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Annexins I and IV inhibit *Staphylococcus aureus* attachment to human macrophages

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Abstract

Annexins are a family of proteins that bind to phospholipids and carbohydrates in a calcium-dependent manner. They are present in a variety of body fluids. Previous studies have shown that annexins have anti-inflammatory activities for lipid A of Gram-negative bacteria.

The present study investigated the effect of annexins on interaction between Gram-positive bacteria and immune cells such as macrophages. Annexins I and IV bound to lipoteichoic acids which are surface molecules on Gram-positive bacteria. Binding of annexins I and IV to whole *Staphylococcus aureus* (*S. aureus*) were observed and these bindings were inhibited by lipoteichoic acid from *S. aureus*. Moreover, annexins I and IV suppressed the attachment of *S. aureus* to phorbol 12-myristate 13-acetate-treated THP-1 cells (human macrophages). These results suggest that annexins I and IV have ligand specificities toward foreign substances, and that the annexins might have some anti-inflammatory property for Gram-positive bacteria.

Keywords: Annexins; Lipoteichoic acid; Anti-inflammatory; Gram-positive bacteria

1. Introduction

Annexins are a family of structurally related proteins that bind to phospholipids and carbohydrates in the presence of calcium ions [1–3]. They are widely distributed in mammals bodies, extracellular annexins have been found in lung [4–9], plasma [10–16], intestine [17], bile [18], and prostatic secretions [19], where infectious agents must be cleared by an efficient host defense system. Moreover the concentration of annexins in extracellular fluids is remarkably increased during various diseases and by treatment with glucocorticoid, which is one of anti-inflammatory hormone [8–11]. For these reasons it has been proposed that annexins might play a role in immune system.

Since annexins were identified as phospholipase A_2 inhibitors [1], the evidences of annexins as modulators of inflammation have been widely provided. Annexin I contributes to the resolution of inflammation through the regulation of interleukin-6 and tumor necrosis factor-α, both of which are considered to be major inflammatory cytokines [20]. Annexin I reduced neutrophil and monocyte infiltration in several animal models [21–23]. In addition, cell-surface receptors for annexins on immune cells such as monocytes, macrophages and neutrophils have also been found [23–26]. Therefore, annexins potentially participate in immunological process at multiple levels from cellular to systemic, although

Abbreviations: PMA, phorbol 12-myristate 13-acetate; GST, glutathione *S*-transferase; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FACS, fluorescence-activated cell sorter

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the mechanism still remains unclear. More recent studies have shown that annexins I and II themselves bind to lipid A of Gram-negative bacteria and suppress cellular responses to endotoxin [27], suggesting that annexins function as modulators of anti-inflammation via recognition of foreign substance.

These lines of evidence have led us to hypothesize that annexins might also have some anti-inflammatory property for Gram-positive bacteria stimuli in host defense. In this study, to gain insight into anti-inflammatory cellular mechanisms of annexins against Gram-positive bacteria, we have investigated the interaction of annexins I and IV, which are known to be especially abundant in lung and blood, with lipoteichoic acids and Gram-positive bacteria. In addition the effects of annexins I and IV on attachment of macrophages and Gram-positive bacteria are reported.

2. Materials and methods

2.1. Preparation of recombinant annexins I and IV

The total RNA was extracted from HT29 (a human colon cancer cell line) by the method using guanidine-thiocyanate and cesium chloride [28]. The cDNAs were generated with oligo (dT)-primers by using recombinant MMLV reverse transcriptase. Human annexin I cDNA encoding complete open reading frame (ORF) was amplified by a PCR method with synthesized primers based on the sequence reported by Wallner et al. [29]. The PCR-amplified human annexin I cDNA was subcloned into pGEX-5X with *Bam*HI and *Xho*I restriction enzymes. A full length cDNA encoding human annexin IV was derived from HT29, previously reported by Satoh et al. [30]. The sample was subcloned into pGEX-3X with *Bam*HI and *Eco*RI restriction enzymes, respectively. The expression vectors, pGEX-3X and pGEX-5X were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Recombinant annexins I and IV were expressed as glutathione *S*-transferase (GST) fusion proteins in *E. coli* HB101. Expression, purification and enzymatic digestion of the recombinant GST-annexins I and IV were performed as previously described [2].

We also prepared untagged annexin I by treatment of GST-annexin I with factor Xa. The untagged annexin I thus obtained was recognized by a monoclonal antibody directed against the N-terminal portion (N-19) of human annexin I (Santa Cruz Biotechnology, Santa Cruz, CA).

2.2. Coupling of biotin hydrazide to lipoteichoic acids

Lipoteichoic acids were labeled with biotin using biotin hydrazide (Pierce, Rockford, IL). Two mg of lipoteichoic acid from *Streptococcus mutans* (*S. mutans*), *Enterococcus faecalis* (*E. faecalis*), *Bacillus subtilis* (*B. subtilis*) or *S. aureus* (Sigma, St. Louis, MO) were added to 2 ml of 3.3 mM $NaIO_4$/0.1 M NaOAc and subjected to periodate oxidation for 1 h at room temperature in the dark. To stop the oxidation, glycerol was added to reach a final concentration of 15 mM, and incubation continued for 5 min at 0 °C and then 10 mg/ml biotin hydrazide dissolved in dimethylsulfoxide, which was prepared just before use, was added to allow reaction for 2 h at room temperature. The excess biotin hydrazide was removed by ultrafiltration through an Ultrafree-4 Centrifugal Filter Unit (Millipore, Bedford, MA). To purify lipoteichoic acids, the solution was separated with gel filtration on a NAP-5 column pre-packed with Sephadex G-25 (Amersham Pharmacia Biotech). Absorbance of each fraction from 200 to 300 nm was measured, and lipoteichoic acid-rich fractions were collected. The fractions were blotted onto a PVDF membrane and stained with toluidine blue or further incubated with HRP-avidin and 4-chloro-1-naphthol. The fractions, which were positive for both the toluidine-blue staining and biotin detection, were used as biotin-labeled lipoteichoic acids for the following assays.

2.3. Binding of annexins I and IV to lipoteichoic acid

A 96-well microtiter plate (Immulon1; Dynatech Laboratories, Chantilly, VA) was first coated with varying concentrations of GST, annexin I or GST-annexin IV and then incubated overnight at 4 °C. For interactions between annexin I and lipoteichoic acids, we used untagged annexin I prepared as described above. The wells were washed three times with 10 mM Tris–HCl, pH 7.6, 150 mM NaCl (TBS) and then blocking of unoccupied sites was achieved by using TBS containing 3% BSA for 2 h at room temperature. Subsequently, the blocking solution was removed and biotin-labeled lipoteichoic acid was incubated in the presence of 1 mM $CaCl_2$ for 2 h at room temperature. All subsequent incubations and washing solutions used TBS contained 1 mM $CaCl_2$. The wells were washed three times as described above. Bound biotin-labeled lipoteichoic acid was analyzed using HRP-avidin (1:1000 dilution in 1% BSA/TBS). The wells were washed, and the color was developed by the addition of 200 μl 0.04% (w/v) *o*-phenylenediamine and 0.01% (v/v) H_2O_2 in citrate-phosphate buffer (pH 5.0). Color development was stopped by addition of 4 M H_2SO_4. The absorbance of each well was measured at 490 nm.

2.4. Binding of annexins I and IV to S. aureus bioparticles

S. aureus bioparticles were obtained from Molecular Probes (Eugene, OR). *S. aureus* (1.5×10^8 cells/500 μl) were suspended in TBS containing 1 mM $CaCl_2$ or 1 mM EDTA, and centrifuged at 2000 × *g* for 1 min. All subsequent incubation and washing solutions contained 1 mM $CaCl_2$ or 1 mM EDTA. The pellet was added to 2.5 μg GST (control), GST-annexin I or IV, in the presence or absence of 7 μg lipoteichoic acid from *S. aureus* to make a total volume of 20 μl and then incubated for 2 h at 4 °C. After centrifuging at 2000 × *g* for 1 min, the supernatant was saved and the pellet was washed three times with 500 μl TBS. The supernatant and the pellet