Characterization of Keratinocyte Plasminogen Activator Inhibitors and Demonstration of the Prevention of Pemphigus IgG-induced Acantholysis by a Purified Plasminogen Activator Inhibitor

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To investigate the mechanisms by which cutaneous plasminogen activator (PA) may be regulated, we have tested cultured keratinocytes for the presence of PA inhibitors. Using biosynthetic labeling experiments with $^{35}$S-methionine in conjunction with specific antibody precipitation, we have shown that human keratinocytes in culture synthesized and secreted both PA inhibitor 1 and PA inhibitor 2. PA inhibitor 1 was present in conditioned media in the inactive form, but it could be detected with reverse phase autography. PA inhibitor 2 was detected by its ability to form complexes with $^{125}$I-uPA. Potential therapeutic relevance for cutaneous PA inhibitor 2 was suggested in skin organ culture experiments which demonstrated that purified PA inhibitor 2 from human placenta was able to prevent the acantholytic changes induced by pemphigus IgG. J Invest Dermatol 92:310–315, 1989

The serine protease plasminogen activator (PA) is found in numerous cell types and appears to have a role in many diverse physiologic functions. Regulation of the PA system is complex and multifaceted, involving both precursor enzyme synthesis and production of at least three different inhibitors [1,2].

Cultured human epidermal cells produce and secrete precursor urokinase type PA (uPA), which can be converted to the active form by cleavage with plasmin [3]. Extracts of normal human epidermis contain tPA [4], at least some of which is in the precursor form [3], as well as a smaller amount of tissue type PA (tPA) [5]. To investigate further the regulation of PA activity in keratinocytes, we have characterized the type of PA inhibitors present in keratinocyte culture. Our results indicate that keratinocytes in culture produce two types of PA inhibitors: PA inhibitor 1 and PA inhibitor 2. (The International Committee on Thrombosis and Haemostasis has assigned the name PA inhibitor 1 to the PA inhibitor initially found in endothelial cells and the name PA inhibitor 2 to the PA inhibitor purified from placenta and U937 cells [12].) PA inhibitor 1, which inhibits both uPA and tPA, is made by a variety of cells, including endothelial cells [6], HT-1080 fibrosarcoma cells [7], and Hep G2 hepatoma cells [8]. PA inhibitor 1 has also been found in platelets and serum [9]. PA inhibitor 2, which previously has been characterized in placenta [10,11], in the histiocytic lymphoma line U937 [12,13] and in human monocytes (13) and leukocytes (14), inhibits uPA more efficiently than tPA [11]. PA inhibitors 1 and 2 are immunologically distinct and differ in their biochemical properties (including isoelectric point and stability to low pH or denaturants), as recently reviewed (15).

Evidence has accumulated that PA may play a crucial role in the development of intraepidermal dysehesia (acantholysis) in the autoimmune disease pemphigus [4,16]. Patients with this disorder have circulating and cutaneous autoantibodies that react with the epidermal cell surface. In normal human skin organ culture, pemphigus IgG is able to induce acantholysis within 24 to 48 h [17,18]. These antibodies also lead to enhanced PA activity in the Iysate and conditioned medium of cultured human epidermal cells [16]. Pemphigus IgG-induced acantholysis in organ culture is totally prevented by the simultaneous addition of anti-uPA IgG [4], strongly suggesting that uPA activity is crucial in the development of intraepidermal dysehesia. In the present report we demonstrate that purified PA inhibitor 2, isolated from human placenta, is able to block pemphigus IgG-induced acantholysis in organ culture.

MATERIALS AND METHODS

Materials Tissue culture supplies were obtained from the following manufacturers: Dulbecco's modified Eagle medium with high glucose, L-glutamine, and sodium pyruvate (GIBCO Laboratories, Grand Island, NY and Flow Laboratories Inc., McLean, VA); fetal calf serum, HEPES, penicillin, and streptomycin (GIBCO); epidermal growth factor (Collaborative Research, Bedford, MA); cholera

Abbreviations:
- BSA: bovine serum albumin
- DMEM: Dulbecco's modified Eagle medium
- FCS: fetal calf serum
- PA: plasminogen activator
- PBS: phosphate buffered saline
- SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis
- tPA: tissue-type PA
- uPA: urokinase-type PA

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toxin (ICN Biomedicals, Inc., Costa Mesa, CA); hydrocortisone (Calbiochem, La Jolla, CA). Other supplies were purchased from the following companies: 35S-methionine #SJ.204 (Amersham, Arlington Heights, IL); Staphylococcus aureus cells (Pansorbin) and fibrinogen #341573 (Calbiochem); human thrombin #76759 (Sigma Chemical Co., St. Louis, MO); asparagine inducible #8008 (Polysciences, Inc., Warrington, PA); purified uPA (Mochida Pharmaceuticals, Tokyo, Japan); and standard uPA (Leo Pharmaceuticals, Ballerup, Denmark).

**Keratinocyte Culture and Harvest** Human epidermal cells were grown on collagen-coated well plates in Dulbecco's modified Eagle medium with high glucose (DMEM) with 15 mM Hepes (DMEM/Hepes) plus 20% fetal calf serum (FCS), epidermal growth factor, cholera toxin, penicillin-streptomycin, and hydrocortisone as described previously [16,19].

To collect conditioned media, confluent cultures were first incubated in DMEM/Hepes with 20% FCS and penicillin-streptomycin (but no other additives) for 24 h. They were then washed twice in phosphate-buffered saline (PBS) and once in DMEM/Hepes and incubated for 24 h with 1 ml per well of DMEM/Hepes plus penicillin-streptomycin. This medium, referred to as conditioned medium, was centrifuged at 4,000 × g and stored at −70°C until use.

**PA Inhibitor Assays** Amidolytic assay of uPA inhibitory activity. Conditioned medium (120 μl) was mixed with 4 μl of uPA (2000 units/ml) and incubated for 5 min at room temperature. After adding 121 μl of assay buffer (0.1M Tris-HCl pH 8.7, 0.5% Triton X-100) and 5 μl of 0.01M S2444 substrate, the rate of absorbance change at 405 nm was measured. The amount of inhibitor in the medium was calculated by the rate of decrease of absorbance compared to uPA control without inhibitor.

Reverse fibrin autography [6]. Samples were separated with sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide gel. An indicator gel was then prepared by mixing agarose (1.3%), fibrinogen (2.25 mg/ml), thrombin (0.08 units/ml), plasminogen (32 μg/ml), and uPA (75 milliunits/ml) at 42°C and pouring the mixture onto a glass plate. After washing the acrylamide gel for 1 h in 2.5% Triton X-100 to remove SDS, it was overlaid onto the fibrin-gelatinous gel and incubated overnight at 4°C and then for several hours at 37°C. The opaque fibrin-gelatinous gel gradually became clear because of lysis of fibrin, and inhibitor species were detected as opaque areas remaining in the cleared gel.

**125I-uPA Binding** Samples (10 μl of either keratinocyte conditioned medium or cell extract) were incubated with 125I-uPA [5 μl of 0.4 μg/ml in PBS plus 2% bovine serum albumin (BSA)] for 1 h at 4°C. After analysis of the samples on SDS-PAGE, the gel was dried and processed for autoradiography to detect bands of free and complexed 125I-uPA. Urokinase type PA (M, 33,000) was iodinated by the iodogen method and separated from free 125I on Sephadex G-25 minicolumns [19].

**Immunoprecipitation** Fifteen microliters of either conditioned medium alone or conditioned medium preincubated with 125I-uPA were incubated with antiserum to PA inhibitor 1, antiserum to PA inhibitor 2 [11], or normal rabbit serum (2 μl of 1:5 dilution) for 2 h at 4°C. Staphylococcus aureus was washed once with 0.5M NaCl, 1.0 mM EDTA, 50 mM Tris-HCl, pH 8.1 (NET) containing 0.5% NP-40 and once with NET containing 0.05% NP-40, and was finally suspended in NET plus 0.05% NP-40 and 1 mg/ml ovalbumin. Seventeen microliters of the 10% Staphylococcus A suspension was then added to the conditioned medium-antiserum mixture, and incubation was continued for 30 min at room temperature. The complexes were pelleted using an Eppendorf microfuge, and the supernatants analyzed using either reverse fibrin autography or autoradiography.

For determination of apparent molecular weight of the bands, the following protein standards were run in an adjacent lane on the gel: carbonic anhydrase (30 KD), ovalbumin (43 KD), catalase (60 KD), and phosphorylase (100 KD).

**Bioinformatic Labeling** Confluent keratinocyte cultures were incubated with 2 ml/well of methionine-free DMEM/Hepes plus 2% FCS overnight. The culture medium was then aspirated, and 2 ml of methionine-free DMEM/Hepes supplemented with 35S-methionine (83 μCi/ml) was added. Incubation was continued for 16−24 h. Medium was then harvested, centrifuged at 4,000 × g, and stored at −70°C. Aliquots (300 μl) were incubated for 2 h at 4°C with 3 μl of anti-PA inhibitor 1 antiserum, anti-PA inhibitor 2 antiserum, or normal rabbit serum. Then 300 μl of a 10% suspension of washed Staphylococcus aureus was added and incubation was continued for 30 min at room temperature. The complexes were pelleted and washed 3 times in PBS containing 0.5% NP-40, 2 mM methionine, and 0.02% Na3S. The material specifically bound was eluted by boiling for 3 min in sampling buffer (0.14M Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 0.002% bromophenyl blue), and this eluted material was subjected to SDS-PAGE. The gel was soaked in Autofluor (National Diagnostics, Somerville, NJ) for 30 min at room temperature, dried, and processed for autoradiography.

**Organ culture** Two experiments, both of which contained two explants for each experimental condition, were performed to test the effect of PA inhibitor 2 upon acantholysis induced by pemphigus IgG. Normal adult face skin was obtained from routine surgical procedures. Pieces of approximately 2 mm² were placed on lens paper that was supported by an aluminum grid screen in a 6-well culture dish. One milliliter of DMEM/Hepes plus penicillin-streptomycin with 2.5 mg/ml BSA and 4 mg/ml pemphigus vulgaris IgG was added. To some wells, PA inhibitor 2 (100 μg/ml),
revealed a single band of inhibitory activity that migrated with an
apparent molecular weight of 46 kD (lane 1). This inhibitory activity
was shown to be related to PA inhibitor 1 by immunoprecipitation
experiments with specific antisera. Pretreatment of the condi-
tioned medium with antisera against PA inhibitor 1 removed the
inhibitor band (lane 3), but pretreatment with normal rabbit serum
(lane 1) or antisera against PA inhibitor 2 (lane 2) did not.

Detection of PA Inhibitor 2 Using another assay system we also
detected PA inhibitor 2 in cultured human epidermal cells. When
either conditioned media or cell lysates were incubated with Mr
33,000 125I-uPA and the mixtures then analyzed with SDS-PAGE
and autoradiography, we observed a shift of the 125I-uPA to higher
apparent molecular weight, indicating the formation of SDS-resis-
tant complexes. Conditioned media consistently yielded two uPA
complex bands with apparent molecular weights of 65 and 80 kD
(Fig 2, lane E). Cell lysates likewise showed a band at 65 kD and also
a fainter one at approximately 115 kD (lane C); in addition, most
lysates had a very faint third band at 80 kD. All of these uPA com-
plex bands were immunoprecipitated with anti-PA inhibitor 2 anti-
serum, indicating that they represented uPA-PA inhibitor 2 com-
plexes (Fig 2).

purified from human placenta [11], was also added. Culture was
carried out for 24–48 h, after which sections were cut and stained
with hematoxylin and eosin. Multiple fields and sections were ex-
amined, and acantholysis was graded on the following histological
basis. Mild acantholysis was defined as retraction of epidermal cells
and rounding of cell profiles. Moderate acantholysis was defined as
loss of contact between epidermal cells associated with intracellular
edema. Severe acantholysis was defined as clear intracellular blis-
ters containing single round acantholytic cells floating free.

IgG was purified on Lysine-Sepharose and Protein A-Sepharose,
as described previously [16]. Purified inhibitor 2 appeared homoge-
neous by SDS-PAGE as well as immunodiffusion criteria, as pre-
viously described in detail [11].

RESULTS AND DISCUSSION
Identification of PA Inhibitory Activity in Conditioned Me-
edium The presence of inhibitory activity against uPA in human
epidermal cell cultures was demonstrated by incubation of the con-
tioned medium with uPA and subsequent assay of remaining uPA
activity using the synthetic substrate S2444. Conditioned medium
collected after 24 h from confluent keratinocytes contained approx-
imately 11 CIU/ml; one CIU is defined as the amount of inhibitory
activity that inhibits 1 CTA unit of uPA.

In order to determine the identity of the inhibitors in the condi-
tioned media and to establish whether they were synthesized by the
keratinocytes, we performed the following experiments.

Detection of PA Inhibitor 1 PA inhibitor 1 can exist in active or
latent forms [20,21]. The latent form is partially activated by SDS
and can be demonstrated by SDS-PAGE and reverse fibrin autogra-
phy [6,20]. PA inhibitor 2 is labile to SDS and hence is not easily
detected with reverse fibrin autography. As shown in Fig 1, reverse
fibrin autography of epidermal cell culture conditioned medium

Figure 3. Metabolic labeling of PA inhibitors. A: Identification of PA
inhibitor 1. Cultured keratinocytes were incubated for 24 h with 35S-methi-
none. Conditioned medium was harvested and immunoprecipitated with
anti-PA inhibitor 1 antisera (lane A) or normal rabbit serum (lane B) and
Staphylococcus aureus. After washing the complexes, the specifically bound
material was eluted in sampling buffer and subjected to SDS-PAGE. Radio-
abeled bands were visualized by autoradiography. Anti-PA inhibitor 1 anti-
serum specifically immunoprecipitated a single band with Mr, 45 kD. B:
Identification of PA inhibitor 2. Labeled keratinocyte conditioned medium,
prepared as in A, was immunoprecipitated with normal rabbit serum (lane B)
or with two different preparations of anti-PA inhibitor 2 antisera (lanes A
and C) and Staphylococcus aureus. Specifically bound material was eluted
and analyzed as in A. Both anti-PA inhibitor 2 antisera precipitated a band
with Mr, 60 kD. C: Binding to uPA. Conditioned medium, prepared from
biosynthetically labeled cells as described in A, was incubated alone (lanes A,
B, and D) or with Mr, 33,000 uPA (lanes C and E) for 1 h. Immunoprecipita-
tion with normal rabbit serum (lane A), with anti-PA inhibitor 2 antisera
(lanes B and C), or with anti-PA inhibitor 1 antisera (lanes D and E) and
Staphylococcus aureus was then carried out. Specifically bound material
was eluted and analyzed with SDS-PAGE as in A. PA inhibitor 2 formed an
SDS-resistant complex with uPA, as indicated by a shift to higher apparent
molecular weight (arrows, lanes B and C), but PA inhibitor 1 did not form
such a complex (lanes D and E). In this experiment the anti-PA inhibitor 2 anti-
sersum also appeared to precipitate two minor bands, either because of
nonspecific binding or some cross-reactivity.
Metabolic Labeling of Inhibitors To establish conclusively that both PA inhibitors were synthesized by cultured keratinocytes, we incubated cells with 35S-methionine and immunoprecipitated their conditioned media with specific antibodies against the inhibitors. By SDS-PAGE analysis of the immunoprecipitated material, we found that anti-PA inhibitor 1 antiserum specifically immunoprecipitated a band with M,46 kD (Fig 3A), and anti-PA inhibitor 2 antiserum specifically immunoprecipitated a band with M,60 kD (Fig 3B). These molecular weights agree well with previously reported data on PA inhibitor 1 and PA inhibitor 2 from conditioned media of human endothelial cells [22] and lymphoma cells [23], respectively.

To test whether these immunoprecipitable molecules would form complexes with uPA, we preincubated aliquots of the labeled conditioned medium with M, 33,000 uPA. The mixture was then immunoprecipitated with anti-inhibitor antibodies. The apparent molecular weight of the band immunoprecipitated by anti-PA inhibitor 1 antiserum was not altered by preincubation with uPA (Fig 3C, lanes D and E), suggesting that this inhibitor is in an inactive state which is not able to form complexes with uPA. Secretion of PA inhibitor 1 as an inactive molecule which is activated by SDS and other denaturants has been documented in cultured endothelial cells [21,22]. In contrast, the band precipitated by anti-PA inhibitor 2 antiserum migrated with a greater apparent molecular weight (M,95 kD) after preincubation with uPA (Fig 3C, lanes B and C), indicating that this inhibitor formed a complex with the enzyme. PA inhibitor 2 that is capable of binding uPA has previously been detected in conditioned media from a variety of cell lines [24]. These results thus demonstrate that keratinocytes in culture synthesize and secrete both PA inhibitor 1 and PA inhibitor 2.

Effect of PA Inhibitor 2 on Pemphigus IgG-induced Acantholysis We have previously suggested that PA may play a crucial role in the development of intra-epidermal deshesion (acantholysis).
that is characteristic of the autoimmune disease pemphigus [4,16].

To determine if a natural PA inhibitor could prevent pemphigus IgG induced acantholysis, we incubated normal skin in organ cultures with pemphigus vulgaris IgG in the presence and absence of purified PA inhibitor 2. Incubation of skin with nonimmune, control IgG revealed some swelling of epidermal cells. As illustrated in Fig 4, organ cultures incubated with pemphigus IgG alone showed severe acantholysis; i.e. there were intraepidermal blisters and swollen acantholytic eosinophilic cells throughout the sections, including the hair follicles. By contrast, explants incubated with pemphigus IgG and PA inhibitor 2 demonstrated only spotty areas of mild acantholysis, i.e., with occasional retraction of basal epidermal cells. Thus the addition of PA inhibitor 2 dramatically decreased acantholysis induced by pemphigus IgG in organ cultures of normal human skin.

We recently have detected (unpublished observations) the presence of PA inhibitor type 2 in extracts of normal human epidermis.

We have shown in the present study that cultured human keratinocytes produce and secrete two plasminogen activator inhibitors. It is likely that these inhibitors are important in the regulation of cutaneous PA activity in vivo. The finding that PA inhibitor 2 can prevent pemphigus IgG-induced acantholysis in organ culture raises the possibility of using PA inhibitors for clinical management of pemphigus. Recent cloning of the genes for both PA inhibitor 1 [25–28] and 2 [29] will allow the production of large quantities of these proteins in the near future. Alternatively, chemical or hormonal modulation of the production of the PA inhibitors in the skin may provide effective and selective therapy.

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