Induction of Neutral Lipid-Containing Granules by Staphylococcal Lipase from Clinical Isolates

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Abstract

Culture supernatant of *Staphylococcus aureus*, isolated from a patient who died after surgery induced granulation in several cultured cell lines of human and animal origin. Histochemical study revealed that the granules contained neutral lipid. A granule-inducing substance was partially purified by DEAE- TOYOPEARL column chromatography. The final preparation revealed two major proteins (45 and 43 kDa) in SDS-PAGE, both of which were cytopathic. Sequencing of the N-terminal and internal amino acids of both proteins demonstrated that the 45-kDa protein is a mature staphylococcal lipase and the 43-kDa protein is a truncated form of this mature lipase. Recombinant lipase also showed cytopathicity. A mutant lipase, constructed by substitution of a serine residue in the catalytic triad of the lipase into alanine, lost both cytopathic and enzymatic activities, indicating that the lipase activity is critical for the cytopathicity. Our results indicate that staphylococcal lipase induces granule formation presumably by impairing lipid metabolism of the cultured cells. Our results raise the possibility that the lipase may play a role in bacterial infection by affecting the cellular metabolism of host cells.

Keywords: *Staphylococcus aureus*; Lipase; Cytopathicity; Recombinant; Methicillin-Resistant *S. aureus* (MRSA); Granule

Introduction

Staphylococcus aureus is an important pathogen causing a wide range of infections in humans and other animals. Possible virulence factors, including various proteinaceous toxins and enzymes, and their roles in the bacterial infection have been the main foci of investigations [1,2]. Among them, alpha-toxin, which is one of the major virulence determinants of microorganisms, and enterotoxins have been extensively examined and characterized biochemically and biologically [2]. However, a precise understanding of the enzymes secreted by *S. aureus* upon bacterial infection has yet to be obtained.

Staphylococcal lipases have been purified from several *Staphylococcus* spp. and their enzymatic properties have been well documented [3-6]. Molecular biological studies and three-dimensional structure analysis revealed that amino acid residues

Ser-Asp-His form the active center of the catalytic activity [3,7] and the serine of this catalytic triad is part of the pentapeptide Gly-X-Ser-X-Gly, which is extremely conserved among serine hydrolases [7]. Staphylococcal lipase-2 of *S. aureus* (SAL-2) encoded by the *geh* gene is produced as a 76-kDa preproprotein, which is processed to a 43-45-kDa mature protein extracellularly [8].

The association of lipase with bacterial pathogenesis has been described as follows. The enzymatic digestion of lipids can benefit bacterial growth [9], and the resultant free fatty acids may impair several immune system components [10-15]. The enzyme itself may interfere with human leukocyte function [16, 17]. Various types of staphylococcal lipase has been highly purified [4-6,18-22], but their biological role remained unclear, mainly because a small amount of detergent contaminated in the purified preparation might have affected their observations.

In this study, we identified novel cytopathicity in culture supernatant of *S. aureus* clinical isolates. This cytopathicity was shown to be caused by staphylococcal lipase, and the active site of the lipase was shown to be necessary for both enzymatic and cytopathic activities.

Materials and Methods

Bacterial culture

A methicillin-resistant *Staphylococcus aureus* (MRSA) strain, 10-123, was isolated from a stool of a patient suffering from severe diarrhea after post-operative antibiotic treatment, who eventually died. MRSA strains No. 1, 2, 3, 4, 5, 6, and Methicillin-Sensitive *S. aureus* (MSSA) strains No. 1, 2, 3, 4 were also clinically isolated. The stock cultures kept at -80°C were inoculated onto nutrient agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and incubated overnight. A single colony on the agar plate was picked up, inoculated into 5 ml of nutrient broth (Nissui), and cultured overnight with shaking. A portion of 1.5 ml of the seed culture was then inoculated into 150 ml of nutrient broth in a 500-ml flask and the flask was incubated at 37°C for 24 h with shaking. To purify a cytopathic substance, 50 ml of the culture was

inoculated into 1.5 L of nutrient broth in a 5-L flask and incubated 24 h at 37°C with shaking. For the lipase and cytopathicity assays, the bacterial culture was centrifuged at 10,000 x g for 20 min, and filtered through cellulose acetate filters (0.22 µm pore size, Sartorius AG, Goettingen, Germany). The culture filtrate was stored at -20°C until use.

Characterization of the strain

The strain MRSA 10-123 was characterized as follows. Many kinds of drug sensitivity including for oxacillin were tested by MIC determination, disk diffusion and agar screen methods, respectively. Hemolytic activity by α -toxin was detected on rabbit blood plate [23]. Producibility and typing of staphylococcal enterotoxin, TSST-1 and coagulase were investigated by using, respectively, SET-RPLA, TST-RPLA and Coagulase Antisera (Seiken, Tokyo, Japan). Specific primers for the amplification of *tox*A and *luk*S genes were designed:

5'-CAT TGC TGG TCA ATA TAG AG-3' and 5'-GTT GGG CTC TCT AAA ATT GT-3' (for *tox*A), and 5'-CTA CAA CTT TAT CTG TGA GC-3' and 5'-TTA TAT TGG AAT GGC CAT CG-3' (for *luk*S). The *mec*A gene was identified according to Murakami *et al.* [24]. The *nuc*A gene was detected by the method of Brakstad *et al.* [25]. Detection of genes for enterotoxins and toxic shock syndrome toxin 1 was performed on the basis of the method of Johnson *et al.* [26].

Cytopathicity assay

UV⁻-1 human embryonic clonal cells [27], Chinese hamster ovary (CHO) cells, were maintained with Eagle's minimum essential medium (MEM) supplemented with 10% newborn calf serum throughout the experiments. The cells were inoculated at 1.5 x 10³ cells/well (150 µl/well) in a 96-well plate. A total of 50 µl of the supernatant or chromatography effluents was added into the well and the mixture was incubated at 37°C for 24 h. Morphological changes of UV⁻-1 cells were examined under phase-contrast light microscopy.

Lipase assay

Lipase activities in the supernatant were determined with Lipase Kit S (Dainippon Pharmaceutical, Co. Ltd., Osaka, Japan) with a colorimetric substrate. The supernatant was serially diluted with a dilution buffer supplied in the kit and subjected to the lipase assay in according with the manufacturer's instructions. The reaction was terminated with termination buffer, and optical density at 412 nm (OD₄₁₂) of the reaction mixtures was determined. One unit was defined as the activity that shows an increase of OD₄₁₂ of 1.0 for 30 min at 30°C. The experiments were performed at least three times and the data are representative of three experiments and each point represents the mean of duplicate measurements.

Purification of cytopathic substance

MRSA10-123 culture supernatant (10 L) was precipitated by adding 75 mM ZnCl₂ and centrifuged at 8,000 x g for 15 min at 4°C. The precipitate was dissolved in 2 L of 0.4 M Na₂HPO₄ and centrifuged at 8,000 x g for 15 min. The supernatant was dialyzed

against 20 L of TEN buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM Na₃N₃) overnight. The dialysate was applied to a DEAE-TOYOPEARL column (3 x 90 cm). After washing away unbound materials with TEN buffer, cytopathic fractions were eluted with TEN buffer containing 0.5 M NaCl. The effluent was collected and dialyzed against TEN buffer, and re-applied to DEAE-TOYOPEARL. Elution was performed with TEN buffer containing a 0-0.3 M NaCl gradient. The cytopathic fractions were concentrated with ultrafiltration membrane (PM-10 membrane, Amicon) to a final volume of 1.0 ml. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the partially purified preparation revealed two major protein bands with molecular masses of 43 and 45 kDa. In order to identify the proteins, ZnCl₂-imidazole staining was performed [28] and then recovery was carried out by electroelution (The Centrilotur Micro-Electroelutor, Amicon).

Analysis of amino acid sequence

N-terminal sequences of the 43-and 45-kDa proteins were determined with a protein sequencer (PSQ-1, Shimadzu, Kyoto, Japan) after electro blotting onto a PVDF membrane (Transblot, BioRad) by the method of Towbin [29]. To determine the internal sequences, the 43-and 45-kDa proteins extracted from the polyacrylamide gel were digested with lysyl-endopeptidase (LEP; Wako, Tokyo, Japan) [30]. Peptides purified by reverse-phase chromatography (LC-6A, Shimadzu, Kyoto, Japan; ODS-HG-5 column, Nomura Chemical, Tokyo, Japan) were sequenced.

Preparation of the recombinant lipase and mutant lipase

MRSA10-123 was grown with shaking overnight at 37°C in 5 ml of tryptic soy broth and recovered by centrifugation. After washing, the chromosomal DNA was prepared as described previously [31]. For cloning of the mature part of the lipase gene *geh* [32], PCR primers with *Bam*HI or *Eco*RI sites were used: 5'-CT GGA TCC AAA GCG AAT CAA GTA CAA CCA CTT A-3' (LIP-S1) and 5'-GC GAA TCC TTA ACT TGC TTT CAA TTG TGT TCC TT-3' (LIP-A1). A 1,188-bp DNA fragment was amplified by PCR and digested with *Bam*HI and *Eco*RI. The digested fragment was cloned into pGEX-2T, a glutathione-S-transferase (GST) fusion vector plasmid (Pharmacia, Sweden). BL21 (DE3) cells transformed with *pGH* 26 (a plasmid with an insert encoding the GST-lipase fusion protein) were incubated until the early log phase and the recombinant lipase was induced with 0.1 mM IPTG. After 3-5 h, the cells were collected by centrifugation and lysed by sonication in PBS-1% Triton X-100. After centrifugation and filtration, the recombinant GST-lipase fusion protein was absorbed to a Glutathione Sepharose 4B column, and cleaved by using thrombin at 4°C overnight. Recombinant lipase was eluted with 0.5 M NaCl-50 mM Tris HCl, pH 8.0. The plasmid expressing the recombinant mutant lipase was constructed by site-directed mutagenesis. Mutated primers containing a *Sph*I site are: 5'-CCA CCC ATA GCA TGC CCT AC-3' (LIP-MA1) and 5'-GTA GGG CAT GCT ATG GGT GG-3' (LIP-MS1). A 362-bp DNA fragment for LIP-S1/LIP-MA1 and an 850-bp DNA fragment for LIP-MS1/LIP-A1 were amplified and each PCR product was digested with *Sph*I. These two fragments were ligated into pGEX-2T. The recombinant mutant lipase was purified by the same method.

Preparation of anti-lipase antibody

BALB/C female mice were injected with either 5 or 25 µg of the purified cytopathic fraction. Several injections were performed over 2 months. The serum was recovered after 3 months and the anti-recombinant lipase titer was checked. The anti-recombinant lipase antibody raised in rabbit (Japanese White) was purified by protein A-coupled Sepharose column chromatography 3 months after immunization. A total of 5 mg of protein A (EY Laboratories, Inc., San Mateo, CA) was coupled with a pre-packed HiTrap NHS-activated (Pharmacia Biotech) column according to the manufacturer's instructions. The column was equilibrated with PBS before use and 1 ml of rabbit serum (anti-recombinant lipase serum or pre-immune serum) was applied to it. After the column was washed with 5 ml of PBS, the bound antibody was eluted with 3 ml of 0.2 M glycine - HCl, pH 2.8-0.5 M NaCl. The effluent was neutralized with 1 M Tris-HCl, pH 8.0, immediately, concentrated by ultrafiltration and dialyzed against PBS.

Western blot analysis

The specificity of the antibodies purified by protein A-coupled affinity chromatography was examined by Western blot analysis. The effluent from DEAE-TOYOPEARL chromatography or the recombinant lipase was run in an SDS-PAGE gel (12%) and the separated proteins were transferred onto a PVDF membrane [29]. After blocking with 3% bovine serum albumin and 2% skim milk in washing buffer (PBS-0.1% Tween 20), the membrane was incubated for 2 h with 5 µg of either affinity-purified anti-recombinant lipase antibody or pre-immune antibody per ml of washing buffer. The membrane was probed with peroxidase-conjugated anti-rabbit IgG antibody (Organon Teknica Corp., West Chester, PA) for 2 h at room temperature, followed by enzyme detection with ECL Western blotting detection reagents (Amersham International plc, Buckinghamshire, England).

Neutralization study

A total of 100 µl of the supernatant from MRSA 10-123 was incubated for 30 min at room temperature with 100 µl of affinity purified anti-recombinant lipase antibody or antibody from pre-immune serum. The mixture was subjected to the cytopathicity assay. Briefly, 5×10^3 cells were treated with the mixture and incubated. The experiments were performed three times and the data are representative of three experiments and each point represents the mean of duplicate measurements.

Determination of protein concentration

Protein concentration was determined by the Bradford method [33] (Protein Assay, BioRad Laboratories, Hercules, CA). Bovine serum albumin served as a standard.

Results

General properties of *S. aureus* MRSA (methicillin-resistant *S. aureus*) 10-123 strain

MRSA 10-123 showed TSST-1, enterotoxin C and type II coagulase activities, but not α-toxin and β-lactamase activities, by bacteriological examinations. Genes of *nucA*, *mecA*, *sec*, *lukS* and *toxA* were detected in MRSA 10-123 by PCR (data not

shown). The filtered supernatant from overnight culture of MRSA 10-123 was examined for cytotoxicity to UV^r-1 [27] and CHO cells. Both cells were severely damaged by the supernatant at high concentration (4- to 16-fold dilution), and then lysed by 24 h after incubation. However, at low concentration (more than 32-fold dilution), intracellular granules accumulated inside of cells. Oily vesicles accumulated in UV^r-1 cells after 12-h incubation with the supernatant (Figure 1B) in contrast to the case for PBS (Figure 1A). After 24-h incubation, the cells had become round and detached from the plate. Histochemical examination by Sudan III staining [34] showed that neutral lipid was present in the vesicles (Figure 1C). The morphological changes of the cells treated with the supernatant were caused by the formation of neutral lipid vesicles. The cytopathic substrate of the supernatant was heat-stable because the supernatant did not lose cytopathicity after heating at 80°C for 30 min (data not shown). Hereafter, this vesicle formation was regarded as the source of cytopathicity in this study.

The cytopathic substance is a staphylococcal lipase

Next, we attempted to purify and identify the cytopathic substance in the bacterial culture supernatant. Cytopathicity was recognized in the fractions eluted with 0.30-0.35 M NaCl by DEAE-TOYOPEARL chromatography (Figure 2A). SDS-PAGE revealed that the fractions included two major proteins with molecular masses of 43 and 45 kDa (Figure 2B). The protein bands visualized by ZnCl₂-imidazole staining were then recovered by electroelution and tested for cytopathicity. Both 43- and 45-kDa proteins showed cytopathicity to UV^r-1 cells. The 45 kDa protein was digested with lysyl-endopeptidase. Two peptides separated by high-performance liquid chromatography (HPLC) were then sequenced by using a protein sequencer. The amino acid sequence of one peptide was identical to the N-terminal sequence of *Staphylococcus aureus* lipase-2 (SAL-2), and the sequence from the other peptide corresponded to the internal sequence of the same lipase (Figure 3), indicating that

Figure 1: Cytopathicity by the supernatant of MRSA 10-123 and by recombinant lipase to the human embryonic UV^r-1 cells. Cells were passaged at 1.5×10^3 cells/well. A total of 50 µl of either PBS (A) or the supernatant (B) was added to the well. After 24-h incubation, the cell viability was examined by light microscopy. Furthermore, the cells treated with the supernatant were stained for neutral fat with Sudan III (C). UV^r-1 cells were treated with 50 µl of recombinant lipase (D).

the 45-kDa cytopathic protein is a lipase. The 43-kDa protein was also subjected to the digestion by a proteinase and peptide sequencing. The N-terminal and internal amino acid sequences of the 43-kDa polypeptide were identical to those of the 45-kDa protein. As previously reported the staphylococcal lipase is secreted into the culture supernatant as a 76-kDa proenzyme and activated extracellularly to produce a 45-kDa mature protein [8]. These factors indicates that the 45-kDa protein identified as a cytopathic substance is staphylococcal lipase and that the 43-kDa cytopathic protein is presumably generated by degradation of the 45-kDa lipase by limited proteolysis at its C-terminal portion because the N-terminal and internal sequences of the two proteins were shown to be identical.

Recombinant staphylococcal lipase is cytopathic

To obtain a further confirmation that the cytopathic substance in the supernatant was staphylococcal lipase, the gene encoding the lipase, *geh*, from MRSA 10-123 was cloned and its gene

Figure 2: The elution profile of DEAE-TOYOPEARL chromatography and the migration profile on SDS- PAGE. (A) DEAE-TOYOPEARL chromatography was performed. The cytopathicity was recognized in the fractions eluted with 0.3 to 0.35 M NaCl. The cytopathicity is described in units; the number of units in fractions is numerically equal to the dilution at CD₅₀. Protein concentration was revealed by OD₂₈₀ (open circle) and the cytopathicity was scored (closed circle). (B) The fractions with cytopathicity were analyzed by 10% SDS-PAGE.

Figure 3: Homology search for the cytopathic substrate versus staphylococcal lipase. Homology search was performed by using Blast. Upper sequence is deduced from the *geh* gene (Genbank accession number: M12715). Lower sequence is from DNA sequencing of the recombinant lipase. Three boxes indicate the amino acid sequences identified by the peptide sequencing analysis of the cytotoxin and the amino acid sequence highlighted with yellow indicates the consensus sequence of lipase catalytic domain.

product was expressed in *Escherichia coli* as a glutathione-S-transferase (GST) fusion protein. The fusion protein was purified with a glutathione-Sepharose 4B column, and its GST portion was cleaved by thrombin digestion. The recombinant lipase induced morphological changes of UV⁻1 cells as well as 45- to 43- kDa cytopathic protein from MRSA 10-123 (Table 1; Figure 1D). Next, we examined whether the cytopathicity in the supernatant was neutralized by the rabbit polyclonal antibody against the recombinant lipase. A total of 40 µg of the antibody completely neutralized the cytopathicity of the supernatant, whereas the same amount of affinity-purified antibody from pre-immune serum failed to do so (data not shown).

These results suggested that the cytopathicity in the supernatant mostly involved the lipase. The specificity of this

antibody against staphylococcal lipase was confirmed by western blotting (Figure 4). In addition, mouse polyclonal anti-lipase antibodies obtained upon immunization with purified native lipase completely inhibited the cytopathicity (Figure 5), whereas pre-immune serum did not.

Lipase activity was essential for the cytopathicity

In general, the catalytic site of lipase consists of Ser-Asp-His amino acid residues. To examine whether the lipase activity was essential for the cytopathicity, the serine residue in the highly conserved pentapeptide sequence GX SXG was replaced with alanine by site-directed mutagenesis of the wild-type recombinant lipase. As shown in (Table 1), the mutant lipase lost the lipase activity. In contrast, the wild-type recombinant lipase and purified staphylococcal lipase were more active than human pancreatic lipase or Rhizopus lipase. The mutant lipase showed no cytopathicity to the UV⁻1 cells (Table 1), whereas Rhizopus lipase and human pancreatic lipase were slightly cytopathic.

Table 1: Comparison of the cytopathicity and the lipase activity of various types of lipases.

	CD ₅₀ (ng/μl)	Lipase Activity (units/μg)
Staphylococcal lipase	0.1	n.t. ^a
r-lipase ^b	0.068	4800
r-m-lipase ^c	>200	<10
Rhizopus lipase	63	800
human pancreatic lipase	73	300

n.t.^a: not tested, r-lipase^b: the recombinant lipase, r-m-lipase^c: the recombinant mutant lipase

Figure 4: The specificity of the antibody against staphylococcal lipase: Anti-recombinant lipase antibody raised in rabbit (Japanese White) was purified by protein A-coupled Sepharose column chromatography. Western blotting was performed with the anti-lipase antibody (lanes 1, 2) or pre-immune sera (lanes 3, 4) against the supernatant from MRSA 10-123 strain (lanes 1, 3) and the recombinant GST-lipase (lanes 2, 4).

Figure 5: Neutralization of the cytopathicity with anti-lipase antibody: The cytopathicity assay was performed as described in Material and Methods. The recombinant lipase was previously mixed with either normal serum or anti-lipase antibody for neutralization assay. Neutralizing effects of normal serum (closed square) and anti-lipase antibody (open square) are shown. The experiments were performed at least three times and the data are representative of three experiments and each point represents the mean of duplicate measurements.

These results indicate that the cytopathicity is identical to the lipase activity.

Lipase activity in the supernatant from S. aureus clinical isolates

To investigate the lipase productivity of various types of S. aureus strain, ten clinical isolates including four strains of methicillin-sensitive S. aureus (MSSA) and six strains of MRSA were examined for the lipase activity of each supernatant (Table 2). Three strains of the four MSSA and one strain of the seven MRSA showed lower lipase activities (<3 units); in contrast, one strain of the four MSSA and six strains including MRSA 10-123 of the seven MRSA had higher lipase activities (>3 units). These results indicate that S. aureus strains were classified into two different groups possessing high or low lipase activities.

Discussion

We identified granule-inducing activity of the staphylococcal culture supernatant on human and animal cell lines. Our observation clearly demonstrated that cytopathicity in the supernatant from MRSA 10-123 was caused by the staphylococcal lipase. Staphylococcal lipase has both chemotactic and chemokinetic properties for human granulocytes [35] and causes monophasic aggregation accompanied by the release of lactoferrin [17]. Moreover, lipase interferes with human granulocyte phagocytic killing of Staphylococcus aureus but not Staphylococcus pneumoniae or Streptococcus agalactioae [16,36,37]. Staphylococcal strains from deep infections produce more lipase than strains from superficial locations [38]. Such strains produce lipases in excess of the amounts necessary to elicit an aggregation response in granulocytes. In this study, the staphylococcal lipase was purified without using a detergent such as Triton X-100. Therefore, the biological effect of staphylococcal lipase was precisely confirmed. Others reported that patients with bacterial endocarditis showed positive reactivity with antibody

Table 2: Lipase activity in the culture supernatant from *S. aureus* clinical isolates.

Strain	Lipase Activity (units/μg)
MSSA	
MSSA1	<1
MSSA2	<1
MSSA3	2.9
MSSA4	>64
MRSA	
MRSA1	5.5
MRSA2	13
MRSA3	5
MRSA4	20
MRSA5	15
MRSA6	2.3
MRSA10-123	7

to staphylococcal lipase or a significant change in antibody titer [39]. These reports suggest that lipase serves as a virulence factor in severe infection of *S. aureus* including septicemia. Our data also showed that there are two groups of *S. aureus* with high and low lipase activities. More recently, in an intraperitoneal challenge mouse model of *S. aureus*, peritoneal abscess formation was reduced by the lipase-deleted mutant [40]. Taking these findings together, staphylococcal lipase may have a role in determining the survival of *S. aureus* in lesions and the cytopathicity may be involved in the pathogenesis of staphylococcal disease.

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