Antimicrobial and Lipopolysaccharide-Binding Activities of C-Terminal Domain of Human CAP18 Peptides to Genus *Leptospira*

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ABSTRACT

Human CAP18 (18-kDa cationic anitimicrobial protein: hCAP18) is a protein originally identified from leukocytes on the basis of its capacity to show antimicrobial activity. We report here that 27-amino acids in C-terminal domain of hCAP18 immobilize and kill *Leptospira interrogans* and *Leptospira biflexa*. The domain binds leptopsiral lipopolysaccharide as well as enterobacterial LPS. Analogue peptides also show the activities.

INTRODUCTION

Leptospires are known to be causative bacteria of an acute and febrile illness, leptospirosis.[1] It has been suggested that phagocytosis is one of the important defense mechanisms against leptospirosis.[2-5] There is evidence that leptospires are digested and do not survive long or persist in the phagocytic cells.[5,6] Once inside a phagocyte vacuole, leptospires can be seen to lose their helical shape and assume the spherical form resembling a spheroplast, believed to be nonviable in that site.[7]

In mammals, the cytoplasmic granules of polymorphonuclear leukocytes are a major store of defense polypeptides, which show diverse primary structures and a varied spectrum of

Table 1. Antimicrobial Activity* of Synthetic CAP18 Peptides

Strain of leptospires used			IC50 (MIC): μg/mL		
			$hCAP18_{109-135}$	LL/CAP18	FF/CAP18
L. biflexa	patoc	PatocI	7.2 (20)	9.0 (20)	3.7 (10)
L. interrogans	canicola	Moulton (V)	2.1 (5)	>20 (>20)	10.1 (20)
L. interrogans	canicola	UT IV	3.5 (10)	4.2 (10)	6.6 (10)
L. interrogans	copenhageni	Shibaura(V)	7.2 (20)	9.9 (20)	4.3 (20)
L. interrogans	lai	Lai(V)	2.2 (5)	13.1 (>20)	2.9 (10)
L. interrogans	copenhageni	Shibaura	4.7 (10)	9.0 (20)	3.6 (20)
L. interrogans	autumnalis	Akiyami A	5.3 (20)	14.4 (>20)	12.2 (>20)
L. interrogans	birkini	Birkin	5.1 (10)	8.4 (20)	14.0 (>20)
L. interrogans	hardjo	Hardjoprajitono	3.4 (10)	4.5 (10)	6.5 (10)
L. interrogans	naam	Naam	2.2 (10)	12.4 (>20)	12.7 (20)
L. interrogans	hebdomadis	Hebdomadis	6.0 (10)	8.2 (20)	6.9 (20)
L. interrogans	icterohemorrhagiae	Okinawa	2.5 (10)	4.8 (20)	3.7 (10)
L. interrogans	australis	Ballico	5.8 (10)	12.1 (10)	13.4 (10)
L. interrogans	pyogenes	Salinem	2.0 (5)	5.4 (10)	4.8 (10)

*Control without each peptide: 100% viable cells. After incubation with chlorhexidine gluconate (1000 μg/ml), no motile bacteria were seen (viable cell %: <0.1).

antimicrobial activity.[8,9] CAP18 (an 18-kD cationic protein) is a granulocyte-derived protein.[10,11] The hCAP18 cDNA encodes a protein composed of a 30-amino-acid signal peptide, a 103-amino-acid N-terminal domain of unknown function, and a 27-amino-acid C-terminal domain that binds to LPS, neutrizes LPS-mediated activation of monocytes, and reduces the lethal toxicity of LPS in mice.[11-13] Injection of CAP18 peptide reduced TNF levels in a model with endotoxemia.[14,15] Thus, the innate immunity such as CAP18 (cathelicidin family) provides the crucial first line host defenses.

LPS associated with leptospires has been described as a virulence factor.[16,17] Chemical and serological studies of LPS from leptospires have demonstrated that it has uncommon constitutes in the outer envelope.[1] We showed leptospiral LPS induced inflammatory cytokines such as TNF, IL-1, and IL-6 after injection of it in a mouse model.[17] Recognition of leptospires by phagocytic cells leads to cell activation and production of cytokines, which in itself induces further activation and cytokine production in a complex process of regulation and cross-regulation. This cytokine network could play a crucial role in the inflammatory responses and outcome of leptospiral infection. We expect that innate immunity such as induction of CAP18 can reduce the severity of the disease.

We hope to discover whether active domain of hCAP18 acts to genus *Leptospira*. Additionally, new synthetic analog peptides were used for the experiments. These synthetic peptides were shown to bind erythrocyte coated with leptospiral LPS, and to kill various leptospires within a short time.

MATERIALS AND METHODS

Bacterial Strains

A strain of *Leptospira biflexa* and 13 strains of *L. interrogans* were listed in

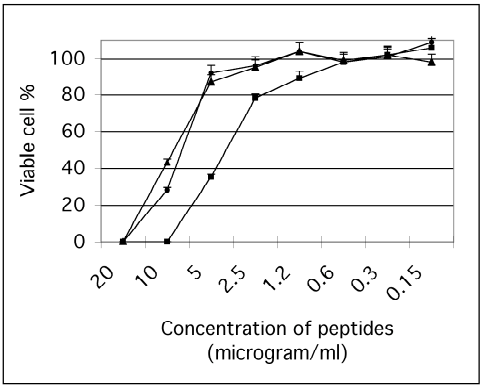

Figure 1. Susceptibility of *L. biflexa* patoc to CAP18 peptides. The points and error bars are expressed as the mean and standard deviation of 3 independent assays. ●: $hCAP18_{109-135}$, ▲: LL/CAP18, ■: FF/CAP18

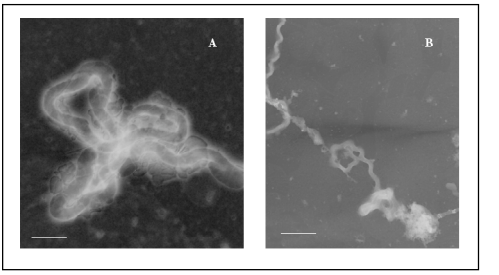

Figure 2. Transmission electron micrograph showing *L. interrogans* copenhageni Shibarura after treatment with $hCAP18_{109-135}$. The leptospiral cells aggregate itself, bar: 0.2 μm (A) and develop a ruffled surface with appearance of bleb-like structure, bar: 1 μm (B) after treatment with $hCAP18_{109-135}$ (10μg/mL) after 15 minutes of incubation.

Table 1. Viable counts were determined under the dark-field microscope with Petrov-Hauzer counting Chamber.

hCAP18 Peptide and Analog Peptides

Peptides were synthesized, purified, and characterized by Peptide Institute, Inc. (Osaka, Japan), according the method previously described.[8] Briefly, CAP18 was synthesized as a C-terminal amide of 27 amino acids ($hCAP18_{109-135}$: FRK SKEKIGKEFK RIVQRIKDFL RNLV). Solid-phase synthesis of this molecule and analogues (LL/CAP18: FRK SKEKIGKLFK RIVQRILDFL RNLV and FF/CAP18: FRK SKEKIGKFFK RIVQRIFDFL RNLV, substituted to phenylalanine or leucin, respectively) was carried out a model ABI 430A (Applied Biosystems, Foster City, Calif) peptide synthesizer. The peptides were purified by reverse-phase high-performance liquid chromatography (Model LC-8A, Shimazu Co., Kyoto, Japan) on a YMC-Pak ODS column (YMC Co. Ltd., Kyoto, Japan). Purity level was more than 95%.

Leptospiricidal Assay

The leptospiricidal assay was carried out at the end of the MIC and IC50 determinations by in vitro immobilization assay.[18] All strains were grown in Leptospira EMJH medium (Difco Co, Detroit, Mich) with 10% normal rabbit serum. Before the experiment, motility in the cultures was confirmed. Bacterial cultures were collected at logarithmic phase and washed twice with HBSS, pH 7.4, and adjusted to a final concentration of 1 X 10^4 cells/mL. To 50μL of bacterial suspension, 50 μL of peptide was added and incubated at 37°C for 30 minutes and examined motility under the dark-field microscope. A non-motile leptospiral cell is regarded as dead. In the experiments, the viable percent was determined by comparison with the control. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide causing at least a 99.9% reduction of the number of viable leptospires (active motile organisms) presented at the beginning of the MIC determination. Fifty percent inhibitory concentrations (IC50s) were determined by least-squares linear regression. The antimicrobial control for all bacteria was chlorhexidine gluconate (1000 μg/mL).

LPS Preparation

After cultivation of *L. biflexa* patoc Patoc I and *L. interrogans* copenhageni Shibaura and *L. interrogans* hebdomadis Hebdomadis in EMJH medium contain-

Table 2. Agglutination of LPS-Sensitized Erythrocytes by Synthetic CAP18 Peptidea

LPS from	Hemagglutination: MAC(μg/mL)		
	$hCAP18_{109-135}$	LL/CAP18	FF/CAP18
L. biflexa patoc Patocl	2.5	0.6	0.15
L. interrogans copenhageni Shibaura	2.5	0.6	0.15
L. interrogans hebdomadis Hebdomadis	5.0	0.6	0.15
S. minnesota R595	1.2	0.15	0.07
E. coli O111:B4	5.0	0.6	0.15
S. flexneri serotype 1A	20.0	2.5	0.6

ing 10% rabbit serum, the leptospiral cells were harvested by centrifugation, were treated with hot phenol by the method previousl y described.[16,17,19] Purified LPSs were used for sensitization of erythrocytes. LPSs from *Salmonella minnesota* R595 (Sigma Chemical Co., St Louis, Mo), *Escherichia coli* O111:B4 (Difco Labs, Detroit, Mich), and *Shigella flexneri* serotype 1A (Sigma Chemical Co., St Louis, Mo) were used as controls.

Erythrocyte Agglutination Assay

LPS-binding activity was examined by the method described previously.[11] One milliliter of 1% erythrocytes (human O type) was sensitized by incubation with 0.2 mL of various LPS solution (100 μg/mL in HBSS). Fifty microliters of a 1.0% suspension of sensitized erythrocytes was mixed with equal volume of a twofold serial dilution of CAP18 peptides in a U-bottom microtiter plate and incubate at 37°C for 1 hour. Activity of CAP18 peptides was expressed as the minimum agglutinating concentration (MAC).

Examination by Transmission Electron Microscopy

Leptospires were incubated with $hCAP18_{109-135}$ at 37°C for 15 minutes. After washing the $hCAP18_{109-135}$-treated leptospiral cells once in HBSS, they were resuspended to distilled water. Samples were mixed in an equal volume of 1.0% v/v neutralized phosphotungstic acid to pH 6.8 with sodium hydroxide) on the surface of a glass slide. A drop of the specimen was transferred to collodion-coated copper grid (200A mesh, Oken Co., Tokyo, Japan). After 30 seconds, excess fluid was removed by touching drop with filter paper. Grids were placed in a desiccator before use. Specimens were examined in a Hitachi HU-12A electron microscope.

RESULTS

Activity of CAP18 Peptide Versus Leptospires

The antimicrobial activity of CAP18 peptides was evaluated versus various strains of genus *Leptospira*. Synthetic peptides demonstrated bactericidal effects against *L. biflexa* patoc (Figure 1). The bacterium was susceptible to $hCAP18_{109-135}$ and the MIC was 20 μg/mL. By replacement of hydrophobic and cationic amino acid residues, bactericidal activity was similar to that of $hCAP18_{109-135}$. MIC of LL/CAP18 was 20 μg/mL and MIC of LL/CAP18 was 10 μg/mL (Figure 1). Table 1 shows that $hCAP18_{109-135}$ killed all the *Leptospira* strains tested and IC50 was ranging from 2.0 to 7.2 μg/mL. The MIC was ranging from 5 to 20 μg/mL. Synthetic peptides, LL/CAP18 and FF/CAP18 also killed various Leptospiral strains (Table 1). After incubation with chlorhexidine gluconate (1000 μg/mL, control), *Leptospira* showed no motility (Viable cell %: <0.1).

LPS Binding by CAP18 Peptides

Leptospiral LPS binding activity, defined as the MAC, is the lowest concentration of peptide that will agglutinate LPS coated red blood cells. The MAC of CAP18 peptides was 2.5 or 5.0 μg/mL for $hCAP18_{109-135}$, 0.6 μg/mL for LL/CAP18, and 0.15 μg/mL for FF/CAP18, respectively (Table 2). CAP18 peptides showed similar binding activity to LPS from *L. biflexa* (non-pathogenic leptospires) and LPS from *L. interrogans* (pathogenic leptospires). In controls, the peptides had MACs of 1.2, 0.15, 0.07 for R595 coated red blood cells for $hCAP18_{109-135}$, LL/CAP18, and FF/CAP18, respectively. To LPS derived *E. coli* O111, $hCAP18_{109-135}$, LL/CAP18 and FF/CAP18 had MACs of 5.0, 0.6 and 0.15μg/mL. To LPS derived *S. flexneri*, $CAP18_{109-135}$, μLL/CAP18 and FF/CAP18 had MACs of 20.0, 2.5, and 0.6μg/mL.

Morphological Alterations in Leptospires After CAP18 Treatment

The interactions between $hCAP18_{109-135}$ and leptospires were examined by transmission electron micrograph. The leptospiral cells aggregated itself and developed a ruffled surface with appearance of bleb-like structure after treatment with $hCAP18_{109-135}$ (10 μg/mL) after 15 minutes of incubation (Figure 2). These alterations were observed in all of letpspires at the concentration of 10 μg/mL.

DISCUSSION

The present studies define the anti-leptospiricidal activity of synthetic peptide CAP18. It has been reported that the C-terminal 37-amino acid domain ($CAP18_{104-140}$) has broad antimicrobial activity versus both gram-positive (IC50=2.5 μg/mL) and gram-negative bacteria (IC50=0.5-5 μg/mL), but not versus *Mycobacterium* species and *Candida albicans*.[11] Genus *Leptospira* was sensitive to $CAP18_{109-135}$, similar to those of Gram-positive and negative bacteria. Recently, *Borrelia burgdorferi* are susceptible to killing by a variety of human polymorphonuclear leukocyte components.[20] The system can inhibit these spirochecal infections.

The outer membrane of *Leptospira* provides an effective permeability barrier against external noxious agents, including antibiotics. It has shown that antimicrobial agents such as polycations weaken the molecular interactions of the LPS with the outer membrane.[21,22] Polycations can, under certain conditions, bind to the anionic phosphates of LPS. Many molecules disorganized and cross the outer membrane to render it permeable to drugs that normally penetrate the intact outer membrane very poorly. Such polycations include polymyxins. However, leptopires were resistant against polymyxin B and the LPS did not bind polymyxin B.[1] These findings were well known in Gram-positive bacteria. CAP18 peptide is cationic leukocyte peptide (polycation) and have α-helix structure. It is likely that the cationic charge mediates membrane/LPS interaction followed by insertion of the amiphipathic helix into the lipid phase of membrane. The activity against leptospires can be mediated by binding to LPS bearing a negative charge and the effect is more broad and strong than polymyxin. In conclusion, peptides released from the C-terminal end of human CAP18 have antimicrobial activity versus genus *Leptospira*. The peptide can attenuate the activity of LPS and induce membrane destruction.

It has been reported that Bac5 and Bac7 (antimicrobial peptides of bovine neutrophils) immobilize and kill *L. interrogans* and *L. biflexa*.[18] It is interesting that the antibacterial peptides from bovine neutrophils are active to leptospires similar to that from human neutrophils (hCAP18). CAP18 proteins are

comprised of 2 domains: a highly conserved N-terminal domain and a less conserved C-terminal domain.[12] C-terminal domain of CAP18 (LLGDF FRK SKEKIGKEFK RIVQRIKDFL RNLVP RTES) as the functional domain is quite different from Bac5 (RFRPPIRRPP IRPPFYPPFR PPIRPPIFPP IRPPFRPPLG PFPGRR). Although these peptides in cathelicidin family are unique in various mammals, they can effectively contribute to reduction in *Leptospira* viability and neutralization of the LPS.

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