth, intact plasminogen and purified plasmin fragments were tested for the presence of endotoxin utilizing a colorimetric modification of the limulus amebocyte assay. When corrected for a false positive reaction because of the presence of enzymatically active plasmin in the various preparations, intact plasminogen, macrophage-derived angiostatin fragments, and K1–3 fragment contained ≤ 0.05 ng/ml endotoxin. Because intact plasminogen
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consistently had no effect on the endothelial cell numbers, it is unlikely that the anti-proliferative effect of the plasmin fragments was because of contaminating endotoxin.

**DISCUSSION**

The observation that angiostatin is generated in mice with Lewis lung carcinoma (5) suggested that similar angiostatic fragments of plasminogen may form under non-neoplastic settings in which angiogenesis is commonly observed. In studies reported here, angiostatin-like plasmin fragments were recovered from the peritoneal cavities of mice following thioglycollate-induced inflammation. This is the first in vivo demonstration that angiostatin forms in mice in the absence of neoplasia.

The mechanism by which angiostatin-like plasmin fragments form was studied in vitro. We previously reported that proteolysis of membrane-bound plasmin was a mechanism by which macrophages localize and regulate pericellular plasmin activity. A byproduct of this regulatory mechanism was the generation of an inactive plasmin fragment, which was bound to the cell surface and found in the fluid-phase (13). Utilizing affinity chromatography, we have isolated lysine binding plasmin fragments from cultures of RAW264.7 macrophages and thioglycollate-elicited peritoneal macrophages. The plasmin fragments are enzymatically inactive, contain kringle 1–4, and inhibit bFGF-induced endothelial cell proliferation. Thus, in addition to regulating pericellular plasmin activity, proteolysis of membrane-bound plasmin generates inactive kringle-containing fragments expressing angiostatic properties.

The proteolysis of membrane-bound plasmin is either auto-proteolytic or plasmin activates metalloproteinases, which are responsible for plasmin proteolysis. Supporting the later hypothesis are the observations that cleavage of plasminogen by several metalloproteinases has been identified as a mechanism for the formation of angiostatin (7–9). However, incubation of RAW264.7 macrophages with plasminogen and inhibitors of metalloproteinases (TIMP-1 and EDTA) did not effect the loss of membrane-bound plasmin activity or plasmin fragmentation. Consistent with these data is the observation that proteolysis of membrane-bound plasmin was not observed when macrophages were incubated with active site-inhibited plasmin (13). Taken together, we conclude that autoproteolysis of plasmin is responsible for the generation of angiostatin-like fragments.

The ability of angiostatin to block tumor angiogenesis may be because of its anti-proliferative effect on endothelial cells (5, 6). Both purified and recombinant angiostatin inhibit bFGF-induced endothelial cell proliferation (5, 6). The anti-proliferative property of angiostatin is also expressed by individual recombinant kringle 1, 2, and 3 (6). Kringle 4 was not effective. Moreover, the removal of kringle 4 from angiostatin enhanced its anti-proliferative activity. These data suggest that the anti-proliferative activity of angiostatin can be shared by similar but non-identical kringle-containing plasmin fragments. Supporting this conclusion is the observation that lysine-binding plasmin fragments isolated from macrophage-conditioned media, which are larger (42–50 kDa) than angiostatin, inhibited bFGF-induced endothelial cell proliferation. The observed inhibition between experiments was variable, but the macrophage-derived plasmin fragments were as effective as a fragment of plasmin containing kringle 1–3. Recently, the anti-proliferative activity of angiostatin has been reported to be because of increased apoptosis ((20). Whether the decrease in endothelial cell number induced by macrophage-derived angiostatin fragments is because of increased apoptosis or decreased cell division is the subject of ongoing studies.

These studies have not addressed what significance, if any, the generation of plasmin fragments by macrophages might have on angiogenesis in non-neoplastic settings. However, macrophages are a prominent cellular component of chronic inflammation and are often associated with wound healing. Macrophages are capable of expressing a variety of angiogenic and angiostatic factors (21). Recent experiments utilizing mice deficient in plasminogen have verified the important role of plasminogen activation in the ability of macrophages to migrate to sites of injury and inflammation, and their removal of necrotic debris and fibrin (22–25). In studies reported here, we have demonstrated that macrophages produce angiostatin-like plasmin fragments as a byproduct of proteolysis of membrane-bound plasmin in vitro. In addition, we demonstrate for the first time that angiostatin-like plasmin fragments are generated in a non-neoplastic inflammatory setting in vivo. Thus, proteolysis of membrane-bound plasmin may contribute to the regulation of angiogenesis observed in chronic inflammation and wound healing.

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