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Inhibition of growth and cysteine proteinase activity of *Staphylococcus aureus* V8 by phosphorylated cystatin α in skin cornified envelope

Masae Takahashi[a], Tadashi Tezuka[a,*], Nobuhiko Katunuma[b]

[a]*Department of Dermatology, Kinki University School of Medicine, 377-2, Ohno-Higashi, Osaka-Sayama, Osaka 589, Japan*
[b]*Institute for Health Sciences, Tokushima Bunri University, Yamashiro, Tokushima, Tokushima 770, Japan*

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Abstract The activity of a cysteine proteinase purified from *Staphylococcus aureus* V8 (SAV8) was inhibited by phosphorylated cystatin α (P-cystatin α) and by purified cornified envelope protein of newborn rat, a conjugated form of P-cystatin α. Immunohistochemical analysis demonstrated a marked decrease in P-cystatin α content in cornified envelope treated with sphingosine. The inhibition of papain activity by proteins from sphingosine-treated skin was much weaker than that exerted by proteins from the untreated skin. The suppression of SAV8 colony formation inoculated on the sphingosine-treated skin was examined. Colony formation on the sphingosine-treated skin was enhanced compared to that on normal skin. These findings suggest that P-cystatin α in the cornified envelope may have a bacteriostatic barrier function against bacterial infection, such as that with SAV8.

Key words: Phosphorylated cystatin α; Cornified envelope; Epidermal barrier function; *Staphylococcus aureus* V8

1. Introduction

Cystatin α is specifically located only in the epidermis [1]. In our previous studies, we reported that cystatin α was phosphorylated by protein kinase C [2] and that phosphorylated cystatin α (P-cystatin α) was incorporated in the keratohyalin granules of the stratum granulosum [3]. P-cystatin α is conjugated with other glutamine-rich skin protein components, such as filaggrin, in the cornified envelope [4]. These findings suggested that P-cystatin α in the cornified envelope would have an inhibitory effect against bacterial and viral cysteine proteinases. Korant et al. [5,6] reported that egg white cystatin inhibited the cysteine proteinase of the picorna virus and inhibited the processing of the virus precursor protein. It was found that the replication of Herpes simplex was blocked by cystatin C [7]. In this study, we found that P-cystatin α in the cornified envelope appeared to inhibit bacterial and viral cysteine proteinases, suggesting that skin P-cystatin α may play a role as a protective barrier against bacterial and viral infections.

2. Materials and methods

2.1. Materials

Newborn Sprague–Dawley rats were purchased from SLC, Japan. *Staphylococcus aureus* V8 was obtained from ATCC, USA. Superdex 75, Superose 12, and Mono Q columns were purchased from Pharmacia-LKB, Sweden. Papain and sphingosine were obtained from Sigma, USA. Aminomethyl-coumarin (MCA) substrate was purchased from the Peptide Institute, Japan. Food stamp 'Nissui' for the specific detection of SAV8 colonies on the skin was obtained from Nissui Pharmaceutical Co., Ltd., Japan.

*Corresponding author.

Abbreviations: P-cystatin α, phosphorylated cystatin α; PoAb, polyclonal antibody; SAV8, *Staphylococcus aureus* V8.

2.2. Purification of P-cystatin α and preparation of polyclonal antibody for P-cystatin α

P-cystatin α was purified to homogeneity by 2-dimensional electrophoresis, as described previously [8]. Three-day-old rat epidermal extract in 50 mM Tris-HCl (pH 7.4) was subjected to preparative isoelectric focusing, to Superose 12 gel filtration and finally to Mono Q anion-exchange column chromatography. The purified P-cystatin α was then injected to rabbits to prepare the polyclonal antibody (PoAb).

2.3. Preparation of cornified envelope from newborn rat stratum corneum

The cornified envelope was prepared from newborn rat stratum corneum with 8 M urea in 50 mM Tris-HCl (pH 9.0), containing 10 mM 2-mercaptoethanol, at 95°C for 10 min and sonicated. After centrifugation, the residue was washed 3 times with 50 mM Tris-HCl (pH 7.5) and then 3 times with distilled water, after which it was freeze-dried. To prepare a sample for immunoelectron microscopy, the stratum corneum was treated with 1% SDS at 95°C for 10 min and sonicated. The sample was then washed with 50 mM Tris-HCl buffer (pH 7.5) containing 1% Triton X-100.

2.4. Partial purification of SAV8 cysteine proteinase

SAV8 was cultured in *Staphylococcus aureus* medium No. 110 for 8 h at 37°C [9]. The culture medium with the cells was sonicated, filtered, and concentrated. Cysteine proteinase activity was determined with Z-Phe-Arg-MCA as a substrate in the presence of 4 mM EDTA and 2 mM dithiothreitol in 0.1 M phosphate buffer (pH 7.4). The reaction mixture was incubated for 20 min at 37°C, and the fluorescence of the MCA released was determined according to [10]. This cysteine proteinase was purified approximately 25 times from the original extract, using a modification of the method reported by Arvidson et al. [11].

The inhibition of SAV8 cysteine proteinase by P-cystatin α or cornified envelope was assayed by incubating P-cystatin α with SAV8 cysteine proteinase for 10 min at 37°C and by incubating the purified cornified envelope suspension with 4% Brij-35 for 10 min at 37°C, respectively.

2.5. Immunostaining of P-cystatin α in sphingosine-treated model skin

Treatment with sphingosine, a natural inhibitor of protein kinase C, leads to a remarkable decrease in the amount of P-cystatin α in keratohyalin granules [2]. Sphingosine was applied to newborn rat skin for 3 days and the skin was then stained with anti-P-cystatin α PoAb. DMSO was used as the control. The skin was embedded in Tissue Tek OCT compound and cryostat sections that had been fixed in cold

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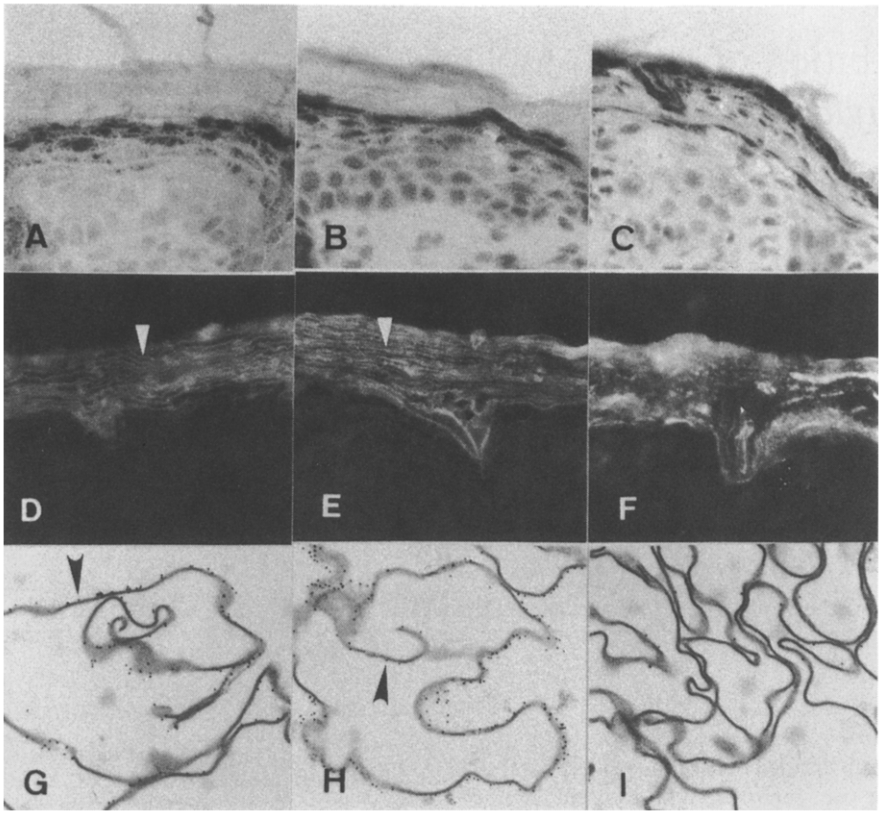


Fig. 1. Morphological demonstration of P-cystatin α contents in various model skins using anti-P-cystatin α PoAb. (A,D,G) Untreated control skin; (B,E,H) DMSO-treated skin; (C,F,I) sphingosine-treated skin; (A,B,C) hematoxylin-eosin staining; (D,E,F) immunofluorescent staining of P-cystatin α contents using anti-P-cystatin α PoAb; (G,H,I) immunoelectron microscopic detection of P-cystain α contents using anti-P-cystatin α PoAb. Arrowheads in D, E, G and H indicate the specific reaction of anti-P-cystatin α PoAb.

ethanol for 4 min were prepared. After treatment with sphingosine or DMSO, the cornified envelope was subjected to heat and SDS treatment and reacted with anti-P-cystatin α PoAb, then with anti-rabbit IgG goat IgG labelled with gold particles. The specimens were embedded in Epon and ultrathin sections were prepared for electron microscopic observation. Papain inhibition of cornified envelope treated with sphingosine or DMSO was compared.

2.6. Suppression of SAV8 colony formation by P-cystatin α in the model skin

The experimental designs are illustrated in the explanatory footnote in Fig. 3. Namely, a bacterial suspension diluted with 0.9% NaCl was incubated with or without P-cystatin α. Filter papers (2 cm in diameter) were soaked in an aliquot of these solutions (0.2 ml) and were placed on the back skin of newborn rats for 15 h at 15°C. Bacterial suspension that had been preincubated with P-cystatin α was inoculated on the sphingosine-treated skin shown in Fig. 3a. As a control, bacterial suspension that had not been pretreated with P-cystatin α was also inoculated on the sphingosine-treated skin, the DMSO-treated skin, and the non-treated skin, as shown in Fig. 3b, c and d, respectively. After the incubation of these inoculated skins for 15 h at 15°C, the number of SAV8 colonies on the respective skin specimens was determined by the stamp agar method.

3. Results

The activity of SAV8 cysteine proteinase was inhibited by P-cystatin α and by the cornified envelope; P-cystatin α was solubilized in phosphate buffer, the cornified envelope was used as a suspension. The inhibitory activity of the cornified

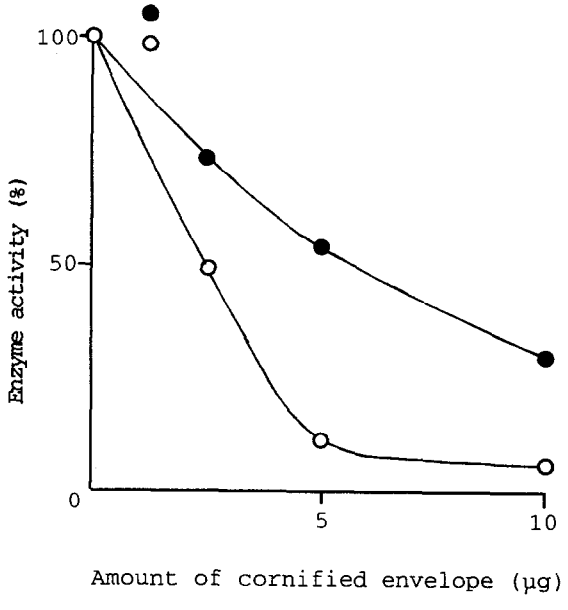
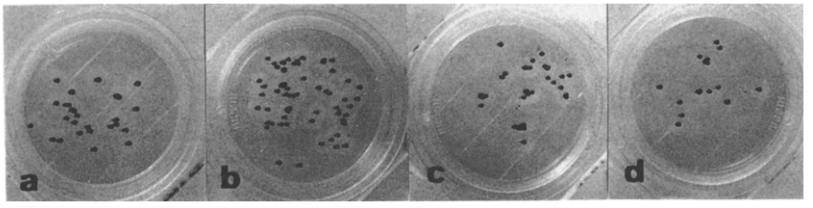


Fig. 2. Inhibition of papain by cornified envelope purified from skins treated with DMSO or sphingosine. Open circles and closed circles indicate cornified envelope purified from skins treated with DMSO or sphingosine, respectively.



a. Sphingosine-treated skin
b. Sphingosine-treated skin
c. DMSO-treated skin
d. Untreated skin

S. aureus V8 preincubated with P-cystatin α
S. aureus V8 preincubated in saline
S. aureus V8 preincubated in saline
S. aureus V8 preincubated in saline

A filter paper soaked with S. aureus V8 solution was placed on the back skin of newborn rats for 15 hours at 15°C

The number of colonies was counted by the stamp agar method.

Fig. 3. Comparison of SAV8 colony formation on the surfaces of sphingosine-treated or untreated skin. (a) Preincubated SAV8 with P-cystatin α; (b,c,d) preincubated SAV8 without P-cystatin α; (a,b) sphingosine treated skin (low P-cystatin α level); (c) DMSO treated skin (high P-cystatin α level); (d) normal skin without any treatment (high P-cystatin α level). The procedure described above shows the experimental design for suppression of SAV8 colony formation by P-cystatin α in the cornified envelope.

envelope appeared to be weaker than that of P-cystatin α. Since the cornified envelope is insoluble, the inhibitory reaction was carried out with a suspension. Immunofluorescent and immunoelectron microscopic studies showed a marked decrease in P-cystatin α content in the cornified envelope treated with sphingosine, as shown in Fig. 1. The amount of P-cystatin α in the DMSO-treated and untreated control skins was much greater than that in the sphingosine-treated skin. The inhibitory activity of cornified envelope proteins against papain was compared; the protein prepared from sphingosine-treated skin showed much weaker inhibition than that shown by the protein prepared from DMSO-treated skin as shown in Fig. 2. To test the bacteriostatic effects of P-cystatin α, the SAV8 suspension was inoculated on the sphingosine-treated model skin, which served as a solid medium. The number of SAV8 colonies on the sphingosine-treated model skin was much greater than the numbers on the skin treated with DMSO, and an untreated skin (Fig. 3). There were significant differences among the 4 groups in the numbers of colonies on the stamps, as shown in Fig. 3a,b,c,d. However, when SAV8 was preincubated with P-cystatin α, colony formation was markedly suppressed, to the normal levels, as a of Figs. 3 and 4. There were significant differences in colony formation between the sphingosine-treated model skin as shown in b of Figs. 3 and 4 and the control skins as shown in c and d of Figs. 3 and 4.

4. Discussion

We found here that both the cornified envelope and P-cystatin α inhibited the activity of SAV8 cysteine proteinase. As we have reported previously, the cornified envelope seems to be formed by the conjugation of P-cystatin α, mediated by epidermal transglutaminase with a suitable glutamine-rich protein [12], such as loricrin [13], involucrin [14], or filaggrin [15], in the stratum corneum. Since P-cystatin α is a lysine-rich protein, it is a good substrate for epidermal transglutaminase. P-cystatin α is a protein component of the cornified envelope [4]. It is therefore possible that the inhibitory function of the cornified envelope was due to P-cystatin α. We have also found that the inhibitory function of the cornified envelope against cathepsin L was heat-stable (data not shown). It is interesting to note that free P-cystatin α inhibited the activity of cathepsins B, H, and L [3], but that the conjugated form of P-cystatin α did not inhibit against cathepsin B [16]. The biological significance and chemical mechanisms underlying this difference are not yet known; however, these features may be related to the fact that the most bacterial cysteine proteinases are of the cathepsin L type (papain type). In this experiment, we developed a model skin system in which P-cystatin α was decreased by sphingosine treatment [4]. Colony formation was increased in sphingosine-treated model skin and was suppressed by the

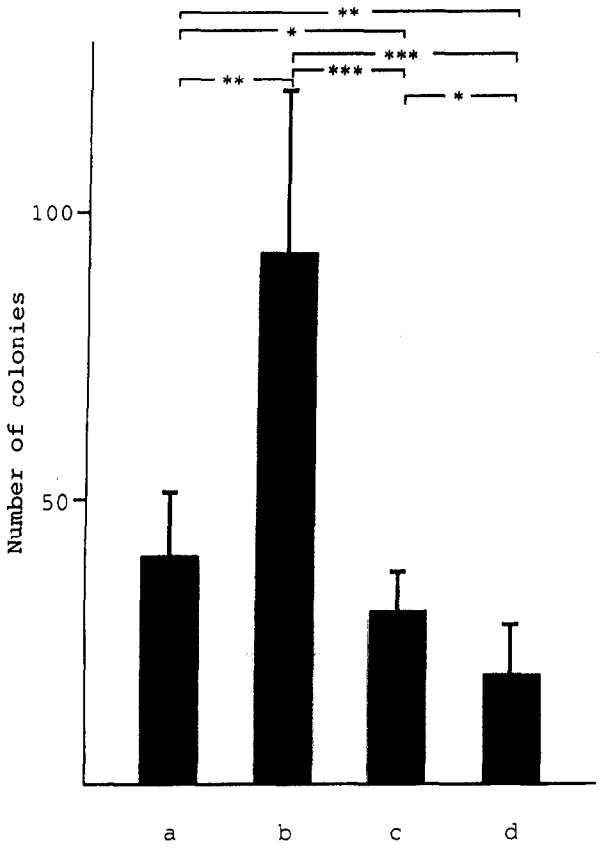


Fig. 4. Quantitative illustration of the SAV8 colony formations on various experimental skins. The explanations of column a, b, c or d are described in Fig. 3. *No significant difference; $^{**}P < 0.05$; $^{***}P < 0.01$. The experiments were performed 4 times.

addition of P-cystatin α, indicating that P-cystatin α in the cornified envelope of the skin may act as a barrier against some bacteria that contain cysteine proteinases. Sphingosine and H1 histone in the epidermis have been reported to have antimicrobial and antifungal activities [17,18]. However, proteinase inhibitors have also been reported to have antiviral, antimicrobial, and antifungal activities [5–7,19,20]. Moreover, it is well known that *Staphylococcus aureus* is increased on the skin surface of the lesions of atopic dermatitis. Further, we have found, using immunohistochemical techniques, that the immunohistochemical reactivity of cystatin A was decreased in the lesions of atopic dermatitis (unpublished data). Our findings indicated that the P-cystatin α contained in the cornified envelope inhibited bacterial cysteine proteinases; thus, it would appear that P-cystatin α may suppress the growth of bacteria.

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