

Levels of Proelafin Peptides in the Sera of the Patients with Generalized Pustular Psoriasis and Pustulosis Palmoplantaris

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The levels of proelafin peptides in the sera of patients with pustulosis palmoplantaris, a unique type of localized pustular psoriasis, and generalized pustular psoriasis were determined by competitive enzyme-linked immunosorbent assays using antibodies against synthetic proelafin polypeptides corresponding to the elastase inhibitor (elafin) and transglutaminase substrate domains. The sera of patients with pustulosis palmoplantaris (9 cases) exhibited a normal range of proelafin peptide levels. The sera of patients with generalized pustular psoriasis (3 cases) showed high titres of proelafin peptide. There were no large differences in the titres between the 2 antibodies. The antibodies for 2 different domains of proelafin showed a similar immunoreactivity for the non-pustular region of the epidermis in all pustulosis palmoplantaris tested. The results indicate that serum proelafin peptides in pustular psoriasis may depend on the extent of the involved area, and that proelafin peptide level in pustulosis palmoplantaris remains normal despite enhanced local expression in the lesional skin. Since the skin lesions of patients with pustulosis palmoplantaris are limited to the palms and soles, enhanced expression of proelafin in the lesional skin may not lead to elevation of proelafin peptides in the serum. **Key words:** proelafin; elastase inhibitor domain; transglutaminase substrate domain; elafin; generalized pustular psoriasis; pustulosis palmoplantaris.

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INTRODUCTION

Elafin, also termed skin-derived anti-leukoproteinase (SKALP), is a potent elastase inhibitor isolated from psoriatic skin (1–4). The inhibitor is found in the subcorneal differentiated keratinocytes of psoriatic epidermis, but not in the normal epidermis (5, 6). Proelafin, a precursor of elafin, has a 9.9-kDa mature protein with a unique structure of 4 repeats of putative transglutaminase substrate motifs at the NH₂-terminal end and an elastase inhibitor domain (elafin) at the COOH-terminal end (7, 8). In plaque type psoriasis, expression of elafin in the upper spinous layers of lesional skin and serum level of elafin have been shown to be elevated, and the latter can be a potential marker for disease activity of psoriasis (5, 9). Elafin has been suggested to play a crucial role in the pathophysiology of psoriasis by protecting lesional tissue against proteolysis. Activated polymorphonuclear leukocytes (PMN) secrete a variety of proteases, including leukocyte elastase. One of the putative functions of leukocyte elastase is to facilitate the migration of PMN through the

connective tissue towards inflammatory foci. In fact, local elafin deficiency was shown in various types of pustular psoriasis, which may promote pustular formation (10, 11).

Pustulosis palmoplantaris (PPP) is a chronic recurrent pustular dermatosis localized on the palms and soles. Histologically, it is characterized by subcorneal pustules filled with neutrophils. Because clinically and histologically these conditions cannot be distinguished from pustular psoriasis, this disease is considered to be localized pustular psoriasis (12). This unique type of localized pustular psoriasis (PPP) is relatively common in Japan, compared with generalized pustular psoriasis and even with plaque type psoriasis (13).

Elafin content in the scales of pustular psoriasis including PPP has been shown to be less than in plaque type psoriasis, despite the same level of elafin mRNA in pustular psoriasis and plaque type psoriasis (11). This suggests that elafin clearance in pustular psoriasis may be accelerated. Since elafin content in sera may be considered to reflect the clearance rate of tissue elafin, we attempted to measure serum level of elafin in generalized and localized pustular psoriasis.

It has been shown that the major proelafin form *in vivo* has lower molecular fragment containing the elastase inhibitor domain (elafin) than the intact molecules (14), and PMN elastase-elastase inhibitor domain (elafin) complex in the lesion may be cleared and excreted into circulating blood and urine (15). This suggests that proelafin may be cleared in a domain-dependent manner. In this study, we produced 2 different antibodies raised against synthetic proelafin peptides corresponding to the N-terminal transglutaminase substrate domain and the C-terminal elastase inhibitor domain (elafin), and attempted to determine the serum level of each proelafin peptide in the patients with pustular psoriasis.

MATERIALS AND METHODS

Patients and sera

Nine patients with PPP, 3 patients with generalized pustular psoriasis and 4 patients with plaque type psoriasis without the involvement of other diseases were selected for analysis. Eight healthy volunteers (4 men, 4 women; age range 26–59, mean 38.3 years) served as controls (Table I). Disease activity was expressed by PASI score (16).

Peptide synthesis and antibody production

A peptide of 57 amino acids corresponding to the C-terminal elastase inhibitor domain of proelafin (AQEPVKGVPSTKPGSCPIILIR-CAMLNPPNRCLLDTDCPGIKKCEGSCGMACFVPQ) was chemically synthesized using an automated peptide synthesizer (model 990, Beckman Instrument Inc.) using Fmoc chemistry. To produce the antibody against the N-terminal domain of proelafin (VPVKGQDTVKGKRVFNGQ DPVKGQVSVKGQDKVK), a tetrameric 34 amino acid proelafin peptide was synthesized according to

Table I. Serum proelafin peptide level of the patients with pustulosis palmoplantaris and generalized pustular psoriasis

Case	Sex/age	Involved site	PASI	Proelafin peptide level (ng/ml)	
				Elastase Inhibitor Domain*	Transglutaminase Substrate Domain**
Pustulosis palmoplantaris and generalized pustular psoriasis					
1	M/57	Palms/Soles	3.2	12.1	18.2
2	F/34	Palms/Soles	2.6	12.1	16.4
3	F/52	Palms/Soles	4.0	15.4	12.1
4	M/80	Soles	2.4	10.5	20.9
5	M/46	Palms/Soles	3.1	13.4	17.2
6	M/50	Palms/Soles	4.4	16	23.1
7	F/51	Palms/Soles	3.6	14.3	9.9
8	F/25	Palms/Soles	3.4	11.6	16.5
9	F/56	Soles	2.0	7.7	14.3
			Mean \pm SD	12.6 \pm 2.6	16.5 \pm 4.1
10	M/56	Generalized	43.0	115.0	63.3
11	M/45	Generalized	36.0	109.0	71.1
12	M/48	Generalized	33.5	97.4	58.2
			Mean \pm SD	107.1 \pm 8.9	64.2 \pm 6.5
Plaque type psoriasis					
1	M/43	Generalized	25.9	74.3	71.0
2	M/57	Trunk/legs	13.2	39.6	19.8
3	M/48	Scalp/trunk	7.2	30.3	39.6
4	M/65	Buttock	1.8	14.3	11.0
			Mean \pm SD	39.6 \pm 25.4	35.4 \pm 26.6
Healthy control			Mean \pm SD	10.4 \pm 1.4	12.1 \pm 3.1

*Elastase inhibitor domain represents a 6-kDa C-terminal part of proelafin molecule (elafin) which is a potent inhibitor specific for neutrophil elastase and proteinase 3.

**Transglutaminase substrate domain indicates a 3.9-kDa N-terminal part of proelafin molecule which mediates covalent cross-linking of elafin to extracellular matrix and cornified envelope proteins in the presence of transglutaminase.

the procedure of Tam (17), using multiple antigenic peptide resin (Applied Biosystems, Foster City, CA, USA), since the 34 amino acid size is not large enough to produce a high titre antiserum on direct injection into rabbits (18).

The peptides were purified with reverse phase HPLC (Beckmann Instrument, Inc.) with C-18 (250 \times 4.6 mm) semi-preparative column. The synthetic peptide was coupled to hemocyanin in 20 mM sodium phosphate buffer pH 7.0 for 3 h. The peptide-hemocyanin complex was mixed with complete Freund's adjuvant (Life Technologies, Grand Island, NY, USA) and injected intraperitoneally into rabbits. Antibodies were purified from the antiserum by protein A-TSK gel (Amersham, Little Chalfont, UK). The peptide-agarose affinity gel was prepared by coupling the peptide to Affi-Gel 15 (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's protocol. The peptide-specific antibody was eluted from the column with 0.1 M citrate buffer, pH 3.0.

Enzyme-linked immunosorbent assays (ELISA)

Patient sera (200 μ l) were mixed with 0.1N HCl (200 μ l) and heat-denatured at 100°C for 2 min as described previously (9) to remove the cross-reactivity of α 1-antitrypsin. After neutralization with one tenth volume of 0.5 M Tris, 0.5% Tween-20, 5% BSA, the solutions were centrifuged at 8,000 rpm for 30 min. Two aliquots of the resulting supernatant (40 μ l) were mixed with 10 μ l of the corresponding antibodies (1:4000 dilution in a final concentration), then incubated at 4°C overnight. The mixtures were placed on the microtitre plates (96 wells) for 2 h. The polystyrene ELISA plates (Serocluster U plates, Costar, Cambridge, MA) were coated with

0.1 μ g/ml synthetic proelafin polypeptides corresponding to elastase inhibitor domain and transglutaminase substrate domain dissolved in the solution (0.05 M Tris-HCl, pH 7.6, 0.15 M NaCl, 0.02% NaN₂) at 4°C overnight. In order to prevent a non-specific reaction, PBS/0.05% Tween/0.1% BSA (PTB) solution was used for washing or dilution buffer. After washing with PTB solution, the plates were incubated with peroxidase-conjugated anti-rabbit IgG (E-Y Laboratories, San Mate, CA, USA) diluted 1:1000 for 1 h. 0-Phenyldiamine was used as a substrate and the colour reaction was stopped by adding 2 M H₂SO₄. The optical density was examined with a microtitre plate spectrophotometer (Tosoh Microplate Reader, MPRA4, Tokyo) at 492 nm.

Immunohistochemical study

Specimens were taken from the lesional skins of generalized pustular psoriasis and PPP. Cryostat sections were soaked in methanol containing 5% H₂O₂, then incubated with 10% goat serum in order to block the non-specific protein-binding sites. The sections were incubated with anti-human proelafin polyclonal antibody raised against elastase inhibitor domain (elafin) or transglutaminase substrate domain at 1:1200 dilution for 24 h at 4°C. The sections were washed with Tris-buffered saline for 5 min \times 3, then incubated with biotin-labelled secondary anti-rabbit Ig antibody (Dako, Glostrup, Denmark) for 60 min at 1:500 dilution. The sections were incubated with avidin-biotin-conjugated peroxidase (Dako) for 30 min. Antigen-antibody was visualized by a chromogenic substrate, 3-amino-9-ethyl carbazole (Dako). The sections were counterstained with haematoxylin.

RESULTS

Preliminary experiments to explore the linearity between input proelafin peptides and optical density were performed with a range of proelafin peptides (1.56–100 ng/ml) and fixed amount of the corresponding antibodies (1:4000). The results showed that the ranges of 3.13–50 ng/ml in elastase inhibitor domain (elafin) correlated well with the resulting optical densities (Fig. 1a). Since sera contain numerous proteins which may hinder specific antigen-antibody reactions in the ELISA, we studied the linearity between the input amount of proelafin peptides (0, 20 and 40 µg/ml) and recovered proelafin peptides in the presence of normal serum as previously described by Alkemade et al. (9). The yield of elastase inhibitor domain (elafin) was found to be parallel to the input amount (Fig. 1b).

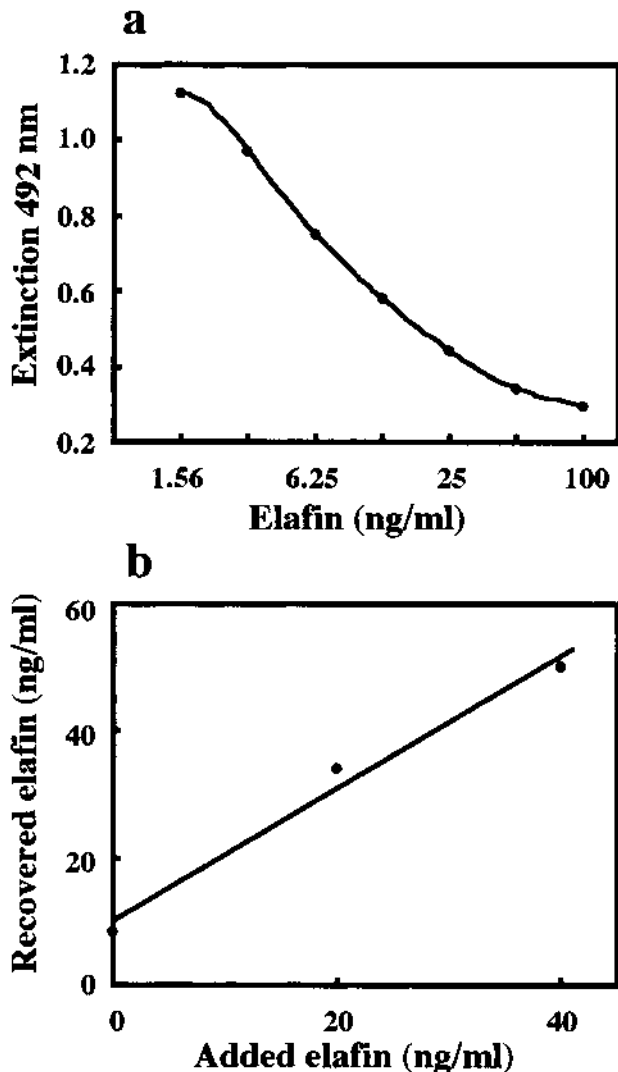


Fig. 1. Correlations between elafin dose and optical density, and between input and recovered amounts of elafin in the presence of normal serum in a competitive ELISA. (a) A range of elastase inhibitor domain (elafin) was added in the ELISA and optical density at 492 nm was measured. (b) A range of elastase inhibitor domain (elafin) was added in the ELISA in the presence of 10 µl of normal serum, then the amount of elastase inhibitor domain (elafin) was measured by the standard curve in (a).

Competitive ELISA using the antibody for elastase inhibitor domain (elafin) demonstrated no significant change in the elafin level of PPP compared with normal controls, but significantly higher titres in generalized pustular psoriasis than in normal controls ($p < 0.01$). The ELISA using the antibody for transglutaminase substrate domain showed similar results to those of the elastase inhibitor domain ($p < 0.01$) (Table I). The ELISA using the antibodies for both domains showed a high proelafin peptide level in the plaque type psoriasis with high PASI scores and low proelafin peptide level in the psoriasis with low PASI scores (Table I).

In PPP, the antibody for elastase inhibitor domain (elafin) demonstrated a weak reaction with the epidermal cells immediately surrounding the pustules (Fig. 2a), whereas a strong elafin immunoreactivity was observed in the subcorneal epidermis adjacent to the pustules (Fig. 2b). Pre-treatment of the antibody with the synthetic proelafin peptide corresponding to the elastase inhibitor domain (elafin) resulted in the disappearance of the reaction (Fig. 2c). Antibody for the transglutaminase substrate domain of proelafin showed similar staining to that of the elastase inhibitor domain (not shown). Skin specimens taken from generalized pustular psoriasis showed similar staining pattern to that of PPP (not shown).

DISCUSSION

Proelafin has 2 distinct domains in the molecule, an elastase inhibitor domain (elafin) and a transglutaminase substrate domain. We produced domain-specific antibodies and measured serum proelafin peptide level in the patients with generalized and localized pustular psoriasis (PPP). The measurement of each domain in patient sera revealed essentially similar results and demonstrated that both domains were elevated in the patients with generalized pustular psoriasis. This finding indicates that both domains are equally excreted from lesional skin into circulating blood, despite the binding ability of transglutaminase substrate

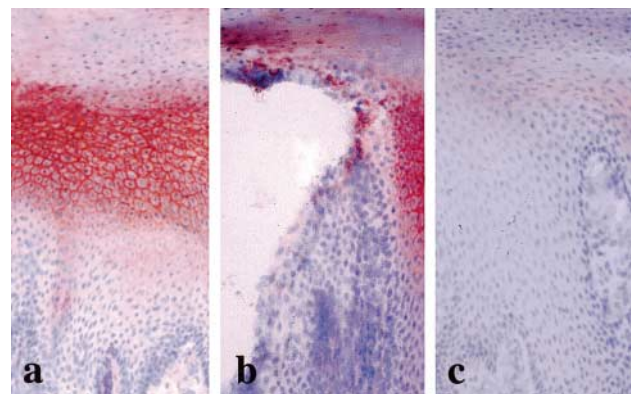


Fig. 2. Expression of elafin in the lesional skin of pustulosis palmo-plantaris (PPP). Cryostat sections of typical cases of PPP were stained with the antibody for elastase inhibitor domain (elafin). (b) A strong immunoreactivity in the non-pustular region of the epidermis (a) and a weak elafin immunoreactivity in the epidermis immediately surrounding the pustules were noted. (c) To show the specificity of the antibody, the antibody was pretreated with corresponding proelafin peptide and stained the non-pustular region of the epidermis (200×).

domain to cornified envelope (19) or extracellular matrix protein (18). Further investigation is needed to determine whether the transglutaminase substrate domain exists in serum as an intact molecule of proelafin or as a fragment.

Immunohistochemical studies on the lesional skins of PPP demonstrated that elafin immunoreactivity was consistently observed in the subcorneal region of epidermis adjacent to the pustules, whereas the immunoreactivity of elafin was lost in the central pustular region as has been described in generalized pustular psoriasis (11).

Kuijpers et al. (11) reported that elafin content in the scales from pustular psoriasis including localized type (PPP) was significantly lower than that in plaque type psoriasis, whereas elafin mRNA level in the epidermis of both types of psoriasis was equally enhanced. This implies that consumption of elafin is enhanced in pustular psoriasis compared with plaque type psoriasis and may explain the mechanism of pustular formation in pustular psoriasis (11). Since elafin in serum is considered to reflect the clearance rate of tissue elafin, we attempted to measure the elafin levels in the patients with generalized pustular psoriasis and PPP. Three cases of generalized type showed high titres of elafin, whereas localized type (PPP) showed normal levels of serum elafin. This suggests that elafin levels in patients with pustular psoriasis may depend upon the extent of the involved area and may explain the lower titre of elafin in PPP patients in spite of the considerable expression of elafin in the non-pustular region. This was confirmed by the ELISA data on plaque type psoriasis that severe plaque type psoriasis with high PASI scores showed a very high titre of serum elafin and mild plaque type psoriasis with relatively lower PASI scores exhibited low levels of serum elafin. The decreased expression of elafin in the central pustular region of generalized pustular psoriasis and PPP appeared to be unrelated to the serum level of elafin.

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