ABSTRACT

Angiostatin4.5 (AS4.5) is a naturally occurring human angiostatin isoform, consisting of plasminogen kringle 1–4 plus 85% of kringle 5 (amino acids Lys578 to Arg529). Prior studies indicate that plasminogen is converted to AS4.5 in a two-step reaction. First, plasminogen is activated to plasmin. Then plasmin undergoes autoproteolysis within the inner loop of kringle 5, which can be induced by a free sulfhydryl donor or an alkaline pH. We now demonstrate that plasminogen can be converted to AS4.5 in a cell membrane-dependent reaction. Actin was shown previously to be a surface receptor for plasminogen. We now show that β-actin is present on the extracellular membranes of cancer cells (PC-3, HT1080, and MDA-MB231), and β-actin can mediate plasmin binding to the cell surface and autoproteolysis to AS4.5. In the presence of β-actin, no small molecule-free sulfhydryl donor is needed for generation of AS4.5. Antibodies to actin reduced membrane-dependent generation of AS4.5 by 70%. In a cell-free system, addition of actin to in vitro-generated plasmin resulted in stoichiometric conversion to AS4.5. Annexin II and α-enolase have been reported to be plasminogen receptors, but we did not demonstrate a role for these proteins in conversion of plasminogen to AS4.5. Our data indicate that membrane-associated β-actin, documented previously as a plasminogen receptor, is a key cell membrane receptor capable of mediating conversion of plasmin to AS4.5. This conversion may serve an important role in regulating tumor angiogenesis, invasion, and metastasis, and surface β-actin may also serve as a prognostic marker to predict tumor behavior.

INTRODUCTION

Tumor growth and development consists of two stages: a prevascular stage and a vascular or angiogenic stage (1–7). In the prevascular phase, tumors are noninvasive and typically not clinically detectable (1, 8, 9). Tumor growth is restricted by the absence of neovascularization, resulting in a balance of cancer cell proliferation and apoptosis (10). Angiogenesis, the process of generation of new microvessels from existing vasculature, is necessary for the vascular phase, which is characterized by rapid tumor growth and metastatic possibility. In tumor development, a nonmetastatic lesion can progress to an aggressive metastatic cancer, in which the switch of tumor cells from a nonangiogenic phenotype to an angiogenic phenotype plays an essential role (9, 11). Angiogenesis is controlled by a balance of angiogenesis promoters, such as vascular endothelial cell growth factor and basic fibroblast growth factor, and angiogenesis inhibitors, such as thrombospondin-1 (9, 12, 13). In invasive cancers, an increase in expression of an angiogenesis promoter, such as vascular endothelial cell growth factor expression, or loss of an antiangiogenic factor, such as thrombospondin-1, initiates an angiogenic cascade (13–15).

O’Reilly et al. (16) described angiostatin, an internal fragment of plasminogen, which is a potent inhibitor of angiogenesis. Plasminogen is the zymogen form of the serine protease plasmin and contains five consecutive kringle domains (17). Plasminogen is cleaved by a plasminogen activator through the hydrolysis of the Arg561-Val562 peptide bond to yield the two-chain serine protease, plasmin, which is the primary fibrinolytic enzyme. As originally described, angiostatin possesses the first three or four of the five kringle domains of plasminogen (16). A variety of proteinases may cleave plasminogen to form angiostatin-related proteins, with a range of NH2 and COOH termini, and varied degrees of antiangiogenic activity (16, 18–23). We and others showed previously that in a human system, plasminogen is converted to angiostatin via plasmin autoproteolysis, which may be mediated by a free sulfhydryl donor (22, 24–26). This reaction results in an intra-kringle 5 cleavage after amino acids Arg530 or Lys531. The resulting angiostatin isoform is referred to as Angiostatin4.5 (AS4.5), because it includes kringle 1–4 plus 85% of kringle 5 (Lys578-Arg529). An angiostatin isoform with identical amino acid sequence has been generated by Cao et al. (27, 28), using plasmin autoproteolysis in an alkaline pH, and Stathakis et al. (25, 26), also by plasmin autoproteolysis.

AS4.5 has been shown to be a potent angiogenesis inhibitor, inhibiting Lewis lung carcinoma and hemangiendothelioma (EOA) tumors in mice (22, 29). In addition, with Mauceri et al. (30), we have shown that AS4.5 may potentiate the cytotoxic effect of ionizing radiation in murine tumor models. In our studies, as well as those of Cao et al. (27, 28), AS4.5 has been shown to be significantly more potent than angiostatinK1–3 and angiostatinK1–4 (24), suggesting the functional importance of the amino acid sequences/structures within kringle 5.

We now show that plasminogen can be converted to AS4.5 on the surface of human cancer cell lines, including PC-3 prostate, HT1080 fibrosarcoma, and MDA-MB231 breast cancer, in the absence of a small molecule-free sulfhydryl donor. Annexin II (and annexin II tetramer), α-enolase, and actin, have all been shown previously to be on the extracellular surface of cells and have been reported to be plasminogen receptors (31–37). In the following studies we demonstrate that β-actin is present on the extracellular surface on all three of the cell lines tested and that it serves a key role in conversion of human plasminogen to AS4.5.

MATERIALS AND METHODS

Materials

Plasminogen purification was described previously (22–24). Inhibitors of plasmin formation/action were as follows: Pefabloc (Roche, Indianapolis, IN), α2-antiplasmin (Sigma, St. Louis, MO), plasminogen-activator inhibitor-1 (American Diagnostica Inc., Greenwich, CT), and e-aminoacapropic acid (Acros Organics, Morris Plains, NJ).

Cell Culture

PC-3 (Human Prostate adenocarcinoma cells), HT1080 (Human fibrosarcoma cells), and MDA-MB231 (human breast adenocarcinoma cells) were from American Type Culture Collection (Manassas, VA). PC-3 cells were cultured in DMEM with 10% (v/v) fetal bovine serum, 100 units/ml penicillin G, 100 units/ml streptomycin, and 2.5 μg/ml amphotericin B (Invitrogen, Carlsbad, CA). HT1080 cells (fibrosarcoma) were cultured in DMEM with 10% heat-inactivated FCS. MDA-MB231 cells (breast cancer) were cultured in RPMI 1640 with 10% heat-inactivated FCS. HMVEC (human neonatal dermal microvascular endothelial cells; Passage 6) and NHDF (human neonatal dermal fibroblasts; Passage 8) were from BioWhittaker, Inc. (Walkersville, MD).
HMVEC were cultured in EGM-MV Bulletkit (BioWhittaker, Inc.). NHDF were cultured in FGM-2 Bulletkit (BioWhittaker, Inc.).

### Plasminogen to AS4.5 Conversion on Fixed Cells

Confluent PC-3 cells in 24-well-plates were fixed with 0.5% glutaraldehyde in PBS for 10 min. Cells were then washed with PBS and used for analysis of plasminogen conversion to AS4.5. Forty μg/ml (440 nm) of human plasminogen in 300 μl of HEPES-buffered saline (pH 7.5; HBS) was added to the wells. At various time points, aliquots of the supernatant were removed and assayed by Western blot or ELISA for AS4.5 and plasminogen.

### PC-3 Membrane Fractions

Confluent PC-3 cells were scraped from plates, washed three times with PBS, and lysed by three freeze-thaw cycles. After each freeze-thaw cycle, the membrane fraction was pelleted by centrifugation (1500 × g), and the pellet was washed with PBS. The membrane pellet was resuspended in HBS. Annexin II (p36) was removed from the cell membrane as described (38). Membrane aliquots were suspended in HBS with 30 mM CHAPS [3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Bio-Rad, Her-Cules, CA] and 5 mM EGTA (Sigma) at 4°C for 1 h. Control aliquots were processed using HBS alone. Membranes were pelleted at 15,000 rpm for 5 min in an Eppendorf microcentrifuge to separate supernatant and pellet. Pellets were washed and resuspended in HBS. Plasminogen was incubated at 37°C with the control PC-3 membrane fraction or CHAPS:EGTA-treated PC-3 membrane fraction from which annexin II was removed. At various time points, aliquots of the supernatant were removed and assayed for AS4.5 content.

### AS4.5 Generation in Fluid Phase

AS4.5 was generated by plasminogen, urokinase and N-acetyl-l-cysteine (Sigma) as described previously (22).

### Western Blot Assay

The Western blot assay was performed as described previously (22, 23), using anti-kringle 1-3 monoclonal antibodies (VAP, a gift from V. Ploplis and F. Castellino, Notre Dame University, Notre Dame, IN, and GMA086 from Green Mt. Antibodies, Burlington, VT).

### AS4.5 ELISA

**COOH-Terminal Peptide Antiserum.** An 18 amino acid peptide of the COOH terminus of AS4.5 was generated with the sequence NH2-RNPDGD-AVVAYAYNNPR-COOH, and was purified by reversed-phase high-performance liquid chromatography to >95% purity (Multiple Peptide Systems, San Diego, CA). The peptide was conjugated 1:1 (w/w) through the α-amino group to keyhole limpet hemocyanin using glutaraldehyde as the cross-linking agent. Polyclonal antiserum was generated in New Zealand White rabbits.

**Preparation of AS4.5-Specific Antibody.** AS4.5-Sepharose was used to affinity purify the antibodies to the COOH-terminal peptide. This antibody bound both AS4.5 and plasminogen. To remove the subpopulations of the antibodies that reacted with plasminogen (but presumably not with AS4.5), 20-fold mass excess of plasminogen was added to the antibody and incubated overnight at 4°C. The plasminogen:antibody complex was the excess plasminogen was removed with lysine-Sepharose chromatography. The COOH-terminal-specific antibodies in the flow-through from the lysine-Sepharose were collected and concentrated. Approximately 70% of the antibody was removed by this procedure, but the remaining anti-AS4.5 antibody did not cross-react with human plasminogen by Western blot and subsequent ELISA.

**ELISA Assay.** Microtiter plates (Fisher Scientific, Hanover Park, IL) were coated with AS4.5 (10 μg/ml) in binding buffer (0.2 M NaHCO3, pH 9.2) for 1 h and washed three times with TBT (10 mM Tris, 150 mM NaCl, 0.2% BSA, and 0.1% Tween 20). Samples or standard AS4.5 sources were incubated with AS4.5-specific antibody at room temperature for 1 h and then mixtures added to the AS4.5-coated wells. After incubation for 1 h, the wells were washed with TBT. Goat-antibrit IgG-conjugated with alkaline phosphatase (0.002 mg/ml; Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and incubated for 1 h. After washing three times with TBT, 200 μl/well of phosphatase substrate solution [1 mg/ml p-nitrophenyl phosphate (Sigma), in 0.05 M NaHCO3 and 1 mM MgCl2] was added to the wells and incubated for 6 h and A405 measured.

### Endothelial Cell Proliferation Assay

Bovine aortic endothelial cells were plated in 24-well culture dishes at 3.3 × 10^3 cells/well in DMEM supplemented with 2.5% heat-inactivated calf serum, 1% penicillin-streptomycin, and 1% amphotericin B. The following day, fresh medium supplemented with 3 ng/ml human basic fibroblast growth factor (Becton Dickinson, Bedford, MA) alone or basic fibroblast growth factor supplemented with 200 nm of PC-3 cell-generated AS4.5 was added. For positive control, 200 nM affinity purified cell-free produced AS4.5 was used. After 72 h of treatment, the cell number was determined by counting from duplicate wells, using an automated cell counter (Beckman Coulter Corp., Miami, FL).

### Plasmin Activity on Fixed Cell Surface

Glutaraldehyde-fixed PC-3 cells in 24-well plates were incubated with 5 μg/ml (55 nm) plasminogen in HBS at 37°C. At 1, 3, 7, and 20 h, aliquots of cells were washed with PBS twice and chromogenic plasmin substrate [0.33 μg/ml n-Val-Leu-Lys p-nitroanilide (Sigma) in HBS] added at 37°C. A405 was read for these solutions at the end of 1 h.

**Generation of AS4.5 by Actin.** Fifty μM human nonmuscle actin (Cytoskeleton, Inc., Denver, CO) was incubated with 440 nm plasminogen with and without urokinase. The AS4.5 generated was measured by ELISA.

### Inhibition of AS4.5 Generation by Antiactin Antibody

Plasminogen (440 nm) was incubated with PC-3 cell membrane fraction or glutaraldehyde-fixed PC-3 cells in the presence or absence of an antibody to the COOH terminus of actin (Sigma). AS4.5 generation was measured by ELISA.

### Detection of Actin on Cell Membrane

Membrane lysates from PC-3 cells, HT1080 cells, and MDA-MB231 cells were prepared by freeze-thaw lysis (see above). Membranes from ~10^5 cells were loaded onto SDS-PAGE gels and transferred to polyvinylidene difluoride paper. These Westerns were developed with monoclonal antibodies to α-actin, β-actin, or the COOH terminus of actin (common to all isoforms; Sigma). Parallel Westerns were performed with PC-3 cells grown for 72 h in serum-free medium to eliminate possible sources of exogenous actin.

### Immunofluorescence Staining

The surface and intracellular immunofluorescence staining was done using a similar protocol to that described previously (39). For surface proteins on nonpermeabilized cells, PC-3 cells grown on gelatin-coated coverslips were washed three times in PBS and then incubated for 3 h at 4°C with FITC-conjugated monoclonal antibody to the COOH terminus of actin (Sigma) or with FITC-conjugated monoclonal anticytokeratin 7 antibody (Sigma). Cells were washed three times with PBS and fixed in 3.7% formaldehyde for 5 min. Coverslips were washed in PBS and mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA). For intracellular staining, PC-3 cells grown on gelatin-coated coverslips were washed with PBS and fixed in 3.7% formaldehyde for 5 min. The fixed PC-3 cells were permeabilized in −20°C acetone for 3 min, and then incubated for 3 h at 4°C with FITC-conjugated monoclonal antibody to the COOH terminus of actin or with FITC-conjugated monoclonal anticytokeratin 7 antibody. Coverslips were washed in PBS and mounted in Vectashield mounting medium.

### Urokinase (uPA) Activity Measurement on Cell Membrane Fractions

Cell membrane fractions were collected from 5 × 10^6 cells after three freeze-thaw cycles. Cell membrane fractions were incubated with 30 μl of 3 mM S2444 (Chromogenix, Franklin, OH) and 270 μl of PBS at room temperature for 1 h. Two hundred μl of liquid was removed to measure A405 upA from Abbott Laboratories (Abbott Park, IL) was used to make the standard curve.
RESULTS

To determine whether the factors necessary and sufficient to convert plasminogen to AS4.5 are present on the surface of PC-3 cells, plasminogen was incubated with glutaraldehyde-fixed PC-3 cells at 37°C and aliquots were tested for conversion to AS4.5 by Western blot, and there was a complete conversion of plasminogen to AS4.5. When plasminogen was added to a suspension of the PC-3 plasma membranes from which all of the cytoplasmic and nonmembrane bound proteins and solutes had been removed, plasminogen was again completely converted to AS4.5 (Fig. 1A). The protein product was confirmed to be angiostatin based on binding to two different monoclonal antibodies to the kringle domains of human plasminogen (VAP and GMA086), by binding to lysine-Sepharose, and ability to inhibit proliferation of vascular endothelial cells (Fig. 1B). The angiostatin isoform was determined to be AS4.5 based on the molecular size (Mr 55,000) as well as the Western blot analysis and ELISA (see below) with a polyclonal antibody directed to the COOH-terminal 18 amino acids of AS4.5. No angiostatin isoforms other than AS4.5 were observed by Western blot. These data indicate that constituents necessary and sufficient to convert plasminogen to AS4.5 are on the cell membrane, and that nonmembrane-associated, small molecule-free thiol donors are not necessary for the plasmin autoproteolysis.

An ELISA specific for AS4.5 was developed using an affinity-purified polyclonal rabbit antiserum to an 18 amino acid peptide from the COOH terminus of AS4.5. The COOH terminus of AS4.5 is within the inner loop of kringle 5 (24, 25), and the specificity of the ELISA derived from the fact that the antibody does not bind to the peptide sequence within the intact kringle 5 of plasminogen, but does bind to the cleaved kringle 5 of AS4.5 (24). The assay was sensitive to AS4.5 from 0.5 to 50 μg/ml (10–1000 nm) and insensitive to plasminogen at up to 50 μg/ml. The ELISA was used to measure AS4.5 generated from plasminogen by cancer cell membranes as well as in cell-free, fluid-phase reactions (Fig. 2). Note that at the 0-h starting time point, in the presence of 40 μg/ml (440 nm) plasminogen, the ELISA result was 0 nm. This confirmed that the human plasminogen substrate did not cross-react and interfere with the assay. By 6 h, 330 nm of AS4.5 was detected of the theoretical maximum of 440 nm, based on amount of plasminogen added. By 20 h, the reaction reached stoichiometric conversion of plasminogen to AS4.5. This ELISA, based on the specific antibody for AS4.5, also confirmed that the AS isoform being generated was AS4.5 and not the kringle 1–3 or 1–4 isoforms observed in murine models (16).

Whereas we and others have published previously that plasminogen may be converted to AS4.5 by plasmin autoproteolysis mediated by disulfide reduction (22, 24, 26–28), other groups have reported that plasminogen may be converted to other angiostatin isoforms (i.e., K1–3 and K1–4) by other proteolytic mechanisms (16, 18–23). If plasmin autoproteolysis is occurring on the cell surface, a plasminogen activator must be present to generate the plasmin intermediate. Western blots of PC-3, MDA-MB231, and HT1080 cell-conditioned media, as well as washed cell membranes all demonstrated the presence of uPA. In contrast, tissue plasminogen activator antigen was not detected in the conditioned media or washed cell membranes (data not shown). Plasminogen activation to plasmin by fixed PC-3 cell membrane preparations was demonstrated (Fig. 3). Within 1 h, PC-3 surface-bound plasmin reached a maximum. A gradual decline in

Fig. 1. A, plasminogen conversion to angiostatin4.5 (AS4.5) by suspension of PC-3 cell membrane fraction. Plasminogen (440 nm) was added to the PC-3 membranes and incubated at 37°C. Aliquots were taken out at 0, 1, 6, and 18 h. for Western blot analysis with GMA086, an antikringle 1–3 monoclonal antibody. There was efficient conversion of plasminogen to AS4.5, with the majority of plasminogen consumed and AS4.5 generated by 1 h. The observed AS4.5 runs as a doublet on the Western blot, reflecting glycosylation isoforms, as described previously (22). B, cell proliferation assay. Early passage bovine aortic endothelial cells were stimulated with 3 μg/ml basic fibroblast growth factor. AS4.5 (200 nm), generated from human plasminogen in a cell-free system of urokinase and N-acetyl-D-cysteine (22), or from the PC-3 membrane lysates, was added to aliquots of the cells. Control cells received basic fibroblast growth factor alone. The cell-free AS4.5 reduced the cell number by 84%, and the PC-3 membrane-derived AS4.5 reduced the cell number by >98%. Data are mean of duplicate wells; bars, ±SD. Similar results were obtained from three independent experiments.

Fig. 2. Quantitation of generation of AS4.5 from plasminogen by PC-3 cell membrane lysates by ELISA. Human plasminogen (440 nm) was added to the PC-3 membrane preparations and aliquots removed at designated times for quantitation of AS4.5 levels. Membranes from ~107 of PC-3 cells were used per 1 mg plasminogen. As indicated above, the ELISA did not register the plasminogen substrate, but did show progressive generation of AS4.5. This is a representative experiment of numerous similar experiments. Membrane lysates from PC-3, HT1080, and MDA-MB231 cells all showed comparable, stoichiometric conversion of human plasminogen to AS4.5.
surface plasmin was observed over the subsequent 24 h, which was associated with an increase in AS4.5 levels, as shown in Fig. 2. Specific inhibitors of plasminogen activators and/or plasmin were shown to inhibit AS4.5 generation by the PC-3 membranes indicating that plasmin generation and activity is necessary for the membrane-dependent conversion of plasminogen to AS4.5 (Table 1). Pefabloc, a broad-spectrum serine proteinase inhibitor, reduced PC-3 membrane-dependent conversion of plasminogen to AS4.5 by 94%. Plasminogen-activator inhibitor-1, at 150 nM, reduced AS4.5 generation by 94%, confirming the essential intermediate role of plasmin generation. H9251-2-Antiplasmin inhibited AS4.5 generation by 87% when in equal molar concentration to plasminogen and by 97% when in molar excess to plasminogen. The lysine analogue, H9280-aminocaproic acid, reduced the PC-3 membrane generation of AS4.5 by 93% suggesting that kringle binding plays a key role in the plasminogen conversion to AS4.5, possibly by disrupting plasminogen/plasmin binding to a membrane receptor.

Cell surface actin has been shown previously to be a plasminogen binding site (34, 39, 40). Actin was associated with the cell membrane fractions of PC-3, MDA-MB231, and HT1080 cells as determined by Western blot (Fig. 4). Various actin isoforms exist, each differing in NH2-terminal sequence (41), which allows identification of the isoforms. Cell surface actin can be recognized by specific antibodies to the NH2-terminus of β-actin (Fig. 4), but not by specific antibodies to α-actin (Fig. 4) or γ-actin (data not shown). Whereas Fig. 4 showed the presence of membrane-associated β-actin, direct immunofluorescent staining showed that the β-actin was present on the extracellular surface of PC-3 cells (Fig. 5). Cytokeratin 7 staining was abundant in the cytoplasm in permeabilized cells and absent on the nonpermeabilized cells, serving as a control. Furthermore, the β-actin signal on the nonpermeabilized cells was punctate, distinct from the cytoplasmic signal in permeabilized cells.

An antibody to the COOH terminus of actin decreased the generation of AS4.5 by ~70%, both on the fixed cell surface and on cell surface plasmin was observed over the subsequent 24 h, which was associated with an increase in AS4.5 levels, as shown in Fig. 2.

Specific inhibitors of plasminogen activators and/or plasmin were shown to inhibit AS4.5 generation by the PC-3 membranes indicating that plasmin generation and activity is necessary for the membrane-dependent conversion of plasminogen to AS4.5 (Table 1). Pefabloc, a broad-spectrum serine proteinase inhibitor, reduced PC-3 membrane-dependent conversion of plasminogen to AS4.5 by 94%. Plasminogen-activator inhibitor-1, at 150 nM, reduced AS4.5 generation by 94%, confirming the essential intermediate role of plasmin generation. α-2-Antiplasmin inhibited AS4.5 generation by 87% when in equal molar concentration to plasminogen and by 97% when in molar excess to plasminogen. The lysine analogue, ε-aminocaproic acid, reduced the PC-3 membrane generation of AS4.5 by 93% suggesting that kringle binding plays a key role in the plasminogen conversion to AS4.5, possibly by disrupting plasminogen/plasmin binding to a membrane receptor.

Table 1 Influence of inhibitors of plasminogen activators and plasmin on Angiostatin 4.5 (AS4.5) generation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PC-3 cell surface</th>
<th>Fluid phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>εACA (10 mM)</td>
<td>93%</td>
<td>45%</td>
</tr>
<tr>
<td>Pefabloc (3 mM)</td>
<td>94%</td>
<td>99%</td>
</tr>
<tr>
<td>A2AP (1:1 molar ratio to Plasminogen)</td>
<td>87%</td>
<td>30%</td>
</tr>
<tr>
<td>A2AP (10:1 molar ratio to Plasminogen)</td>
<td>97%</td>
<td>97%</td>
</tr>
<tr>
<td>PAI-1 (150 nM)</td>
<td>94%</td>
<td>99%</td>
</tr>
</tbody>
</table>

Fig. 3. Plasmin activity on glutaraldehyde-fixed PC-3 cells. Fixed PC-3 cells were incubated with plasminogen and at 1, 3, 7, and 20 h, and supernatants were removed from the wells. The cells were washed with PBS twice. d-Val-Leu-Lys p-Nitroanilide was incubated in the wells and change of A405 per hour was measured. Plasmin activity was normalized to the peak at 1 h (100%).

Fig. 4. Detection of actin on PC-3, HT1080, and MDA-MB231 cell membranes. Washed cell membranes from PC-3 cells, HT1080 cells, and MDA-MB231 cells were assayed by Western blot for the presence of actin using specific antibodies to α-skeletal actin, β-actin, and an antibody to the COOH terminus of actin, which reacts with all of the isoforms. Pure α-skeletal actin as control. All three of the cancer cell lines demonstrated membrane-associated β-actin.

Fig. 5. Direct immunofluorescence staining for actin and cytokeratin 7. For surface protein staining, nonpermeabilized PC-3 cells were incubated with FITC-conjugated monoclonal antibodies to the COOH terminus of actin, β-actin, or cytokeratin 7. For intracellular protein staining, PC-3 cells were permeabilized in −20°C acetone before staining with the same antibodies. Cytokeratin 7 served as a negative control for surface protein staining. β-Actin was shown both on the PC-3 cell surface and in cytoplasm, whereas cytokeratin 7 was only present in the cytoplasm.
membrane fractions (Fig. 6, A and B). Nonspecific antibodies, such as a monoclonal antibody to α-2-antiplasmin, did not show significant influence on AS4.5 generation (Fig. 6B). This supports the hypothesis that the β-actin on the outer surface of the cell membrane serves as the receptor for plasminogen conversion to AS4.5.

Whereas actin alone had no discernible effect on plasminogen in a cell-free system, when added to uPA/plasminogen (a source of plasmin), actin caused complete conversion of plasminogen to AS4.5, comparable with the effect of a free sulphydryl donor (Fig. 6C). This indicates that actin mediates plasmin autotransformation within kringle 5, yielding AS4.5. Furthermore, AS4.5 generation from plasmin by actin does not need a free sulphydryl donor.

Annexin II has been reported to be a plasminogen and plasmin receptor (32, 33), and the annexin II tetramer was shown to be a reductase, mediating plasmin conversion to an angiotatin-like molecule, designated A61 in the HT1080 system (31, 35, 36). By Western blot, annexin II protein (p36) was detected on the membranes of the PC-3, MDA231, and HT1080 cells, and all of the detectable membrane-bound annexin II was removed by treatment with the detergent CHAPS and EGTA (data not shown). There was an ∼50% reduction in surface-bound plasmin by the CHAPS:EGTA treatment (data not shown), but an insignificant reduction (<20%) in plasminogen conversion to AS4.5. The resistance of the AS4.5 generation to CHAPS:EGTA treatment (i.e., annexin II removal), in the three cancer cell lines tested, indicates that annexin II is not an essential receptor for AS4.5 generation. Similarly, no evidence of a role for α-enolase, another reported plasminogen receptor, was observed for generation of AS4.5. Treatment with carboxypeptidase B was used to remove COOH-terminal lysine residues from surface proteins, including any α-enolase present. The AS4.5 generation was reduced by ∼10% (data not shown) by carboxypeptidase B. However, carboxypeptidase B did reduce cell surface plasmin activity, consistent with the role of α-enolase as a plasmin receptor. These data suggest that α-enolase is not the plasminogen conversion receptor.

To determine whether the conversion of plasminogen to AS4.5 could also occur on primary, normal cell surfaces, HMVECs and NHDFs were tested for cell surface activator, plasminogen activator, and AS4.5 generation. On the basis of Western blot and immunofluorescent staining, HMVEC and NHDF cells express cell surface β-actin comparable with PC-3 cells both (data not shown). However, Western blot of HMVEC and NHDF cell membrane lysates revealed much less urokinase receptor (uPAR) on their surfaces than PC-3 cells (data not shown). Furthermore, HMVEC and NHDF cells had less uPAR activity on the surface than PC-3 cells (Table 2). The two primary cell types did convert plasminogen to AS4.5, but at a reduced rate compared with the PC-3 cells (40%), approximately proportional with the uPA levels on the cell surfaces (Table 2). These data suggest that at least in tissue culture and among the cell types studied, normal cells have surface β-actin and uPAR-type plasminogen activator, and the ability to convert plasminogen to AS4.5. However, the conversion by the normal cells is at a reduced rate compared with PC-3 and other cancer cell lines.

**DISCUSSION**

We and others have shown previously that human plasminogen may be converted to an angiotatin isoform in a two-step reaction, with plasmin
formation and activity as an essential intermediate (22, 25, 26). Furthermore, this process yields an isoform of angiostatin consisting of kringle 1–4 plus 85% of kringle 5 (amino acids Lys78 to Arg529), which has been designated AS4.5 (24, 25). In prior studies, the conversion of plasminogen to angiostatin was characterized in the soluble protein fractions. In this article, we now demonstrate that the conversion of plasminogen to angiostatin can be potentiated by a cell surface receptor for plasmin, supplanting the need for a free sulfhydryl donor to allow for cleavage within kringle 5. Furthermore, the plasmin receptor has been identified as β-actin. The three cancer lines tested all expressed β-actin on the cell membrane, and antibody blockade of the actin with a COOH-terminus antibody inhibited the conversion to AS4.5. Furthermore, in a cell-free system, purified actin was able to efficiently mediate conversion of plasmin to AS4.5.

Phosphoglycerate kinase has been reported to serve as a disulfide reductase for plasmin, which may mediate cleavage to an angiostatin isoform (42, 43). However, phosphoglycerate kinase is distinct from the cell surface reaction mediated by β-actin, because phosphoglycerate kinase is a secreted protein and would not be present at significant levels in the washed cell membranes.

Whereas many proteinases have been reported to cleave plasminogen to an angiostatin isoform, our model is an extension of the initial observation that cancer cells themselves can efficiently convert plasminogen to angiostatin (22, 23). Serine proteinase activity is essential for AS4.5 generation, as the AS4.5 generation can be completely blocked by the broad serine proteinase inhibitor, Pefabloc. More specifically, plasmin formation and activity are both required for AS4.5 generation. Plasminogen-activator inhibitor-1 inhibition of AS4.5 generation showed that uPA activity and plasmin formation is required for AS4.5 generation. As in fluid phase, α2-antiplasmin, a specific inhibitor to plasmin activity, also can inhibit AS4.5 generation on cell surface, suggesting that plasmin autopropeolysis played a critical role in AS4.5 generation on cell surface. ε-Aminocaproic acid, which is an analogue of lysine, is used to disrupt the kringle-lysine interaction between plasminogen and plasmin and their receptors/substrates (17). The fact that ε-aminocaproic acid can reduce AS4.5 generation on the cell surface suggests that the plasmin receptor can provide lysine residues or analogues, and the interaction between the plasmin receptor and plasmin is dependent on lysine and the kringle of plasmin.

There have been a number of prior article describing putative plasmin or plasminogen receptors on cell surfaces. These receptors include annexin II (35, 36), α-enolase (37), and actin (5). Annexin II is a plasminogen/plasmin receptor and was reported to act as a reductase for generation of an angiostatin-like-molecule (31). Kassam et al. (35) also showed that the annexin II tetramer on HT1080 cells could mediate conversion of human plasminogen to an angiostatin-like isoform, consisting of amino acids Lys78-Lys468 and designated A61. Therefore, we considered whether one or more of these receptors contributed to the plasmin(ogen) binding and autopropeolysis to yield AS4.5. We showed that the angiostatin isoform being generated is indeed AS4.5 based on its molecular weight, binding to multiple antibodies to plasminogen kringle domains, and most specifically, binding to an antibody to the COOH terminus of the AS4.5 generated in a cell-free system (24). Our results demonstrate that annexin II is present on the surface of cancer cells and is an effective plasmin receptor, as removal of annexin II reduced plasmin binding to the membranes by ~50%. However, removal of annexin II from all three of the cancer cell lines tested resulted in only a modest reduction in plasminogen conversion to AS4.5.

Similarly, α-enolase has been reported to be a plasminogen receptor, although there has been no evidence that it contributes to generation of angiostatin or an angiostatin-like isoform (34, 37). The COOH-terminal lysine residue plays a critical role in the interaction of α-enolase and plasminogen (37). We treated PC-3 cells with carboxypeptidase B, which proteolytically removes the COOH-terminal lysine on cell surface proteins. Again, whereas the carboxypeptidase B treatment reduced plasmin binding to the cell surface, there was no significant affect on generation of AS4.5.

Whereas we saw evidence that the three cancer lines expressed the three putative plasmin(ogen) receptors on their cell surface, it was only the surface β-actin that was shown to have a functional role in the conversion of plasmin to an angiostatin isoform, specifically AS4.5. There have been a series of studies demonstrating the presence of one or more actin isoforms on the external cell surface. One or more actin isoform was found by several independent groups of researchers on the surface of lymphocytes (44, 45), brain capillary endothelial cells (46), and bovine pulmonary endothelial cells (47–50). Actin was also discovered as a cell surface binding site for plasminogen on endothelial cells (39), monocytoïd cells, blood monocytes (34), and breast cancer cells (40). The mechanism of actin trafficking and binding to the cell surface is not well understood. However, actin can bind to membrane lipids such as phospholipids (51–55), and direct interactions between actin and cell membranes are possible in vivo although actin does not have a transmembrane domain. Furthermore, studies by Hu et al. (50) also showed that cell-surface actin was not derived from the serum in tissue culture experiments.

Two nontransformed cell lines, HMVEC and NHDF, were tested for surface expression of β-actin, uPA, and uPAR, as well as the ability to convert plasminogen to AS4.5. Surface β-actin was present on the normal cell surface in culture comparable with the cancer lines. However, uPAR antigen, and uPA antigen and activity were present in the primary cell lines, but at lower levels than in the PC-3 and other cancer lines, and this correlated with a reduced rate of conversion of plasminogen to AS4.5. These data suggest that the level of uPA and uPAR may be the rate-limiting factor in the cell surface conversion of plasminogen to AS4.5. If and how this represents the in vivo process in normal tissues remains to be determined.

Tumor angiogenesis is a significant prognostic factor (8, 56). The ability of tumors to convert plasminogen to AS4.5 influences the balance of tumor angiogenesis and may be a key prognostic factor. Recognition of the cell surface receptor for conversion of plasminogen to AS4.5 may contribute to the ability to predict the clinical course of cancers. Because cancer cell surface β-actin can serve as a receptor for plasminogen conversion to AS4.5, the generation and activity of AS4.5 may influence tumor angiogenic capability and tumor aggression. Cell surface β-actin may be an important prognostic indicator for human cancer and may correlate with patient survival. Greater levels of β-actin on the cancer cell surface may contribute to the rate of AS4.5 generation and the angiogenic capacity of the tumors. Additional studies, including studies of clinical specimens, may serve to clarify the actual prognostic power of the expression of cell surface β-actin.

Expression of uPA and its receptor uPAR have also been reported to have an important role in cellular invasion and cancer metastasis (57, 58). Interestingly, numerous studies have suggested that increased levels of uPA and uPAR expression on cancer cell membranes serves as a negative prognostic parameter for the cancer (57, 58). Whereas our data do not address the in vivo generation of AS4.5 by normal or cancer cells, they do suggest that the role of the plasminogen system may be more complex than previously thought. Plasmin is known to promote angiogenesis and invasion, by cleaving matrix proteins, such as laminin, and activation of metalloproteinases (57, 58). However, plasmin activity is also essential for the generation of human AS4.5, which inhibits angiogenesis (22, 23).

In discussions on angiostatin, it is often asked why cancer cells would mediate the generation of angiostatin, which would inhibit angiogenesis and cancer growth. Our data, characterizing the related processes of plasmin and AS4.5 formation, which result in opposing effects on an-
giogenesis, suggests the question is far more complex then at first appearance.

REFERENCES

Cell Surface-Dependent Generation of Angiostatin4.5

Hao Wang, Ryan Schultz, Jerome Hong, et al.