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A NOVEL EPIDERMAL CELL DIFFERENTIATION INHIBITOR (EDIN): PURIFICATION AND CHARACTERIZATION FROM *STAPHYLOCOCCUS AUREUS*

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SUMMARY A factor inhibiting the calcium-induced terminal differentiation of cultured mouse keratinocytes was purified to homogeneity from the extracellular products of *S. aureus* E-1 and designated 'epidermal cell differentiation inhibitor' (EDIN). EDIN activity was sensitive to trypsin and heat-labile, suggesting that EDIN is a protein. EDIN gave a single band with a molecular weight of 27,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was found to be a single chain polypeptide, having an isoelectric point higher than 9. The N-terminal amino acid sequence of EDIN was determined as A-D-V-K-N-F-T-D-L. EDIN inhibited the differentiation of not only mouse but also human keratinocytes in culture.

Staphylococcus aureus is an important pathogen in both hospital and community environments and it continues to be the most common cause of skin wound infections (1,2). Skin diseases induced by *S. aureus* have been shown to be caused by its extracellular proteins (3,4). However, the diversity of such proteins, which are produced simultaneously by a single strain, has made it difficult to define their roles in the pathogenesis of staphylococcal skin diseases.

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The epidermis consists of several layers that differ in levels of differentiation, and epidermal cells change dramatically in morphology during the differentiation process. We have employed a mouse keratinocyte culture as an in vitro model of differentiation and studied the biological effect of extracellular products of S. aureus on the differentiation of keratinocytes. With this primary culture system, cell differentiation can be controlled by the calcium concentration in the medium (5). We have previously shown that S. aureus culture supernatants contain a factor(s) that inhibits differentiation of mouse epidermal keratinocytes (6) and designated it 'epidermal cell differentiation inhibitor' (EDIN).

We herein report the purification and some characterization of this novel protein from extracellular products of S. aureus.

MATERIALS AND METHODS

Materials Staphylococcus aureus E-1 was isolated from skin lesions of impetigo with atopic dermatitis (6). S. aureus E-1 exponentially growing in TY medium (10 g of yeast extract, 17 g of Trypticase, 5 g of NaCl, and 2.5 g of K_2HPO_4 per liter of distilled water) was inoculated into 10 liters of fresh medium and incubated for 24 h at 37°C with continuous agitation using a magnetic stirrer under a constant atmosphere of 10% CO_2. The culture was centrifuged at 10 000 x g for 20 min at 2°C and the supernatant fluid was passed through a membrane filter (0.22 µm, Millipore Corp., Bedford, MA). Purification was carried out with concentrated culture filtrate (CCF), which was prepared by 80% saturated ammonium precipitation of the culture filtrate. TSKgel SP Toyopearl 650 and TSKgel HA-1000 were purchased from Tosoh, Tokyo, Japan. VIDAC RP-C_4 was obtained from Cypress International Ltd., Tokyo, Japan.

Assay for differentiation of mouse keratinocytes Epidermal keratinocytes were isolated from newborn CD-1 mouse skin and cultured under low calcium conditions as described previously (5, 6). One million cells were plated on 35-mm plastic dishes. The cells were selectively grown as a monolayer in Eagle's minimum essential medium (Joklik-modified) without calcium, to which was added 10% chelated fetal calf serum, penicillin (75 Unit/ml) and streptomycin (50 µg/ml) (Grand Island Biological Co., Grand Island, NY), and the calcium concentration was adjusted to 0.05 mM. Terminal differentiation was induced in epidermal cells growing in the presence of 0.05 mM calcium by increasing the calcium concentration to the normal level (1.8 mM). The cells then stratified exhibited morphological changes (5, 7) and cornification occurred (8), all characteristic of differentiated epidermal cells. Therefore, inhibition of morphological changes was used as the major criterion in determining EDIN activity during purification. For morphological observation, cells grown for 6-7 days in 0.05 mM calcium medium (low calcium medium) were switched to an identical medium containing 1.8 mM calcium (high calcium medium) and monitored for 48 h by phase contrast microscopy.

Assay for differentiation of human keratinocytes Adult human keratinocytes were cultured as described previously (9). Second-passage cells were plated in 6-well plates at a density of 1.5 x 10^4/well in medium containing

0.1 mM calcium. After reaching subconfluency, cells were refed with medium containing 1.8 mM calcium and EDIN was added to the fresh medium at concentrations of 25, 50, 100 and 500 ng/ml. After 96 h, cells were detached with trypsin/EDTA solution, then fixed in 3.7% formaldehyde for 8 min, followed by cold methanol (-20°C) for 2 min. Cells were washed three times with Tris-buffered saline (10 mM Tris, 0.15 M NaCl, pH 7.6) containing 0.2% Tween 20 (TBS-Tween), and incubated for 15 min in PBS containing 10% (v/v) normal goat serum to block nonspecific binding. Cells were incubated with rabbit anti-human involucrin IgG and then FITC-conjugated goat anti-rabbit IgG at room temperature for 30 min each. After washing with TBS-Tween, cells were filtered through nylon mesh and analyzed by flow cytometry (FACStar, Becton Dickinson, Oxnard, CA). A total of 10^4 cells were used in each analysis.

Other procedures Protein was determined by the method of Lowry et al. (10) with BSA as a standard. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Weber and Osborn (11). The isoelectric point was determined by interpolation from Pharmacia IEF standards (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) with isoelectric focusing using IEF gel (pH 3-9) on the Pharmacia Phast System (Pharmacia LKB Biotechnology AB). Amino acid analysis was performed on a Beckman amino acid analyzer (model 835, Beckman Instruments, Inc., Belmont Drive, NJ) using ninhidrin as a coloring reagent. Samples were hydrolyzed in 6 N HCl containing 0.1% phenol at 130°C for 4 h. Amino acid sequence analysis was performed on an automated gas-phase protein sequencer (model 470A; Applied Biosystems, Foster City, CA, USA) according to operation program 02RPTH provided for the sequencer.

RESULTS

EDIN was purified from a concentrated culture filtrate of S. aureus E-1. The inhibitory activity was monitored by assessing its effect on morphological differentiation of cultured mouse epidermal keratinocytes. CCF dialyzed against 50 mM phosphate buffer (pH 5.0) was loaded onto a cation exchange column of SP-Toyopearl 650 (22 x 200 mm) equilibrated with the same buffer. The column was washed with the starting buffer until the major portion of unbound proteins passed through. EDIN activity bound to the column was then eluted by increasing the NaCl concentration with a linear gradient from 0 to 0.5 M for 120 min (Fig. 1a). Fractions with EDIN activities were pooled and dialyzed against 50 mM phosphate buffer (pH 6.9) and subsequently loaded onto a TSKgel HA-1000 hydroxyapatite column (7.5 x 75 mm). EDIN activity bound to this column was eluted by a linear gradient of phosphate buffer (pH 6.9) from 50 mM to 0.5 M for 30 min. The EDIN activity was eluted in the second major peak (Fig. 1b). The active fraction was then subjected to further purification by RP-HPLC using a column of VIDAC RP-C_4 (4.6 x 250 mm). The solvent system was linear gradient elution from A to B for 30 min.

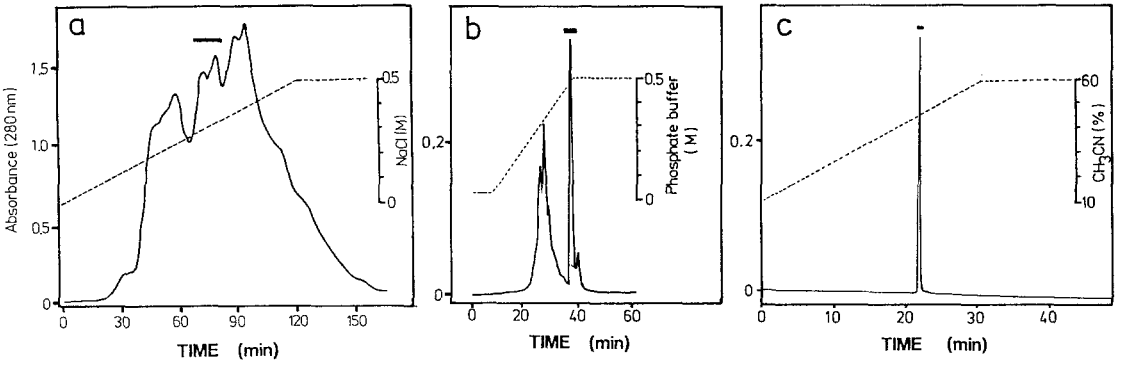

Fig. 1. Purification of Epidermal Cell Differentiation Inhibitor (EDIN) from the culture filtrate of S. aureus E-1. a, Cation exchange chromatography of concentrated culture filtrate. Column size, 22 x 200 mm; flow rate, 240 ml h^{-1}; solvent system, linear gradient elution from A to B for 120 min. A, 50 mM phosphate buffer (pH 5.0); B, 50 mM phosphate buffer containing 0.5 M NaCl (pH 5.0). b, Hydroxyapatite-HPLC of the active fractions obtained from cation exchange chromatography. Column size, 7.5 x 75 mm; flow rate, 60 ml h^{-1}; solvent system, linear gradient elution from A to B for 30 min. A, 50 mM phosphate buffer (pH 6.9); B, 0.5 M phosphate buffer (pH 6.9). c, RP-HPLC of the active peak obtained by Hydroxyapatite-HPLC. Column size, 4.6 x 250 mm; flow rate, 60 ml h^{-1}; solvent system, linear gradient elution from A to B for 30 min. H_2O:CH_3CN:5% TFA (trifluoroacetic acid) for A was 90:10:1, for B was 40:60:1 (v/v). The horizontal bars indicate pooled fraction of EDIN.

H_2O:CH_3CN:5% TFA (trifluoroacetic acid) for A was 90:10:1, for B was 40:60:1 (v/v). The EDIN activity eluted as a single peak was pooled (Fig. 1c). The yield of EDIN was estimated to be about 1.3 mg, starting from 195.7 mg protein in the CCF.

The protein nature of EDIN was demonstrated by its loss in activity with digestion by trypsin and with heating at 60°C for 30 min. The purified EDIN

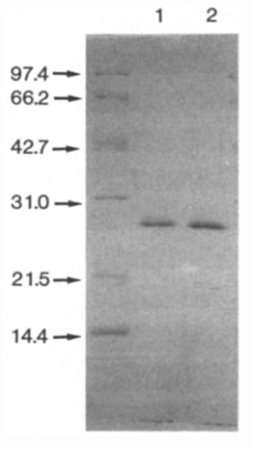

Fig. 2. Analysis of purified EDIN on SDS-PAGE. SDS-PAGE was performed in a 15% gel by the method of Weber and Osborn (11). Sample (1 µg of protein) was treated with (1) or without (2) 2-mercaptoethanol at 100 °C for 3 min prior to electrophoresis. Standard Mr values are indicated by the arrows ($x10^3$). Proteins were stained with Coomassie brilliant blue.

was found to be a single protein band by SDS-PAGE with the same mobility with or without a reducing agent (Fig. 2). Thus EDIN appeared to be a single peptide with a molecular weight of 27,000 (Fig. 2). Its isoelectric point was higher than 9 (data not shown). Table 1 shows the amino acid composition of purified EDIN. The results of the automated sequence analysis of the EDIN suggested the following N-terminal sequence: A-D-V-K-N-F-T-D-L. This sequence has no homology with other staphylococcal proteins, and no identical sequence was found in the known protein sequences stored in the data bank at NBRF (National Biomedical Research Foundation).

When the optimum concentration (50-100 ng/ml) of purified EDIN was added to the medium together with 1.8 mM calcium, terminal differentiation of mouse keratinocytes was completely inhibited, since no stratified cells were observed in EDIN-treated cells as was the case in CCF-treated cells as described previously (5).

We examined the effect of EDIN on differentiation of human epidermal keratinocyte by measuring involucrin-positive cells in culture. Involucrin is a precursor protein of detergent-insoluble cornified envelope and one of

Table 1
Amino acid composition of EDIN[a]

Asx	29.22
Thr	14.01
Ser	16.52
Glx	19.67
Pro	4.49
Gly	13.94
Ala	12.36
1/2Cys	0.00
Val	13.10
Met	0.86
Ile	11.09
Leu	24.00
Tyr	12.97
Phe	3
His	0.00
Lys	20.62
Arg	9.75
Trp	ND[b]

a Figures for each residue calculated assuming three phenylalanines per molecule.
b ND, not determined.

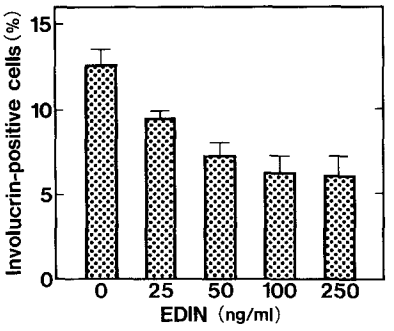

Fig. 3. Effect of EDIN on differentiation of human keratinocytes. Experiment was performed in triplicate, and results of one of the three experiments are presented.

the typical markers of terminal differentiation of keratinocytes (12). The control population contained 12.7 ± 1.0% involucrin-positive cells. Addition of EDIN dose-dependently decreased the percentage of involucrin-positive cells, being 6.3 ± 1.1% at 100 ng/ml (Fig. 3). This indicates that EDIN inhibits differentiation of human keratinocytes.

DISCUSSION

From the deduced partial amino acid sequences, EDIN appears to be a novel protein. The amino acid composition of the EDIN resembles those of staphylococcal alpha haemolysin (4) and beta haemolysin (4), i.e. absence of half-cysteine and relatively high ratios of lysine, aspartic acid and glutamic acids, which suggests that EDIN is a member of the haemolysin family. However, EDIN has no haemolysin or leucocidin activity suggesting that EDIN is not a membrane-damaging toxin (data not shown). The mechanism by which EDIN inhibits terminal differentiation of epidermal keratinocytes is not known. Further studies with purified EDIN are in progress to clarify the mechanism of inhibition of keratinocyte differentiation and the possible role of EDIN in staphylococcal skin diseases.

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