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Epidermal plasminogen activator inhibitor (PAI) is immunologically identical to placental-type PAI-2

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Plasminogen activator inhibitor (PAI) purified from human epidermis [(1986) FEBS Lett. 408, 273–277] was immunologically identified as placental-type PAI-2. In both fibrinolytic and synthetic substrate assays inhibitory activity of epidermal PAI was neutralized by anti-PAI-2, but not by anti-endothelial type PAI-1. Immunoblotting technique confirmed that the purified epidermal PAI is reactive with anti-PAI-2, but not with anti-PAI-1. Consequently PAI in human epidermis was demonstrable by immunohistochemical technique.

Placental-type plasminogen activator inhibitor-2; Endothelial-type plasminogen activator inhibitor-1; Urokinase; Tissue-type plasminogen activator; (Human epidermis)

1. INTRODUCTION

Tissue-type [1] and urokinase-type plasminogen activators (PAs) [2], have been found in various tissues and body fluids [3–5]. On the other hand, recent studies have demonstrated that PA activities are controlled by, at least, two classes of specific PA inhibitors (PAIs), endothelial-type PAI-1 [6–9] and placental-type PAI-2 [9–12]. Recently Hibino et al. [13] have purified PAI from cornified cells of human epidermis. The epidermal PAI, with M_r 43000 and p*I* 5.2, inactivated both urokinase and tissue-type PAs but did not inhibit plasmin, thrombin, glandular kallikrein or trypsin. It was postulated that PA activity in the normal epidermis is under control of the epidermal PAI [13,14], while exceeding PA activity in the skin becomes pathogenic in acantholytic bulla formation [15], abnormal keratinization [16], and cancer metastasis [17]. The present study investigated immunologic cross reactivity of epidermal PAI using monospecific antibodies against PAI-1 and PAI-2. The results benefit investigators to apply immunohistochemical technique to the skin.

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2. MATERIALS AND METHODS

2.1. *Purification of epidermal PAI*

Epidermal PAI was purified from cornified cells scraped from the foot of healthy individuals as previously reported [13] with modifications. Briefly, human cornified cells were extracted with 0.1 M Tris-HCl (pH 8.0) + 0.14 M NaCl. Supernatant was fractionated by Sephacryl S-200 and DEAE Sepharose chromatography. Inhibitor fraction was then applied to hydroxyapatite HPHT (Bio-Rad) and FPLC anion-exchanger Mono Q (Pharmacia) chromatography at pH 7.2 and 8.0.

2.2. *Preparation of PAI-1 and PAI-2*

Since platelet PAI belongs to endothelial-type PAI-1 [8,9], we extracted platelet PAI for PAI-1. Platelet-rich plasma was obtained from 100 ml of blood containing 1/10 volume of 3.8% sodium citrate by centrifugation at 250 × *g* for 15 min. Platelets were collected after centrifugation at 800 × *g* for 15 min and washed 5 times in modified Tyrode's buffer [18]. PAI-1 was extracted with 1.5 ml of 0.5% Triton X-100 for 1 h at 4°C [19]. PAI-2 partially purified from human placentas was obtained from Green Cross Co., Ltd, Osaka, Japan. This was further purified by FPLC Mono Q chromatography at pH 8.0, twice.

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2.3. *Anti-PAI-1 and anti-PAI-2 antibodies*

Monoclonal anti-human PAI-1 antibody was prepared from mouse hybridoma cells as reported [9]. Monospecific polyclonal anti-human PAI-2 antibody was prepared from goat sera immunized with purified PAI-2 [11].

2.4. *Immunoblotting*

After SDS slab gel electrophoresis, proteins were transferred to a nitrocellulose sheet electrophoretically as described by Howe and Hershey [20] at 200 mA for 8 h. The nitrocellulose sheet, washed three times for 5 min each with 0.02 M sodium-phosphate buffer + 0.14 M NaCl, pH 7.4 (PBS) containing 0.1% Triton X-100 (TPBS), was incubated with anti-PAI-1 or anti-PAI-2 IgG for 30 min at room temperature. After rinsing with TPBS three times for 5 min each, the sheet was incubated with biotinylated anti-mouse IgG or anti-goat IgG diluted 1:50 in TPBS for 30 min and washed with TPBS three times for 5 min each, according to the avidin-biotin-peroxidase complex (ABC) method [21] using StrAvigen (BioGenex Laboratories, Dublin, USA). After reaction with ABC (1:50 dilution) for 30 min and washing with PBS the sheet was immersed in diaminobenzidine (5 mg/10 ml). Reaction was stopped by rinsing with water.

2.5. *Inhibitor assays*

Colorimetric inhibitor assay for urokinase was done using Glu-Gly-Arg-*p*-nitroanilide (Kabi Diagnostica, Stockholm, Sweden) as substrate [13]. Urokinase (12 IU/0.1 ml) (M_r 55 000; Green Cross, Tokyo, Japan) was mixed with 0.05 ml of sample or PBS (control) and 0.8 ml of Tris-HCl (pH 8.5), and preincubated for 30 min at room temperature. Reaction was started by adding 0.05 ml of 4 mM substrate and change of absorbance at 405 nm was monitored. Inhibitor activity was expressed by the enzyme activity (nmol/min = unit) that was inhibited.

Fibrin plate method was also used according to the method of Astrup and Müllertz [22] with modifications [14]. Urokinase (1.5 IU/ml) was mixed with equal volume of inhibitor sample or PBS (control) and the mixture (20 μl) was placed on plasminogen-rich or plasminogen-free fibrin plates.

In order to study neutralizing effects of antibodies on PAI activity, each PAI sample was incubated with equal volume of anti-PAI-1 IgG (0.2 mg/ml) or anti-PAI-2 IgG (0.5 mg/ml) for 30 min at 25°C before starting inhibitor assays.

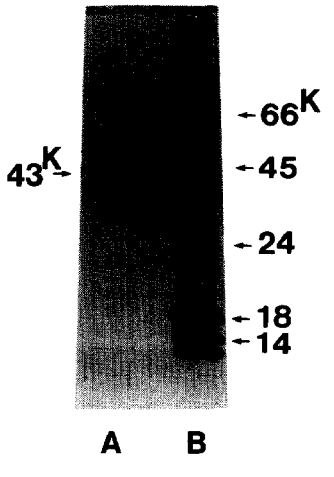

Fig.1. SDS polyacrylamide gel electrophoresis of the purified epidermal PAI (lane A) and molecular mass markers (lane B).

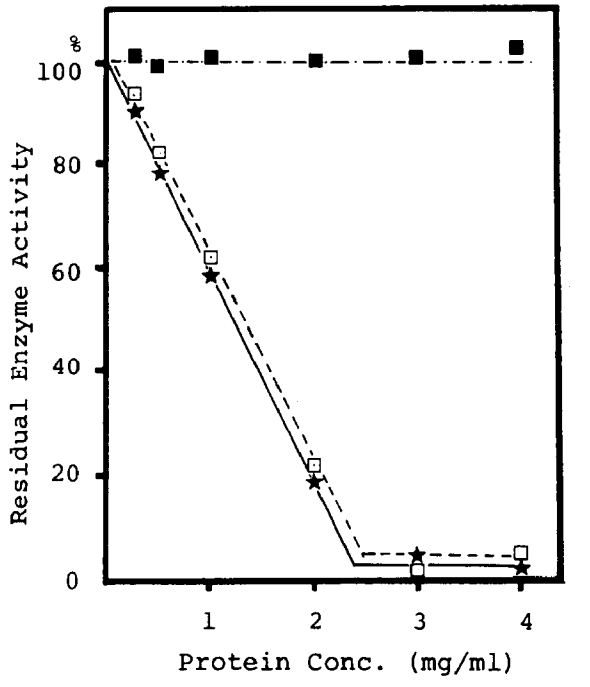

Fig.2. Effect of anti-PAI-1 and anti-PAI-2 antibodies on crude epidermal cell extract. The extract was incubated with PBS (★—★), anti-PAI-1 (□---□), or anti-PAI-2 (■---■) for 30 min at 25°C before mixing with urokinase. Inhibitor assays were carried out using Glu-Gly-Arg-*p*-nitroanilide as substrate.

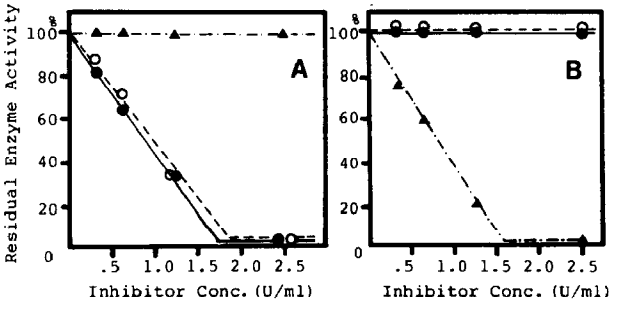

Fig.3. Effect of anti-PAI-1 and anti-PAI-2 antibodies on the reaction between urokinase and platelet PAI-1 (▲---▲), placental PAI-2 (○---○) and epidermal PAI (●—●). Inhibitor fractions were incubated with anti-PAI-1 (A) or anti-PAI-2 (B) for 30 min at 25°C, and placed on plasminogen-rich fibrin plates.

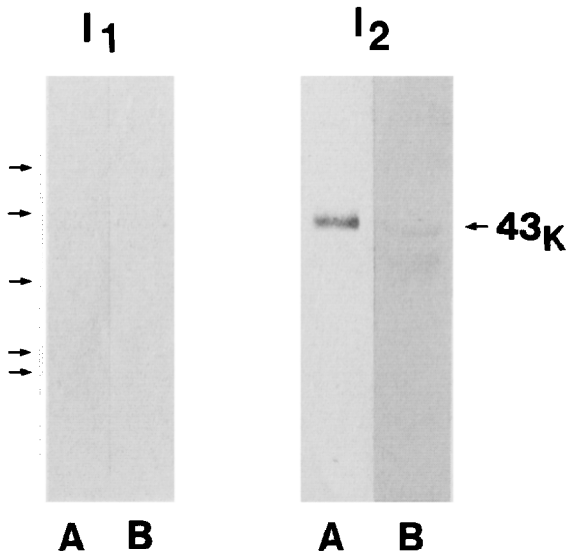

Fig.4. Immunoblots of purified epidermal PAI (lane A) and partially purified placental PAI-2 (lane B). After SDS-polyacrylamide gel electrophoresis, proteins were transferred and immunostained with ABC method by anti-PAI-1 (left panel) or anti-PAI-2 (right panel). Small arrows indicate positions of molecular mass markers as in fig.1.

2.6. *Immunohistochemical localization of PAI-1 and PAI-2*

Human skins from healthy volunteers were fixed in periodate-lysine-paraformaldehyde [23]. Tissues were transferred to 0.1 M phosphate buffer, pH 7.2, containing 5, 10, and 15% sucrose, respectively, each overnight. Cryostat sections on albumin coated slides were air dried for 30 min and reacted with the primary IgG diluted (1:100 to 1:1000) with TPBS overnight. Sections were stained with ABC method using StrAvigen, according to the manufacturer's instructions. Control studies were carried out using nonimmunized antiserum of each species, or by preabsorption of the antibody with cornified cell extract.

3. RESULTS

3.1. *Purification of epidermal PAI*

By the present procedures of purification an electrophoretically homogeneous protein with an M_r of 43000 was obtained (fig.1). Specific activity increased approx. 1500-fold from the starting extracts with a yield of 6%, and the final product showed 20 unit/mg protein.

3.2. *Effect of anti-PAI-1 and anti-PAI-2 on PAI activities*

By the synthetic substrate assay, the crude (before purification) epidermal cell extract demonstrated linear dose-dependent inhibition for urokinase (fig.2). This inhibitor activity was completely neutralized by anti-PAI-2 IgG, while it was not affected by anti-PAI-1. The neutralizing effect of anti-PAI-2, but not of anti-PAI-1, was confirmed with purified epidermal PAI, when inhibition of urokinase was measured by the fibrinolytic assay

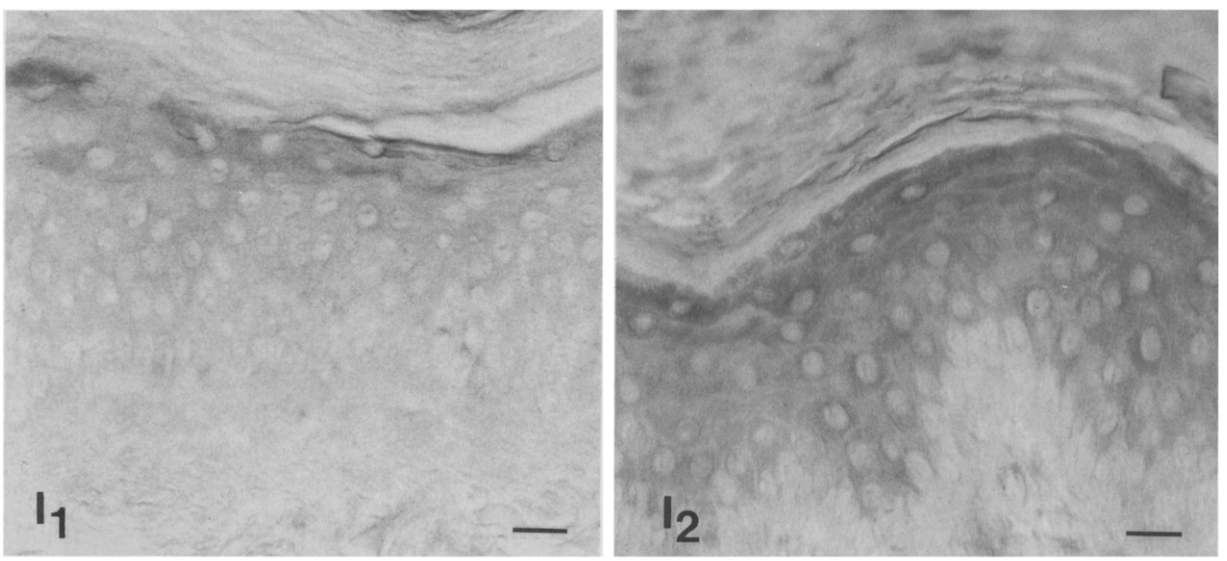

Fig.5. Immunohistochemical staining of normal human foot epidermis by monospecific antibodies against PAI-1 (left) or PAI-2 (right). Bars indicate 20 μm.

(fig.3). Control experiments with platelet PAI-1 and placental PAI-2 showed that anti-PAI-1 neutralized selectively the reactivity of PAI-1, while anti-PAI-2 neutralized selectively the reactivity of PAI-2 (fig.3), confirming previously reported results using the same antibodies [24].

3.3. *Immunoblotting*

It was confirmed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting that purified epidermal PAI, like placental PAI-2, reacted with anti-PAI-2 (fig.4). Neither epidermal PAI nor placental PAI-2 showed immunoreactivity to anti-PAI-1, while control experiments showed that the same anti-PAI-1 antibody reacted in immunoblots with PAI-1 [25].

3.4. *Immunohistochemical localization of PAI-2 in human skin*

Human skin sections demonstrated immunoreactivity to anti-PAI-2 IgG (fig.5). Cornified and granular cells were intensely stained while lower spinous cell layer showed reducing reactivity. Preabsorption of anti-PAI-2 IgG by the epidermal cell extract abolished the reactivity. On the other hand, there is some reactivity to anti-PAI-1 IgG, although PAI-1 activity was not detectable (fig.2).

4. DISCUSSION

The purified epidermal PAI was shown to be immunologically related to PAI-2, since its inhibitory effect was neutralized by anti-PAI-2 and since it reacted with anti-PAI-2 in immunoblots of SDS-polyacrylamide gels. The inhibitor did not crossreact with anti-PAI-1. Immunohistochemical analysis showed that PAI-2 antigen is intensely localized in the granular and cornified cells. The pattern of localization of PAI-2 antigen accounts for that (1) the epidermal PAI-2 was extracted and purified from the cornified cells of foot epidermis, and (2) PAI activity was stronger in the tissue extract of uppermost layer of epidermis than whole epidermis (our unpublished data). Elevated PA activity has been demonstrated in (1) loss of intercellular coherence that results in bulla formation in pemphigus [15] and (2) accelerated turn-over of epidermal cells in psoriasis [16]. Regulation of PA activity by the epidermal PAI-2 may be involved in prevention of such pathogenic conditions in the epidermis, and seems physiologically significant in the upper layer of normal epidermis where cell motility ceases. Monocytes/macrophages also contain PAI-2 [26,27], suggesting its extravascular role in controlling inflammation. Furthermore, immunohistochemical analysis has shown that PAI-2 is present in trophoblastic epithelium of placenta [28].

Although the inhibitor activity for urokinase by the epidermal extract was totally neutralized by anti-PAI-2 antibody but not by anti-PAI-1 antibody, the present immunohistochemistry showed a weak immunoreactivity to anti-PAI-1 remained in the human epidermis. The PAI-1-related antigen in the epidermal cells may not be present in the extractable condition, or may be functionally inactive.

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