inflammation, and also suggests some new therapeutic approaches to inflammatory skin diseases.

Materials and Methods

Antibodies and cytokines Anti-phospho-ERK1/2, anti-phospho-JNK, anti-phospho-p38, anti-phospho-EGFR tyr845, anti-phosphoEGFR tyr992, anti-phosphoEGFR tyr1045, and anti-phospho-EGFR tyr1068 were purchased from Cell Signaling (Beverly, Massachusetts). Rabbit polyclonal anti-human IκBα antibody, monoclonal mouse anti-ERK2, anti-JNK, anti-p38, anti-phospho-STAT1, anti-STAT1α, anti-rabbit IgG horseradish peroxidase (HRP) conjugate, and anti-mouse IgG HRP conjugate were from Santa Cruz (Santa Cruz, California). Recombinant human TNFα and recombinant human IFNγ were from R & D systems (Minneapolis, Minnesota).

Signal transduction inhibitors Several signal transduction inhibitors were added 1 h before stimulation with TNFα and IFNγ. PD98059 was purchased from Alexis Biochemicals (San Diego, California), PD153035, Parthenolide, Bay 11-7085, and SB202190 were purchased from Calbiochem (San Diego, California).

Cell culture HaCaT KC were a generous gift from Dr Kuroki (Showa University, Tokyo, Japan) with the permission of Dr Fusenig (Institute Fur Zell- und Tumourbiologie, Deutsches Kresforschungszentrum, Heidelberg, Germany). They were grown routinely in Eagle's minimum essential medium (MEM, SIGMA, St. Louis, Missouri) supplemented with 10% fetal calf serum (FCS) in a humidified CO_2 incubator. Cells of the 30th to 50th passage were used for experiments.

Normal neonatal foreskin human KC were purchased from Clonetics (San Diego, California), and cultured in keratinocyte-SFM supplemented with BPE and EGF, from Invitrogen (Carlsbad, California).

ELISA HaCaT KC were trypsinized into six-well plates. When they reached subconfluency, the medium was changed to MEM without FCS. After incubation in MEM without FCS for 24 h, RXM (provided by Eisai, Tokyo, Japan) at a concentration of 10^{-4} to 10^{-8} M was added prior to the addition of with 10 ng per mL TNFα and 100 U per mL IFNγ. Supernatants were harvested after 24 h and subjected to ELISA utilizing 96-well plates coated with a murine monoclonal antibody against human CCL17 (TECHNE, Minneapolis, Minnesota). ELISA was performed according to the manufacturer's directions. Briefly, samples and standards were applied to antibody-coated 96-well plates, incubated for 2 h, washed, incubated with secondary conjugated antibodies for another 2 h, washed again, and incubated with substrate for 30 min before the reaction was terminated. The optical density of each well was determined using a microplate reader (Model 550, Bio-Rad, Hercules, California) set to 450 nm.

Recombinant adenovirus Adenovirus vectors containing the genes for HA-tagged wild-type STAT1 (AxCAwtSTAT1) and HA-tagged dominant-negative STAT1 (AxCAdnSTAT1) (Nakajima *et al*, 1996), which contain a CAG promoter (chicken β-actin promoter withCMV enhancer), were prepared by homologous recombination in 293 cells as described previously (Hanakawa *et al*, 2000). DNA encoding wtSTAT1 and dnSTAT1 were kind gifts from Dr Nakajima of Osaka University. HaCaT KC were infected with AxCAwtSTAT1 or AxCAdnSTAT1, at a multiplicity of infection (MOI) of 10 in MEM containing 10% FBS. They were starved of serum for 24 h, and incubated with or without TNFα (10 ng per mL) and IFNγ (100 U per mL) for 24 h. Concentrations of TARC/CCL17 in supernatants were examined by ELISA. The concentration of TARC/CCL17 was normalized with the protein amount because of the anti-proliferative effect of the STAT1 wild-type vector.

Luciferase assay The luciferase construct containing four NFκB consensus sequences in tandem and the CMV–renilla luciferase construct were purchased from Promega (Madison, Wisconsin). HaCaT KCs were trypsinized into 100 mm dishes, and cultured in MEM with 10% FCS until subconfluent. The NFκB–luciferase (NFκB–luc) construct together with the CMV–renilla construct were co-transfected using Fugene6 from Roche Diagnostics (Tokyo, Japan) as recommended by the manufacturer. Six hours after transfection, cells were trypsinized into six-well plates to remove the toxic effect of Fugene6, and incubated overnight before being stimulated with TNFα and IFNγ. RXM (20 μg per mL) was added 2 h before treatment with TNFα (10 ng per mL) and IFNγ (100 U per mL), and the cells were harvested after 16 h of incubation. The cells were disrupted with lysis buffer provided by the manufacturer, and then subjected to a dual-luciferase assay (Promega) with a luminometer (Luminescencer-PSN, AB-2200, ATTO, Tokyo, Japan) as described by the manufacturer.

Western blotting HaCaT KC or normal human KC pre-incubated overnight with or without RXM (20 μg per mL) were stimulated with TNFα (10 ng per mL) and IFNγ (100 U per mL), and disrupted in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM β-glycerophosphate) with 2 mM phenyl methyl sulfonyl fluoride (PMSF) (Boehringer Mannheim, Germany), 1 mg per mL leupeptin (Sigma-Aldrich, St. Louis, Missouri), and 1 mM sodium orthovanadate (Na_3VO_4) from Sigma-Aldrich. The concentrations of the extracted proteins were measured using a BCA Protein Assay Kit (Pierce, Rockford, Illinois). The samples were boiled in sample buffer (50 mM Tris (pH 7.4)/0.14% sodium dodecylsulfate (SDS)/1% β-mercaptoethanol (vol/vol)), and separated by 12.5% SDS-polyacrylamide gel electrophoresis (10 μg of protein per lane). After transfer to an Immobilon-P transfer membrane (Millipore, Billerica, Massachusetts), the membrane was incubated in blocking buffer (5% bovine serum albumin in 25 mM Tris/0.02% KCl/0.8% NaCl (pH 7.4) tris-buffered saline (TBS)) for 1 h at 4°C, followed by an appropriate primary antibody overnight at 4°C. The membrane was washed and incubated with a secondary antibody for 1 h, and the bands were visualized using a chemiluminescence method (Phototope-HRP Western Blot Detection Kit, New England BioLabs, Beverly, Massachusetts).

Supplementary Material

The following supplementary material is available for this article online.

Figure S1
TNFα and IFNγ induced phosphorylation of the EGF receptor, which was completely abolished with the addition of PD153035.

Figure S2
Phosphorylations of JNK and STAT1 were not attenuated by the addition of RXM.

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Susceptibilities of periodontopathogenic and cariogenic bacteria to antibacterial peptides, β-defensins and LL37, produced by human epithelial cells

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***Objectives*: Antimicrobial peptides are one of the factors involved in innate immunity. The susceptibility of periodontopathogenic and cariogenic bacteria to the major antimicrobial peptides produced by epithelia was investigated.**

***Methods*: Synthetic antimicrobial peptides of human β-defensin-1 (hBD1), hBD2, hBD3 and LL37 (CAP18) were evaluated for their antimicrobial activity against oral bacteria. They included *Actinobacillus actinomycetemcomitans* (20 strains), *Porphyromonas gingivalis* (6), *Prevotella intermedia* (7), *Fusobacterium nucleatum* (7), *Streptococcus mutans* (5), *Streptococcus sobrinus* (5), *Streptococcus salivarius* (5), *Streptococcus sanguis* (4), *Streptococcus mitis* (2) and *Lactobacillus casei* (1).**

***Results*: Although the four peptides had bactericidal activity against all bacteria tested, the degree of antibacterial activity was variable against the different strains and species. The antibacterial activity of hBD1 was lower than that of the other peptides. Among the bacteria tested in this study, *F. nucleatum* was highly susceptible to hBD3 and LL37, and *S. mutans* was highly susceptible to hBD3. We measured the Zeta-potential, representing the net charge of whole bacteria, to study the relationship between susceptibility to cationic peptide and the net charge of the bacteria. Although we found some correlation in *A. actinomycetemcomitans* strains, we did not find a definite correlation with all the bacterial species.**

***Conclusions*: These results indicate that β-defensins and LL37 have versatile antibacterial activity against oral bacteria.**

Keywords: oral bacteria, defensins, cathelicidins

Introduction

Mammalian cells produce several kinds of antimicrobial peptides, such as α-defensin in neutrophils, β-defensins in epithelia, histatins in saliva and cathelicidin (CAP18 or LL37) in neutrophils and epithelia.[1–7] These peptides have been reported to function as antimicrobial agents against Gram-negative and Gram-positive bacteria, fungi and viruses.[2,4] Some of them are also implicated as mediators for inflammation (chemotactic factor).[7,8] Therefore, these peptides may be an important component of the innate immune system. Among these peptides, β-defensins are thought to be the first barrier against bacterial infection

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because epithelial cells in the skin and mucosae produce them.[9] Four human β-defensins (hBD1–4) have been identified in several organs.[10–15] It is well accepted that hBD1 is constitutively expressed, and that other peptides show inducible expression by bacterial contact. hBD1 and hBD2 are salt-sensitive, and both act mainly on Gram-negative bacteria,[13–17] whereas hBD3 is salt-insensitive, and effective on Gram-positive and -negative bacteria.[12] CAP18 is an 18 kDa protein that is a member of the cathelicidin family of antimicrobial peptides. It has been reported that CAP18 is processed by a protease, and the last 37 amino acid residues at the C-terminus (LL37) are active against bacteria.[1,18,19] LL37 has been identified in several tissues, such as neutrophils and epithelium.[1,19]

Tooth decay (dental caries) and periodontal diseases are caused by bacterial infection.[20–24] Cariogenic bacteria such as *Streptococcus mutans* and *Streptococcus sobrinus*, and also periodontopathogenic bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia* and *Actinobacillus actinomycetemcomitans*, have been identified as causative agents. These Gram-positive and -negative bacteria tend to aggregate and coexist in dental plaque. Dental plaque is the pathogenic source for dental caries and periodontitis. Many antimicrobial agents, such as histatin, lactoferrin and lysozyme, are known to be produced in the oral cavity. The production of these agents is considered to be one of the roles of innate immunity against bacterial infection.[3,25] Gingival epithelial cells are also reported to produce antimicrobial peptides, such as β-defensins and calprotectin.[16,17,26,27] Gingival epithelial cells, especially non-keratinized cells at the bottom of the periodontal pocket, are considered to produce these antimicrobial peptides in contact with bacteria in the dental plaque. It has been demonstrated that *Fusobacterium nucleatum* induced hBD2 production through the mitogen-activated protein (MAP) kinase pathway.[16] However, little is known about the mechanism of the interaction between these peptides and bacteria. Several reports concerning the activity of antimicrobial peptides, especially cathelicidins,[28–30] have been published, but a detailed investigation has not been conducted so far. Therefore, an investigation into the susceptibility of cariogenic or periodontal bacteria to these peptides is of great interest for understanding the potential role of innate immunity in dental diseases.

In this study, we have investigated the antimicrobial activity of hBD1-3 and LL37 against four periodontopathogenic, five oral streptococci and one *Lactobacillus* sp. containing clinical isolates. Also, an electron microscopic observation of *A. actinomycetemcomitans* exposed to these peptides was performed. Finally, we also assessed the net charge of bacteria to investigate whether the bacterial charge is associated with susceptibility to these cationic peptides.

Materials and methods

Bacterial strains and culture conditions

Bacterial strains used in this study were *A. actinomycetemcomitans* (20 strains), *P. gingivalis* (6), *P. intermedia* (7), *F. nucleatum* (7), *S. mutans* (5), *S. sobrinus* (5), *Streptococcus sanguis* (4), *Streptococcus salivarius* (5), *Streptococcus mitis* (2) and *Lactobacilllus casei* (1). Three *A. actinomycetemcomitans* strains (Y4, IDH781, SUNYaB75), two *P. gingivalis* (WA83, WA50), one *F. nucleatum* (ATCC 25586), one *S. sobrinus* (OMZ176) and one *L. casei* (IFO3983) were standard strains, and other strains were clinically isolated. *A. actinomycetemcomitans* was cultured in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD, USA) supplemented with 1% (w/v) yeast extract (TSBYE) in a 5% CO_2 atmosphere. *P. gingivalis*, *P. intermedia* and *F. nucleatum* were cultured in TSB supplemented with 1% yeast extract, haemin (5 mg/L), vitamin K3 (1 mg/L) and 5% sheep blood (TSBYE-B) in an anaerobic atmosphere using an Anaero Pack system (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). Streptococci and *L. casei* were grown aerobically in brain heart infusion broth (BHI; Difco Laboratory, Detroit, MI, USA).

Synthetic peptides

Synthetic peptides used are listed in Table 1. We constructed hBD1–3 as mature forms and LL37 as a C-terminally truncated form (34 amino acids), which has previously shown antibacterial activity.[31] Peptides were synthesized in a Shimazu peptide synthesizer. Purification of peptides was performed by the method described previously.[31] In brief, peptides were purified by reversed phase high performance liquid chromatography with an octadecyl-4PW column (Tosoh, Tokyo, Japan). Separation was performed with a linear gradient, from aqueous 0.05% trifluoroacetic acid (TFA) to 100% acetonitrile containing 0.05% TFA at a flow rate of 1 mL/min for 30 min. Major peak fractions (absorbance at 230 nm) were collected and lyophilized to completely remove the organic solvent. To confirm the purity and the quality of the peptides, mass spectrometry using MALDI/TOF-MS was performed using Voyager (PerSeptive Biosystems, MA, USA). TOF/MS analysis revealed that

Table 1. Synthetic peptides

Peptide	Amino acid sequence[a] (no. of amino acid residues)	Molecular weight (Da) calculated[b]	observed	S-S bond	Net charge[c]
hBD1	GLGHRSDHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK (42)	4540.1	4533.6	3	4.90
hBD2	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKK (40)	4235.1	4228.7	3	5.82
hBD3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK (45)	5158.7	5152.3	3	10.73
LL37 (CAP18)	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPR (34)	4174.5	4174.1	0	7.0

[a]A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; Y, tyrosine.
[b]Calculated without disulphide bonds.
[c]Net charge of the peptide in pH 7.0 was calculated.

Table 2. Zeta-potential and susceptibility to hBD3 and LL37

Strains	Zeta potential (−mV)	MIC (mg/L)[a]		Percentage of viable cells[b]	
		hBD3	LL37	hBD3 (1 mg/L)	LL37 (1 mg/L)
A. actinomycetemcomitans					
Y4	14.7	200	100	31.1 ± 2.0	37.8 ± 5.4
129	15.5	> 200	200	61.5 ± 8.0	70.4 ± 5.8
2267	19.9	200	100	35.0 ± 2.5	25 ± 1.8
29523	20.5	100	50	12.2 ± 0.5	37.8 ± 2.0
IDH781	27.8	100	100	7.6 ± 0.8	41.0 ± 4.0
SUNYaB75	13.2	> 200	100	83.6 ± 6.2	100 ± 5.8
P. gingivalis					
WA83	13.0	200	100	52.8 ± 3.0	63.0 ± 2.4
WA50	19.9	200	100	57.7 ± 1.2	42.3 ± 3.9
P. intermedia					
163	6.3	100	100	87.8 ± 5.4	78.0 ± 2.4
22	6.3	200	200	92.5 ± 1.2	82.5 ± 3.9
F. nucleatum					
21	11.5	12.5	12.5	5.4 ± 0.6	2.6 ± 0.3
20	13.6	12.5	25	5.7 ± 0.2	2.0 ± 0.1
S. mutans MT403R	8.7	25	25	1.8 ± 0.1	18.9 ± 0.8
S. sobrinus SL-1	10.3	50	25	4.4 ± 0.2	17.3 ± 0.3
S. salivarius H665	7.8	100	25	10.0 ± 1.9	7.7 ± 1.8
S. sanguis H2	5.2	200	50	60.0 ± 2.9	22.4 ± 4.2
S. mitis H65	6.2	200	50	57.1 ± 3.0	57.1 ± 2.4
L. casei IFO3983	6.4	100	50	72.2 ± 6.8	54.1 ± 3.9

[a]MICs were determined by the method of Wu and Hancock.[34]
[b]Percentages of viable cells were determined by the method of Midorikawa *et al.*[31] Values represent means ± SD.

the masses of hBD1, hBD2, hBD3 and LL37 were 4533.6, 4228.7, 5152.3 and 4174.1 Da, respectively (Table 1). The mass of synthetic LL37 was identical to that calculated from the primary sequence, whereas the masses of each of the β-defensins (hBD1, hBD2, hBD3) were 6 Da less than expected from the primary sequence, respectively. Native β-defensins have three disulphide bonds using six cysteine residues that form three β-sheets and one α-helix.[32,33] Our mass spectrometry data suggested that each synthetic β-defensin possesses three disulphide bonds, respectively. We also measured the antimicrobacterial activity of β-defensins using synthetic peptides (Peptide Institute, Inc., Osaka, Japan), which were shown to be structurally similar to the native peptides, by our antimicrobial assay, and confirmed that our synthetic peptides showed comparable antimicrobial activity against *S. mutans*, *Staphylococcus aureus* and *Escherichia coli*.

Antibacterial assay

Two methods were used for the antibacterial assay. One has been described elsewhere.[31] Briefly, for this first method, overnight cultures of bacterial strains were harvested, washed with Dulbecco's phosphate-buffered saline (PBS) and suspended with 10 mM sodium phosphate buffer (PB) (pH 6.8). The bacterial suspension was diluted to 10^7 cells/mL with PB (pH 6.8), and 10 μL of bacterial suspension (10^5 cells) was inoculated into 200 μL of PB with or without various concentrations of antibacterial peptides (final concentration: 0.5, 1, 5, 10, 20, 50 mg/L) and incubated anaerobically for 2 h at 37°C. An appropriate dilution of the reaction mixture in PB (100 μL) was plated on an appropriate agar plate for each species (TSBYE agar for *A. actinomycetemcomitans*, TSBYE-B agar for *P. gingivalis*, *P. intermedia* and *F. nucleatum*, BHI agar for streptococci and *Lactobacillus*), and then incubated at 37°C overnight. Inoculum density (cfu/mL) was calculated from the number of colonies on each plate. The antibacterial effect was estimated as the rate of cells surviving against the total number of cells used. To evaluate the effect of NaCl, 10 mM PB (pH 6.8) containing 10 mg/L of antimicrobial peptides with two different concentrations of NaCl (100 and 500 mM) were used in the antibacterial assay described above. Also, to evaluate the effect of saliva, we used artificial saliva instead of PB to determine the antibacterial activity. Artificial saliva contained 1.2 g of KCl, 0.844 g of NaCl, 0.34 g of K_2PO_4, 0.15 g of $CaCl_2$ and 0.05 g of $MgCl_2$ per 1 L of water (pH 7.0). We measured the antibacterial activity of 10 mg/L of antimicrobial peptides in the presence of artificial saliva.

A slightly modified method of that described by Wu and Hancock[34] was the second method used to the monitor the antibacterial effect. Series of two-fold dilutions of the antibacterial peptides in the range 2000–1.95 mg/L were prepared in 0.2% bovine serum albumin, 0.01% acetic acid buffer in polypropylene microtubes. Each dilution (10 μL) was pipetted into the wells of a 96-well microtitre plate. Overnight cultures of bacterial strains were diluted to 10^6 bacterial cells per mL in half-strength of an appropriate medium for each bacterial species and 90 μL was pipetted into each well. The final concentration of each peptide was from 200–0.195 mg/L. The plate was incubated at 37°C overnight in an anaerobic or aerobic condition for each species. The MIC was measured as the lowest concentration that prevented visible growth.

Electron microscopy

Thin-section electron microscopy was performed to observe the influence of each antimicrobial peptide on cultured *A. actinomycetemcomitans*. An overnight culture of the Y4 strain was harvested, washed with 10 mM sodium PB (pH 6.8) and suspended in

the same buffer. About 10^9 cfu/mL of bacteria were reacted with the antimicrobial peptides at a final concentration of 100 mg/L, and incubated for 2 h at 37°C. Cells were washed with PBS and then were doubly fixed with 2.5% glutaraldehyde. The samples were dehydrated in a series of ethanol concentrations and then embedded in Spurr's Epon. Thin sections were cut on an ultramicrotome with a diamond knife and examined in a JEOL JEM-2000 EX II electron microscope at 80 kV.

Measurement of the Zeta-potential

The Zeta-potential of bacterial cells was measured by particle micro-electrophoresis using the Zeta-potential analyser Zeecom (Microtec, Nition, Funabashi, Japan). Overnight cultures of the bacterial strains were harvested, washed with 10 mM PB (pH 6.8), then resuspended with the same buffer to give a final concentration of 10^9 cfu/mL. Five microlitres of cell suspension was added to 10 mL of PB (pH 6.8) and the bacterial suspension applied to the apparatus for measurement of the Zeta-potential under a voltage of 100 V. The electrophoresis mobility of 100 particles of each strain was automatically measured, and the Zeta-potential calculated from the electrophoresis mobility using the Smoluchowski equation as described elsewhere.[35]

Results

Antibacterial activity of hBD1, 2, 3 and LL37 against periodontopathogenic and cariogenic bacteria

Four Gram-negative periodontopathogenic and six Gram-positive cariogenic strains were analysed (Figure 1). Compared with Gram-positive bacteria, Gram-negative bacteria—except *F. nucleatum*—tended to show low susceptibility to antimicrobial peptides. The strain *F. nucleatum* 21 had a remarkable susceptibility to hBD3 and LL37, having 100% susceptibility in the presence of 1 mg/L of the peptides. *A. actinomycetemcomitans* Y4, *P. gingivalis* WA83 and *P. intermedia* 163 showed an almost similar susceptibility pattern to the peptides; hBD1 and hBD2 were less effective than hBD3 and LL37. Six Gram-positive bacteria, oral streptococci and *L. casei*, showed an almost similar susceptibility pattern to the peptides. Except for hBD1, all peptides demonstrated nearly 100% bactericidal activity with concentrations > 10 mg/L of the peptides.

Table 2 shows the MICs determined by the microdilution method using the bacterial medium. Compared with the results of the assay using the PB, the antibacterial effect was weak. The MICs of hBD3 and LL37 for almost all *P. gingivalis*, *P. intermedia* and *A. actinomycetemcomitans* strains were 100 or 200 mg/L, whereas the MICs of the peptides for *F. nucleatum* showed low values (12.5 or 25 mg/L). As for Gram-positive bacteria, the MICs of the peptides were relatively lower than those for Gram-negative bacteria except for *F. nucleatum*. Comparison among the strains of the antibacterial effect of growing (microdilution method) and non-growing (PB) conditions revealed that there was no difference in terms of antimicrobial activity.

Effect of NaCl and saliva on the susceptibility of bacteria to the antimicrobial peptides

In the presence of 100 mM NaCl, antibacterial activities of hBD3 and LL37 on *A. actinomycetemcomitans* and *S. mutans* were not influenced, whereas that of hBD1 or hBD2 on these two strains was reduced to 50 and 80%, or 80 and 85%, respectively (Figure 2). In the presence of 500 mM NaCl, 20–55% inhibition of antibacterial activity was observed with all peptides against *A. actinomycetemcomitans* and *S. mutans*.

In the presence of saliva, antibacterial activity on *A. actinomycetemcomitans* showed 54% reduction when incubating with hBD1 and hBD2, 20% with hBD3 and 30% with LL37. The antibacterial activity on *S. mutans* showed 23% reduction with hBD1, 11% with hBD2 and no reduction with hBD3 and LL37.

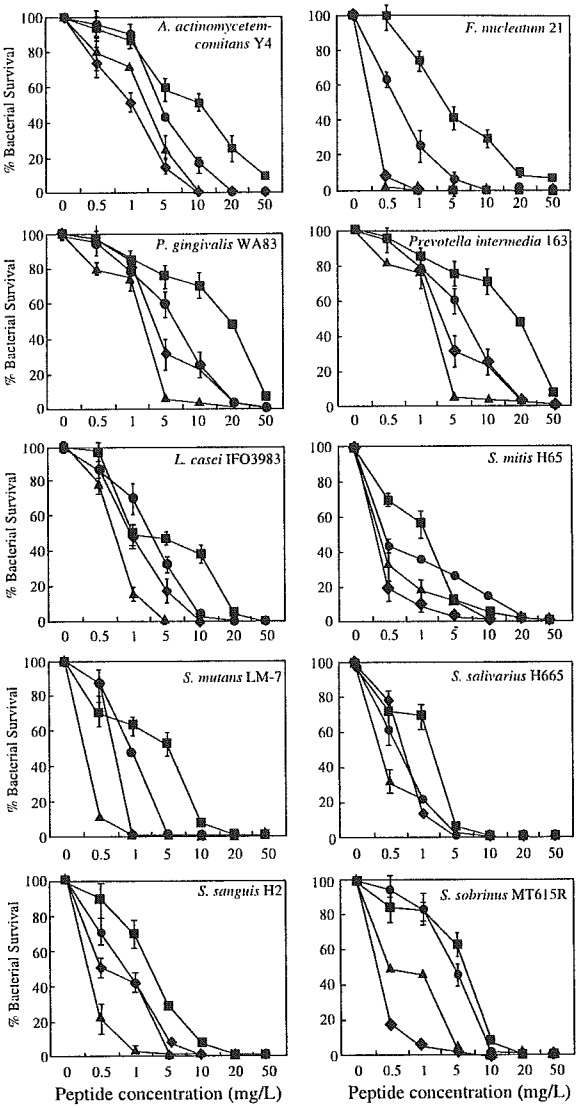

Figure 1. Antibacterial activity of hBD1–3 and LL37 against periodontopathogenic and cariogenic bacterial species. Each peptide was incubated for 2 h at 37°C in 200 μL of 10 mM sodium PB (pH 6.8) containing 10^5 bacterial cells. Serial dilutions were then plated on Trypticase soy agar (TSA), and colony counts were performed after 24 h of incubation at 37°C. The ratio of bacterial survival as a percentage of survival in the presence of peptides compared with that without peptides is represented in the longitudinal axis. The results represent the means ± SD from three independent experiments. Symbols: squares, hBD1; circles, hBD2; diamonds, hBD3; triangles, LL37.

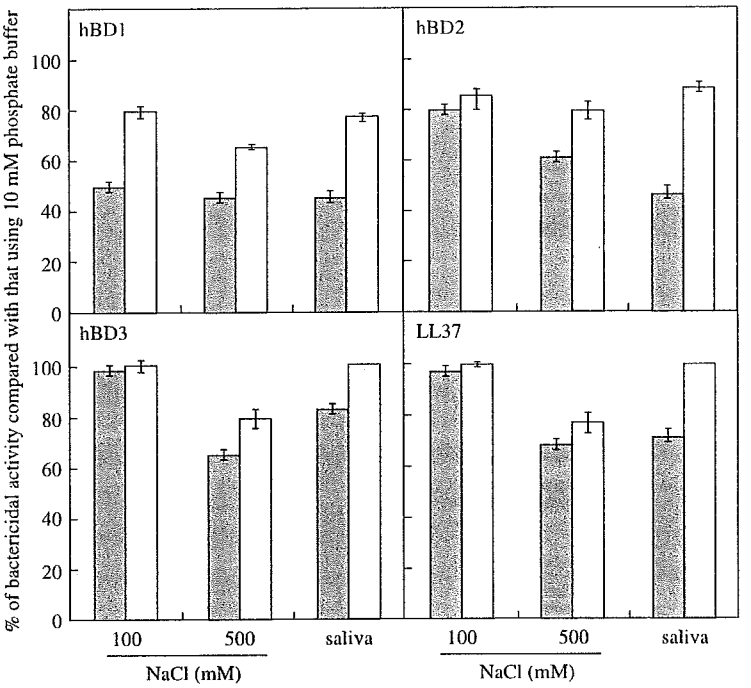

Figure 2. Effect of NaCl and saliva on the susceptibility to hBD1–3 and LL37. Activities of hBD3 and LL37 against *A. actinomycetemcomitans* Y4 (grey bars) and *S. mutans* LM-7 (white bars) were measured in the presence of 100 mM NaCl, 500 mM NaCl and synthetic saliva. Each peptide (10 mg/L) was reacted with bacterial cells by the method described in the Materials and methods section. The *y*-axis indicates the percentage of bactericidal activity compared with that using 10 mM phosphate buffer (pH 6.8). The results represent the means ± SD from three independent experiments.

Susceptibility of clinical isolates to hBD3 and LL37

Forty strains of Gram-negative bacteria, including 20 of *A. actinimycetemcomitans*, seven of *P. intermedia*, six of *P. gingivalis* and seven of *F. nucleatum* were analysed (Figure 3). In Figure 3, the percentage ratio of the bacterial survival is shown when hBD3 (1 mg/L) or LL37 (1 mg/L) was used. The susceptibility of all *F. nucleatum* strains to hBD3 and LL37 was higher than those of other species. *P. intermedia* and *P. gingivalis* strains showed low susceptibility to hBD3, while *A. actinomycetemcomitans* strains showed variable susceptibility to hBD3. The four species showed a variable response to LL37 antimicrobial activity. There was no significant correlation between susceptibility to hBD3 and LL37 in each strain; some strains were highly susceptible to both peptides, whereas others were highly susceptible to only one of them.

S. mutans, *S. salivarius* and *S. sobrinus* strains tested in this study were more susceptible to hBD3 than were other Gram-positive species. The susceptibility of Gram-positive bacteria to LL37 was variable among species.

Electron microscopic features

Electron microscopic observations of *A. actinomycetemcomitans* treated with the four antimicrobial peptides revealed common morphological changes: the cytoplasmic content was released to the outside of the bacterial cells, and only cell walls lacking the inner content were observed (Figure 4). In some bacterial cells, the perforation of the peripheral cell wall shown by arrowheads was observed. The electron microscopic features were no different among bacterial specimens treated with different peptides.

Measurement of the Zeta-potential of whole bacteria

The distribution of the Zeta-potential in 100 particles of various bacterial strains is shown in Figure 5. The mean values of the Zeta-potential were variable among the different species, and it was difficult to see any correlation between the Zeta-potential and susceptibility to the peptides among the tested bacterial species (Table 2). We found some correlation in a limited number of *A. actinomycetemcomitans* strains. In *A. actinomycetemcomitans* strains, the strains (29523, IDH781, 2267) having a higher value (more negative charge) exhibited higher susceptibility to hBD3 and LL37, compared with the strains (129, SUNYaB75) having a lower value of the Zeta-potential. However, the Y4 strain was an exception. Other strains showed various values of the Zeta-potential, and we saw no correlation with the susceptibility to hBD3 and LL37.

Discussion

We have demonstrated the antimicrobial activities of hBD1–3 and LL37 against oral bacteria. The synthetic peptides used in this study all had varied antibacterial activity (Figure 1 and Table 2). Previously, two methods using growing or non-growing conditions have been used to measure the antibacterial activities of peptides.[3,19,28,31,34,36,37] In the method using growing conditions, the MIC value after 24 h of incubation was determined, whereas in the method using non-growing conditions,

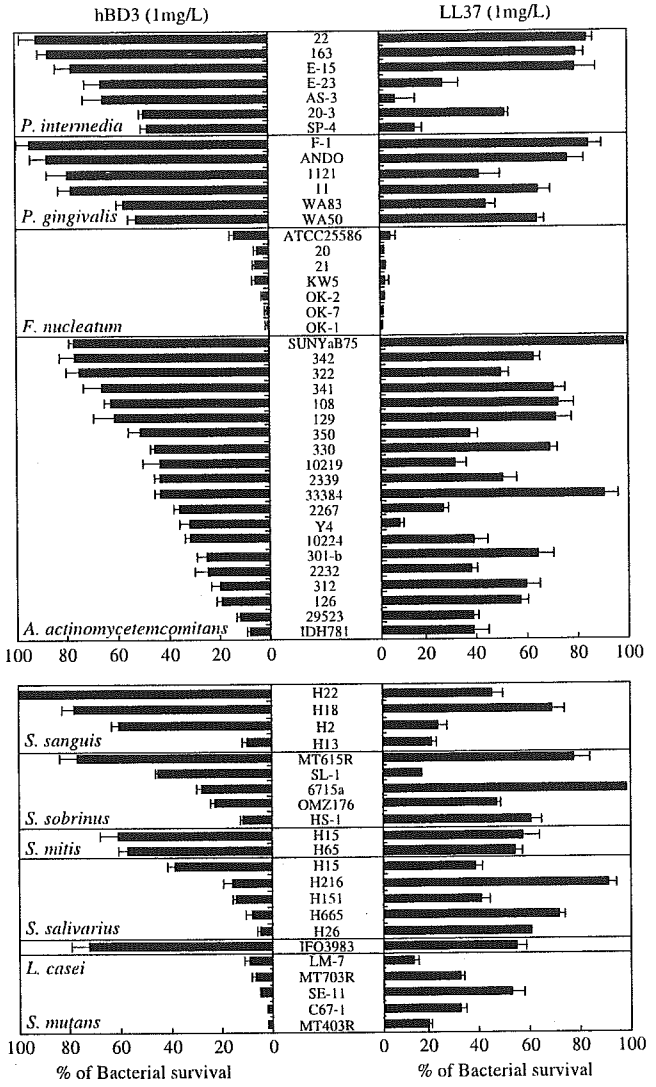

Figure 3. Antibacterial activity of hBD3 and LL37 to various clinically isolated strains. Antibacterial activities of hBD3 (1 mg/L) and LL37 (1 mg/L) against Gram-negative (upper panels) and Gram-positive bacteria (lower panels) were analysed with the method described in the Materials and methods section. The results represent the means ± SD from three independent experiments.

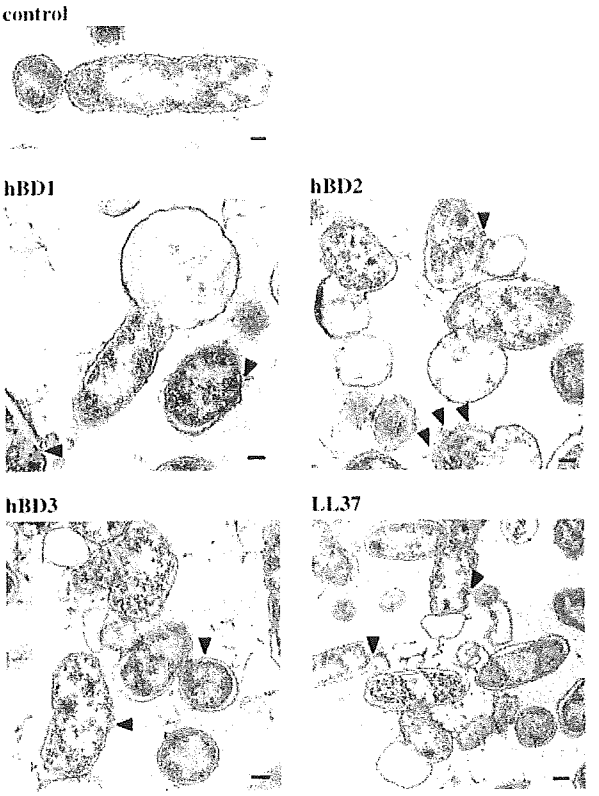

Figure 4. Thin sections of *A. actinomycetemcomitans* Y4 exposed to antimicrobial peptides. *A. actinomycetemcomitans* cells were exposed to PBS, or to 200 mg/L of hBD1, hBD2, hBD3 or LL37. Typical membrane perforation is shown by arrowheads. Bars, 100 nm.

the killing rate after 1–2 h of incubation was determined. We evaluated the antibacterial effect using these two methods. Although we found that there was no obvious difference in terms of antibacterial activity among strains, the concentration of the inhibitory effect was different in both conditions (Table 2). The peptides hBD1 and hBD2 have been reported to be less effective against Gram-positive bacteria.[13,15] Although we detected the antimicrobial activities of hBD1 and hBD2 against Gram-positive bacteria, streptococci and *L. casei*, the antimicrobial effect of hBD3 and LL37 was stronger than that of hBD1 and hBD2, showing a similar tendency with previous results.[13,15] Among β-defensins, hBD3 had the strongest antibacterial activity. This is because hBD3 is the most basic and positively charged peptide among those tested.[38] hBD3 and LL37, due to a strong charge, had less influence with the change in salt concentration. However, the effect of NaCl varied among species, showing a strong effect on *S. aureus*[31] and a weak effect on *S. mutans* and *A. actinomycetemcomitans*. Therefore, NaCl does not affect the peptide itself, but is likely to affect the interaction between the peptides and the bacteria. Among periodontopathogenic bacteria, all *F. nucleatum* strains tested in this study showed the highest sensitivity to hBD3 and LL37 when compared with those of other bacteria. However, the net charge (negative charge) of *F. nucleatum* was not so strong compared with those of other Gram-negative bacteria. Therefore, the high susceptibility of *F. nucleatum* is not only due to the net charge, but also involves other factors.

Electron microscopic observations of *A. actinomycetemcomitans* cells exposed to these peptides revealed the disintegration of the outer and inner membranes, resulting in the perforation of the cell membrane. The target of β-defensins and LL37 is thought to be the bacterial membrane and lipopolysaccharide (LPS).[19,39,40] It has been reported that *Treponema denticola* showed resistance to antimicrobial peptides due to the lack of LPS.[36] Also, the *mprF S. aureus* mutant that had an altered, more negative membrane charge, showed a remarkable increase in susceptibility to antimicrobial peptides.[37] Consequently, the chemical composition of LPS and/or membrane in *F. nucleatum* may contribute to a higher susceptibility to these peptides. Other species of periodontopathogenic bacteria showed variable susceptibility to hBD3 and LL37, implying that the factors affecting the susceptibility to these peptides were different among species and strains.

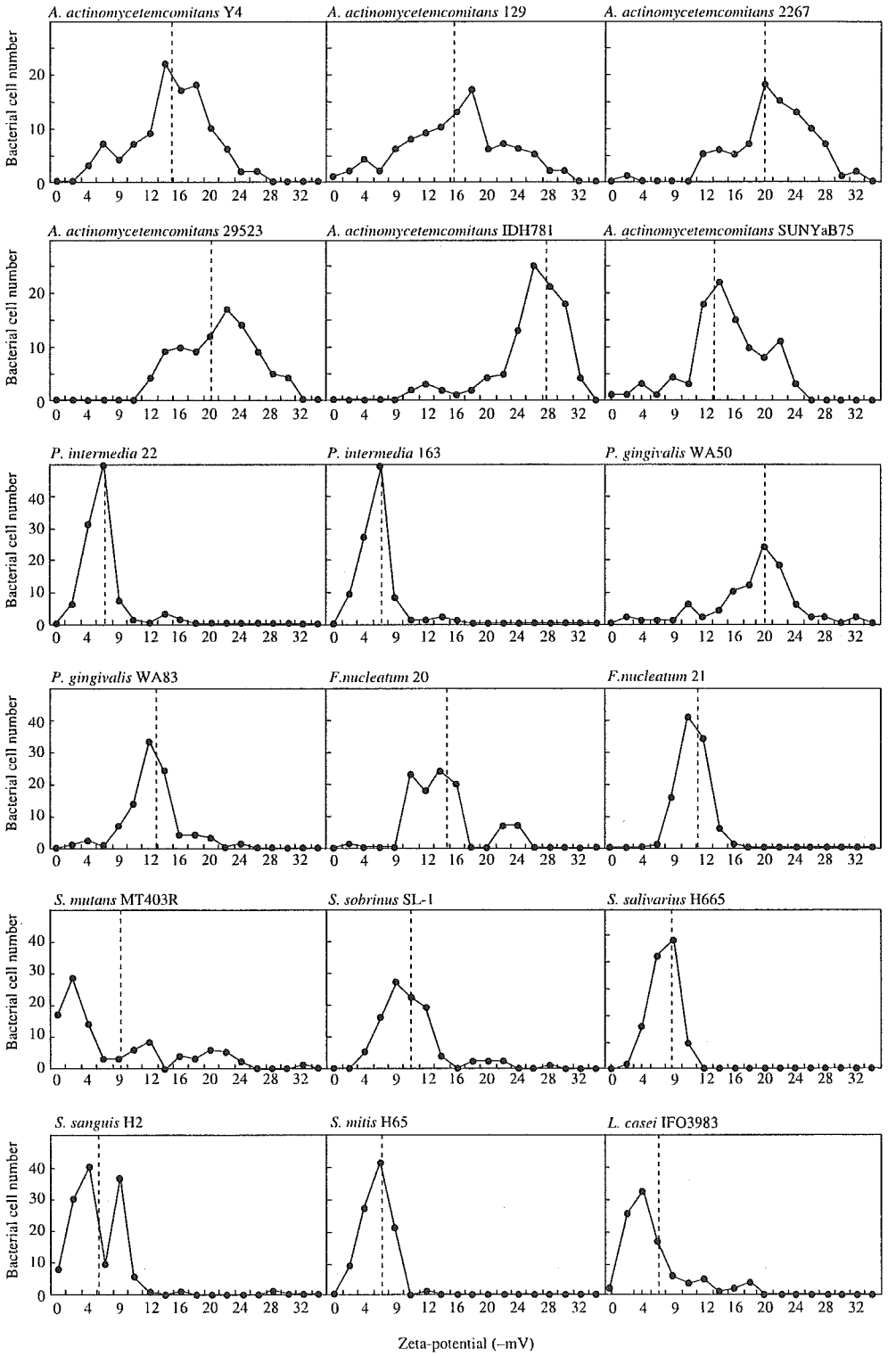

Figure 5. Measurement of the Zeta-potential of various bacterial strains. About 100 particles of bacterial cells were measured with the method described in the Materials and methods section. The distribution for each value of the particles is represented. Longitudinal axis and horizontal axis represent the number of particles and the Zeta-potential (−mV), respectively. The dashed line represents the mean value of the Zeta-potential.

Compared with Gram-negative bacteria, Gram-positive oral bacteria showed relatively high susceptibility to these peptides. Among oral streptococci, *S. mutans* had the highest susceptibility to hBD3, although the susceptibility to LL37 was not as high when compared with other streptococci. The proportion of *S. mutants* and *S. sobrinus* in oral streptococci in saliva and buccal mucosae is very low, and other streptococci, especially *S. salivarius*, *S. sanguis* and *S. mitis*, are dominant.[41,42] Salivary glands and oral epithelia in gums and mucosae were reported to produce β-defensins, LL37, carprotectin and lactoferrin.[16,25,26,27,43] We demonstrated the antibacterial effect of β-defensins and LL37 on oral bacteria in the presence of saliva (Figure 2), indicating that these peptides are active in the presence of saliva. Therefore, antimicrobial peptides in saliva may affect the composition of oral bacteria. In contrast, *S. mutans* and/or *S. sobrinus* in dental plaque are present as aggregates together with other bacterial species. Thus, they are protected by forming a biofilm producing exopolysaccharide, which might prevent exposure to antimicrobial peptides. Therefore, antimicrobial peptides could be one of the selective pressures that bacterial cells need to overcome in order to colonize specific loci in the oral environment, such as dental plaque and saliva.

Since some reports have demonstrated that the bacterial charge affected the susceptibility to these cationic antimicrobial peptides,[37,44] we measured the net charge of whole live bacteria. We have shown that the tested strains possess various levels of negative charge even in the same species. In some *A. actinomycetemcomitans* strains we saw a correlation between the negative charge of strains and the susceptibility to antimicrobial peptides. In *S. aureus*, the *dlt* mutant has a strong negative net charge due to the lack of D-alanine esters in its teichoic acids and showed an increased susceptibility to antimicrobial peptides.[44] These results suggest that the strains with a highly negative charge are more susceptible to antimicrobial peptides among the same bacterial species. However, some strains among the species were highly susceptible to these peptides although their net charge was low, implying that factors other than net charge are involved. The mechanism of antibacterial activity of β-defensins and LL37 seems not to be completely identical because the degree of susceptibility to β-defensin did not always correlate with the degree of LL37 susceptibility (Figure 3). Also, LL37 has been implicated as the LPS neutralizing factor, although few reports were made about the interaction between β-defensins and LPS.[39,40] Although, structural differences between β-defensins and LL37 may result in a difference in bactericidal effect, the microscopic features of the bacterial cells exposed to β-defensins and LL37 are similar.

In conclusion, although we found that synthetic peptides of hBD1-3 and LL37 had antimicrobial activity against oral bacteria, the activity of these peptides is different among species and strains. The net charge of bacterial cells may be one of the factors affecting the susceptibility to these peptides, but involvement of other factors should be considered. These peptides may contribute to the selective colonization of bacterial cells in the oral cavity.

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TGF-beta1, IL-1beta, and Th2 cytokines stimulate vascular endothelial growth factor production from conjunctival fibroblasts

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Abstract

Giant papillary formation containing newly formed vessels is a major characteristic of severe allergic conjunctivitis, such as atopic keratoconjunctivitis (AKC) or vernal keratoconjunctivitis (VKC). We examined production of vascular endothelial growth factor (VEGF) from cultured conjunctival fibroblasts from normal volunteers under stimulation with type 1-, type 2-helper T cell derived and proinflammatory cytokines to investigate the mechanism of giant papillae formation in AKC/VKC. Primary cultured conjunctival fibroblasts were incubated with interleukin (IL)-4, IL-13, IL-1beta, IL-2, tumor necrotizing factor (TNF)-alpha, interferon (IFN)-gamma, or transforming growth factor (TGF)-beta1. Effects of cytokines on VEGF protein secretion in supernatant were assessed by ELISA, and VEGF mRNA expression in cultured cells were assessed by quantitative PCR. TGF-beta1 most effectively increased VEGF concentration with dose- and time-dependent manner IL-1beta, IL-4, and IL-13 significantly increased VEGF concentration. Though IL-2 also showed slight increase of VEGF concentration, it was not statistically significant. TNF-alpha and INF-gamma did not increase VEGF concentration. Quantitative PCR showed significant increase of VEGF mRNA in TGF-beta1, IL-1beta, and IL-4 stimulated fibroblasts. TGF-beta1, IL-1beta, and Th2 cytokines from allergic inflammatory cells induced VEGF production in conjunctival fibroblasts, and may play a crucial role in neovascularization and formation of giant papillae in AKC/VKC.

Keywords: giant papilla; conjunctival fibroblast vernal; keratoconjunctivitis; atopic keratoconjunctivitis

1. Introduction

Giant papillary formation is a major characteristic of severe chronic allergic conjunctivitis, such as atopic keratoconjunctivitis (AKC) or vernal keratoconjunctivitis (VKC). By slit lamp observation, giant papillae appear as reddish elevated formation of the edematous conjunctival mucosa containing enlarged vessels and fibrotic septa. In severe cases, corneal epithelial complications are observed. Epithelial erosion with plaque formation or shield ulcers become treatment resistant, resulting in disturbances of visual acuity (Bielory, 2000).

The mechanism of giant papillary formation has not yet been clarified, although giant papillary formation is thought to play an important role in the genesis of allergic corneal damage. Histopathologically, excised papillae consist of conjunctival epithelium containing goblet cells, a cluster of inflammatory leukocytes (lymphocytes, plasma cells, eosinophils, mast cells, and neutrophils), and newly formed vessels among excess fibrosis (Spencer and Zimmerman, 1985; Völker and Naumann, 1985; Leonardi, 2000; Romagnani, 2000).

Vasculogenesis, the differentiation of mesenchymal cells into hemangioblasts and primitive vessels, and angiogenesis, the formation of new blood vessels from preexisting endothelium, are essential components of tissue growth and wound healing. Vascular endothelial growth factor (VEGF), which is involved in microvascular permeability, is known as a potent multifunctional cytokine that has

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several important effects on angiogenesis (Aiello, 1997; Schlingemann and van Hinsbergh, 1997). In ocular tissue, VEGF has been reported to contribute to the development of corneal neovascularization, proliferative diabetic retinopathy, retinopathy of prematurity, rubeosis iridis, as well as choroidal neovascularizaion (Aiello, 1997; Schlingemann and van Hinsbergh, 1997).

Recently, contribution of VEGF to allergic disorders, especially in bronchial asthma, has been studied. Boesiger et al. (1998) and Grützkau et al. (1998) showed VEGF production and secretion from human mast cells. Hoshino et al. (2001) demonstrated that mRNAs of VEGF and its receptors, flt-1 and flk-1, were significantly increased in asthmatic subjects compared to those of control patients. These data indicate VEGF involvement in allergic tissue reconstruction.

Therefore, we hypothesized that cytokines released in allergic condition upregulate the production of VEGF in subepithelial fibroblasts in conjunctiva. We examined in this study the production of VEGF by primary cultured conjunctival fibroblasts in response to various cytokines relevant to allergic inflammation, and investigated the mechanism of giant papillary formations in severe allergic conjunctivitis.

2. Materials and methods

2.1. Conjunctival fibroblast cultures

After an informed consent was obtained, human conjunctivae were excised from normal volunteers, who were operated due to cataract. Human conjunctival fibroblasts were established in culture, as previously described (Cubitt et al., 1993). Cells were cultured in 35 mm culture dishes (Iwaki Co., Tokyo, Japan) and were studied from third to eighth passage. Purity of each cell type was assessed by cell morphology and immunohistochemical staining for vimentin.

Cells were removed from culture dishes by diluting cultures 1:10 with 0·05% trypsin–0·53 mM EDTA (Gibco BRL, Grand Island, NY) in PBS and incubating for 5 min. Conjunctival fibroblasts were resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS). We investigated dose-dependent effect of cytokines on VEGF production from cultured conjunctival fibroblasts. The cells were cultured in 96-well culture plate (10^4 cells per well) for 72 hr in DMEM supplemented with FCS, and the cultured medium was replaced to serum-free DMEM and further incubated for 24 hr. Then, the medium was replaced again to serum-free DMEM containing 0·3, 3·0, and 30·0 ng/ml of cytokines (recombinant interleukin (IL)-4, IL-13, IL-1beta, IL-2, tumor necrotizing factor (TNF)-alpha, interferon (IFN)-gamma, or transforming growth factor (TGF)-beta1). The cultured supernatants were collected 48 hr after stimulation, and processed for ELISA assay for VEGF concentration. Each experiment was triplicated using fibroblasts from three different donors.

We also investigated time-dependent effect of cytokines on VEGF release from cultured conjunctival fibroblasts. Supernatant of cultured fibroblasts, which was stimulated with 3·0 ng/ml of each cytokines, were collected at 0, 3, 24, 48, and 72 hr after stimulation, and VEGF concentrations were assessed by ELISA. Each experiment was triplicated using fibroblasts from three different donors.

2.2. Cell proliferation assay

When the culture supernatant for time-dependent effect of cytokines on VEGF release was collected, we simultaneously performed cell proliferation assay at 48 and 72 hr using Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan). After the cultured supernatants were collected for assessment for VEGF concentration, each well were replaced with 100 µl of serum-free DMEM. After 1 hr, 10 µl of mixture of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, and 1-methoxy-5-methylphenazinium methyl sulfate was added following the attached protocols. Cells were then incubated in 37°C for 2–3 hr, and then absorbance was measured at wavelength of 415 nm. Each experiment was triplicated using fibroblasts from three different donors.

2.3. Enzyme-linked immunosorbent assay for VEGF protein

Cultured supernatant of each well was stored at −70°C until it was used. The samples were defrosted at room temperature, and diluted five times, and concentration of VEGF was measured with enzyme-linked immunosorbent assay (ELISA) using human VEGF ELISA kit (R & D Systems, Inc., Minneapolis, USA) following the attached protocols.

2.4. Quantitative PCR for VEGF mRNA

The conjunctival fibroblasts were cultured in six-well culture plate ($1·5 \times 10^5$ cells per well) for 72 hr in DMEM supplemented with FCS, and the cultured medium was replaced to serum-free DMEM and further incubated for 24 hr. Then the culture medium was replaced to DMEM containing cytokines (3·0 ng/ml of IL-4, IL-13, IL-1beta, IL-2, INF-gamma, TNF-alpha, or TGF-beta1) in the absence of FCS for 6 hr. The cells were then washed with PBS, and total RNA was extracted with an Rneasy Mini Kit (Qiagen, Valencia, CA). Real-time PCR analysis was performed with the fluorogenic probe-based 5′ exonuclease assay (Taqman; Applied Biosystems, Weiterstadt, Germany) on an automated sequence detection system (model 7700; Applied Biosystems) according to

Table 1
Sequences of the primers and probes for Taqman real-time PCR

VEGF	Forward primer	TGC AGC CTA AAA GGA CCT ATG TC
	Reverse primer	GGA AGG TCA ACC ACT CAC ACA C
	Probe	*FAM*-CAC ACC ATT GAA ACC ACT AGT TCT GTC CCC-*TAMRA*
GAPDH	Forward primer	GAA GGT GAA GGT CGG AGT C
	Reverse primer	GAA GAT GGT GAT GGG ATT TC
	Probe	*FAM*-CAA GCT TCC CGT TCT CAG CC-*TAMRA*

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

the manufacturer's instructions. Reactions were performed with 100Rxn PCR Core Reagents (Applied Biosystems). The manufacturer's quantitative RT-PCR methodology was used to measure VEGF gene copy number, which was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA copy number. Linear standard curves were created for each amplifications, with 10^1–10^7 copies of VEGF or GAPDH plasmids (Nisshinbo, Chiba, Japan). Control amplification (no probe and no template) samples were included in each reaction to set baselines. All quantitation of gene expression was within the linear range of amplification. The sequences of primers and probes for VEGF and GAPDH are as shown in Table 1.

2.5. Statistical analysis

Statistical analysis was carried out by ANOVA, Dunnett's test, and student t test. p values of less than 0·05 were considered statistically significant.

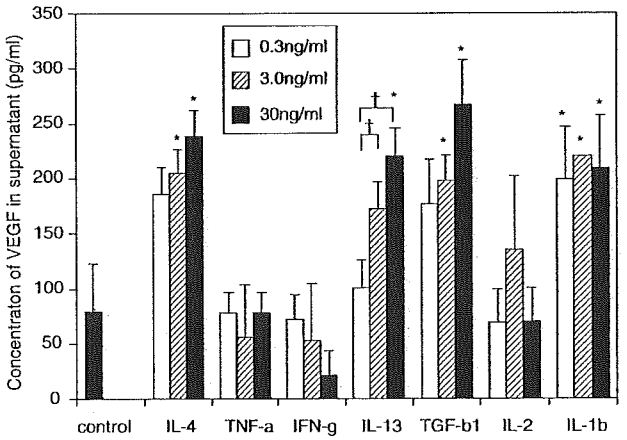

Fig. 1. Dose-dependent effects of cytokines for VEGF concentration from cultured conjuctival fibroblasts (pg/ml). TGF-beta1, IL-1beta, and Th2 cytokines (IL-4, and IL-13) show increase of VEGF concentration. Cont, control; TNFa, TNF-alpha; IFNg, IFN-gamma; IL-1b, IL-1beta; TGF-b, TGF-beta1. The graph shows a representative data using fibroblasts from one donor. Error bars show standard deviation. * shows significant increase compared to control. † shows significant increase compared to lower doses of cytokine stimuli ($p<0·05$).

3. Results

3.1. Effects of cytokines on the release of VEGF from cultured conjuctival fibroblasts

3.1.1. The effect of dose dependent cytokine stimulation on VEGF production

Concentration of VEGF in supernatant of wells without cytokine stimulation was 87·2±8·9 pg/ml. TGF-beta1, IL-1beta, and IL-4 significantly increased VEGF concentration in the culture supernatant at dose higher than 3·0 ng/ml. IL-13 significantly increased VEGF concentration at dose 30 ng/ml. TGF-beta1 increased VEGF concentration most efficiently, then IL-1beta and IL-4 followed (Fig. 1). TNF-alpha and IFN-gamma showed no increase of VEGF concentration. IFN-gamma showed inhibitory effect at dose 30·0 ng/ml, though it was not statistically significant.

3.1.2. The effect of time-dependent cytokine stimulation on VEGF production

VEGF levels were not detectable in the medium at the beginning of culture. Low levels of VEGF were measured at 3 hr after stimulation, increased with time-dependent manner until 48 hr, and then become plateau in TGF-beta1 and IL-13 stimulated wells, whereas IL-4 and IL-1beta kept increasing until 72 hr (Fig. 2). TNF-alpha and INF-gamma showed similar kinetics as TGF-beta1 or

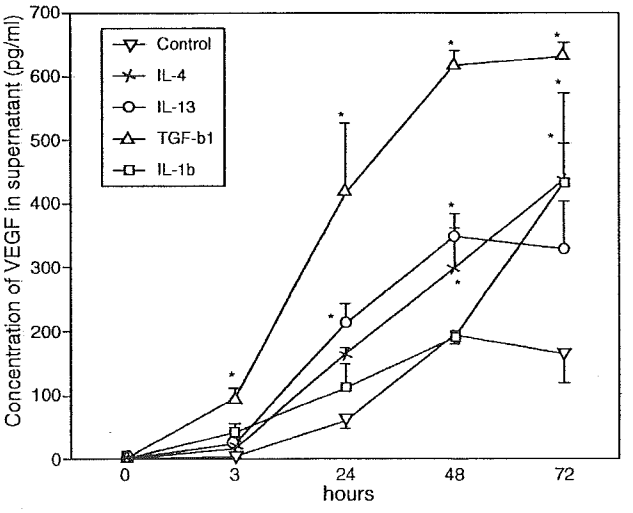

Fig. 2. Kinetics of release of VEGF from cultured conjunctival fibroblasts by cytokine stimulation (pg/ml). TGF-beta1, IL-1beta, and Th2 cytokines (IL-4, and IL-13) show increase of VEGF in cultured supernatant with time-dependent manner. All cytokines were added with dose of 3·0 ng/ml. TGF-beta1 and IL-13 increased VEGF concentration until 48 hr, and then it becomes plateau. IL-4 and IL-1beta keep increasing until 72 hr. Cont; control, IL-1b; IL-1beta, TGF-b; TGF-beta1. The graph shows a representative data using fibroblasts from one donor. Error bars show standard deviation. (* shows significant increase compared to control; $p<0·05$).

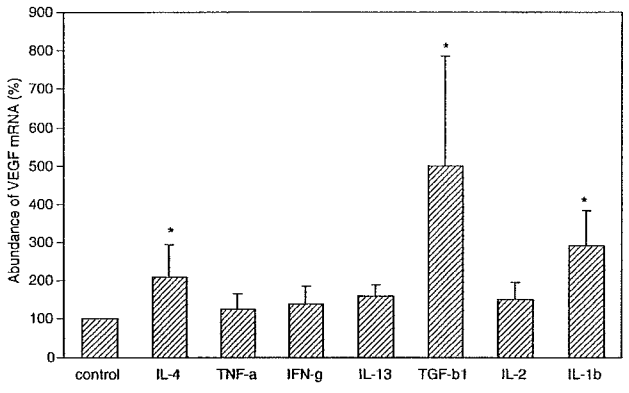

Fig. 3. Effects of cytokines for abundance of VEGF mRNA in conjunctival fibroblasts at 6 hr after stimulation. Quantitative PCR shows increase of VEGF mRNA in fibroblasts stimulated with TGF-beta 1 (*; <0·05). Cont, control; TNFa, TNF-alpha; IFNg, IFN-gamma; IL-1b, IL-1beta; TGF-b, TGF-beta1. Error bars show standard deviation among data of three different donors.

IL-13, however the VEGF quantity was quite low having no statistical significance.

3.1.3. Cell proliferation assay

The proliferation rate at 48 hr ranged 94·3–109·4% in each cytokine stimulated well compared to unstimulated wells, showing no cytokine significantly increased cell proliferation (data not shown). Cell proliferation rate at 72 hr also showed no significant increase, although IL-2, IL-13, and TNF-alpha slightly more increase compared to unstimulated wells (140, 134, and 132%, respectively; data not shown).

3.2. Effects of cytokines on expression of VEGF mRNA by human conjunctival fibroblasts

We examined the effect of cytokines on the abundance of VEGF mRNA in human conjunctival fibroblasts by quantitative PCR analysis. Three individual experiments were performed using fibroblasts from three different donors. The abundance of VEGF mRNA was significantly increased in TGF-beta1 stimulated fibroblasts (499·6% of control). IL-1beta, and IL-4 also showed a significant increase of VEGF mRNA expression (291·2, and 210·8%, respectively; $p<0·05$; Fig. 3).

4. Discussion

In this study, we investigated the effect of representative Th2-, Th1-, and proinflammatory cytokines on VEGF production of conjunctival fibroblasts. The results showed that both VEGF protein and mRNA of human cultured conjunctival fibroblasts were increased by stimulation with TGF-beta and Th2-type cytokines, IL-1beta, and IL-4. IL-13 also showed increase of VEGF protein, although increase of mRNA was below statistical significance.

Fibroblasts have been thought to be mesenchymal cells that exist mainly in the dermis of the skin or interstitial tissue of various organs. During wound healing, fibroblast is capable of synthesizing collagen, glycosaminoglycans, and elastins (Howes, 1985). However, recent several investigations indicated that fibroblasts were able not only to secret collagen fibers but also to mediate inflammation (Smith et al., 1997). Th2 cytokines and TGF-beta1 synergically stimulate human airway fibroblasts to increase production of eotaxin, a potent chemotactic mediator for eosinophils (Wenzel et al., 2002). It has also been reported that IL-4 and TNF-alpha induce eotaxin production of human corneal keratocytes (Fukagawa et al., 2000). These findings support the theory that fibroblasts may play a crucial role in the exacerbation of allergic inflammation.

It is reported that long-standing inflammation in the bronchus thickens the smooth muscle layer and increases the connective tissue layer. This process is known as tissue remodeling, in which bronchial fibroblasts transform into myofibroblasts. In ocular allergic disorders, existence of fibrogenic factors such as basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), and TGF-beta1 in conjunctival biopsy specimens from VKC patients was demonstrated by Leonardi et al. (2000). They also showed an increase in collagen III and decrease of proteoglycans in the conjunctival specimens from VKC patients (Leonardi et al., 1995). In addition, change of equivalence of matrix metalloproteinases in the tear of VKC patients in vivo (Kumagai et al., 2002) and production of collagen types I and III from conjunctival fibroblasts in vitro were demonstrated (Leonardi et al., 1998). These facts indicate that fibroblasts may play a crucial role in the remodeling process of severe allergic conjunctival disorders, directly by producing extracellular matrices and indirectly through various growth factors.

In the present study, the most effective stimulant increasing VEGF production from conjunctival fibroblasts was TGF-beta1. TGF-beta1 is one of the three isoforms of TGF-beta. The fibrogenic roles of TGF-beta in fibroblast proliferation, collagen synthesis, and lattice contraction have been previously reported (Levi-Schaffer et al., 1999; Rochester et al., 1996). Moreover, elaboration of TGF-beta by eosinophils (Gharaee-Kermani and Phan, 1998) and TGF-beta1 expression in human eosinophils increased by IL-4 stimulation were reported (Elovic et al., 1998). Those facts provide further evidence that TGF-beta1 is a key cytokine regulating tissue remodeling in allergic condition.

The cytokine that elevated VEGF production from conjunctival fibroblasts next to TGF-beta1 was IL-1beta. IL-1beta is known as one of the proinflammatory cytokines that play crucial roles in allergic disorders (Reynolds et al., 2002). Bronchoalveolar fluid from asthmatic patients contained significantly elevated level of IL-2beta than that of normal subject (Borish et al., 1992). He also showed IL-1beta transcripts were found to be located within alveolar macrophage. Roca-Ferrer et al. (2001) reported that

IL-1beta increased glandular secretion from human nasal mucosa. In the field of ophthalmology, increased level of IL-1beta in tear from patients with limbal vernal keratoconjunctivitis was reported by Leonardi et al. (1998). Those facts may suggest that IL-1beta play an important role also in mechanism of exacerbation of allergic conjunctivitis.

The third effective cytokine to increase VEGF production from conjunctival fibroblasts was IL-4. IL-13 also increased VEGF concentration in supernatant, however, IL-13 simultaneously upregulated cell proliferation rate. This may indicate the VEGF secretion from individual cells was upregulated only by IL-4, and IL-13 rather contributed only to increase cell proliferation. IL-4 and IL-13 are the representative Th2 cytokines that are predominantly produced by helper T lymphocytes, mast cells, and eosinophils (Baggiolini, 1998; Hingorani et al., 1998; Leonardi, 1999). Th2 cells are thought to aid in humoral responses such as IgE isotype switching and mast cells and eosinophils growth and differentiation. The contribution of Th2 cytokines to ocular allergic disorders has been previously demonstrated by several investigators. Increased Th2 phenotype among local lymphocytes (Leonardi et al., 1999) and elevated concentration of IL-4 in tears from patients with allergic conjunctivitis was reported (Fujishima et al., 1995, 2002; Uchio et al., 2000). Moreover, contribution of IL-4 and IL-13 on airway remodeling processes (Lordan et al., 2002) and on eosinophil recruitment in pathogenesis of VKC/AKC (Leonardi et al., 2003) has also been demonstrated. Those cytokines that induce remodeling may simultaneously stimulate fibroblasts to produce VEGF, resulting in tissue edema, new vessel formation, and consequently papillary formation.

Other proinflammatory cytokines (TNF-alpha, INF-gamma) or Th1 cytokines (IL-2, INF-gamma, and TNF-alpha) showed less effect on VEGF production despite multiple trials with various concentrations in the present experiment. These results suggested that VEGF secretion from conjunctival fibroblasts was increased more effectively by Th2 inflammation rather than Th1 inflammation. This may be consistent with the fact that giant papillary formation is a characteristic feature for allergic conjunctivitis, in which Th2 inflammation is predominant.

In conclusion, the present study showed that human conjunctival fibroblasts were capable to produce and secrete VEGF protein and that this function was increased in response to stimulation with TGF-beta1, IL-1beta and Th2 cytokines. Conjunctival fibroblasts may play an important role in exacerbation of Th2 inflammation and tissue remodeling in conjunctival, resulting in giant papillary formation. Further investigations are required to clarify other factors and mediators in addition to VEGF, which may be involved in the genesis of giant papillary formations and exacerbation of allergic conjunctivitis.

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Tryptase Increases Proliferative Activity of Human Conjunctival Fibroblasts through Protease-Activated Receptor-2

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Purpose. Tryptase that is released by mast cell degranulation has recently been thought to play a key role in wound healing in allergic bronchitis. Conjunctival fibroblasts secrete mediators and extracellular matrices that could exacerbate inflammation and papillary formation in allergic conjunctivitis. This study was conducted to investigate the effect of tryptase on the proliferation of conjunctival fibroblasts and studied whether this effect was mediated by protease-activated receptor (PAR)-2.

Methods. Conjunctival fibroblasts were cultured with or without tryptase (0.1 ng/mL to 1.0 μg/mL), and the proliferation rate was assessed after 48 hours. The effects of tryptase inhibitors (leupeptin, benzamidine) and a PAR-2 agonist (SLIGKV) were examined. The existence of PAR-2 mRNA and protein in conjunctival fibroblasts was examined by RT-PCR and Western blot analysis, respectively. The existence of PAR-2 in cultured conjunctival fibroblasts and conjunctival papillae from patients with vernal keratoconjunctivitis, as well as conjunctival tissue from normal subjects was examined by immunohistochemistry.

Results. Conjunctival fibroblast proliferation was upregulated by tryptase in a dose-dependent manner ($P < 0.001$). Leupeptin and benzamidine inhibited tryptase-induced fibroblast proliferation ($P < 0.05$), and SLIGKV mimicked tryptase's effect. PAR-2 mRNA and protein were detected in cultured conjunctival fibroblasts using RT-PCR and Western blot analysis. PAR-2 immunoreactivity in both the cultured conjunctival fibroblasts and in stromal cells in excised conjunctival tissues was observed.

Conclusions. Tryptase increased conjunctival fibroblast proliferation and this response appeared to be mediated by PAR-2. Mast cells are the most likely source of tryptase in the conjunctiva and may play an important role in chronic exacerbations with conjunctival papillary formation in allergic conjunctivitis. (*Invest Ophthalmol Vis Sci.* 2005;46:4622–4626) DOI:10.1167/iovs.05-0388

Giant papillary formation is a major characteristic of severe chronic allergic conjunctivitis, like atopic keratoconjunctivitis (AKC) or vernal keratoconjunctivitis (VKC). Histopathologically, excised papillae consist of the conjunctival epithelium containing goblet cells, a cluster of inflammatory leukocytes (lymphocytes, plasma cells, eosinophils, mast cells, and neutrophils), and newly formed vessels among excessive fibrosis. Under these conditions, conjunctival fibroblasts are suspected to increase in number and to be activated, secreting soluble inflammatory mediators and extracellular matrix molecules.

An increased number of mast cells[1–5] and elevated tryptase concentrations[6–10] have been reported to exist in the tears of patients with allergic conjunctivitis, including AKC and VKC. These facts may indicate that mast-cell–derived tryptase contributes to not only the IgE-mediated early-phase reaction, but also the exacerbation of late-phase inflammation and papillary formation in chronic allergic conjunctivitis.

Tryptase is a trypsin-like serine protease that is released by mast cell degranulation at inflammatory sites. Tryptase has several in vitro functions that may be important in the pathogenesis of asthmatic inflammation: the inactivation of fibrinogen and the inhibition of fibrinogenesis, the activation of tissue matrix metalloproteinases (MMPs) including MMP-3, the inactivation of neuropeptides (including bronchodilatory vasoactive intestinal peptide), the stimulation of lung fibroblast proliferation and collagen synthesis, eosinophil chemotaxis, and the upregulation of intercellular adhesion molecule (ICAM)-1 expression and of IL-8 synthesis by bronchial epithelial cells.[11]

The mechanism by which tryptase exerts these effects is not fully understood, although recent evidence has suggested that tryptase may activate protease-activated receptor (PAR)-2. PAR-2 is a member of the PAR family, which includes PAR-1, -3, and -4. The distribution of PAR-2 in human tissue is not fully known, although previous investigations have detected mRNA expression in the stomach, intestine, glandular epithelial cells of salivary glands and the pancreas, vascular endothelial cells, tracheal epithelial and smooth muscle cells, liver, kidney, and central and peripheral nervous systems.[12–14]

The endogenous activation of PAR-2 by trypsin or tryptase requires the cleavage of the extracellular NH_2-terminal domain, revealing a new peptide sequence (tethered ligand) that is able to bind to sites within the second extracellular loop of the seven-transmembrane receptor.[14,15] The ability of tryptase to activate PAR-2 appears to differ among tissues or cells expressing PAR-2.[15] Akers et al.[16] reported that the proliferation of lung fibroblasts was mediated by PAR-2 activation by tryptase from human mast cells. Frungieri et al.[17] also reported that both recombinant and human mast cells-derived tryptase increased the proliferation of fibroblasts from human foreskin by PAR-2 activation.

In the present study, we assessed the tryptase-induced proliferation of primary cultured conjunctival fibroblasts to investigate the pathogenesis of giant papillary formation. We also investigated the existence of PAR-2 on conjunctival fibroblasts, because this receptor is thought to mediate tryptase-related effects.

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Investigative Ophthalmology & Visual Science, December 2005, Vol. 46, No. 12

TABLE 1. Sequences of PAR-2 and GADPH Primers

PAR-2	
Forward primer	GTT GAT GGC ACA TCC CAC GTC
Reverse primer	GTA CAG GGC ATA GAC ATG GC
GAPDH	
Forward primer	GTC TTC ACC ACC ATG GAG AAG GCT
Reverse primer	CAT GCC AGT GAG CTT CCC GTT CA

MATERIALS AND METHODS

Human skin β-tryptase was obtained from Promega (Madison, WI). Anti-PAR-2 antibodies (SAM-11) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Leupeptin and benzamidine were obtained from Biogenesis (Poole, UK) and Research Organics (Cleveland, OH). The PAR-2 agonist peptide, SLIGKV (H-Ser-Leu-Ile-Gly-Lys-Val-OH), was obtained from Bachem (Bubendorf, Switzerland). The anti-human mast cell tryptase (MCA1438) was obtained from UK-Serotec, Ltd. (Oxford, UK).

Conjunctival Fibroblast Cultures

Human conjunctival tissue was excised from normal volunteers after obtaining their informed consent. Human conjunctival fibroblasts were established in culture, as previously described.[18] The cells were cultured in 35-mm culture dishes (Iwaki Co., Tokyo, Japan) and were studied from the passages 3 to 9. The purity of each cell type was assessed by cell morphology and immunostaining for vimentin.

Cell Proliferation Assay

Conjunctival fibroblasts were removed from the culture dishes by diluting the cultures 1:10 with 0.05% trypsin-0.53 mM EDTA (Invitrogen-Gibco, Grand Island, NY) in PBS and incubating for 5 minutes. Cells were resuspended in Dulbecco's modified Eagle's medium (DMEM/F12; Invitrogen-Gibco BRL) supplemented with 10% fetal calf serum (FCS). The cells were cultured in 96-well culture plates (5000 cells per well) for 24 hours in DMEM/F12 supplemented with FCS. Then, the medium was replaced with serum-free DMEM/F12 containing tryptase, tryptase with leupeptin or benzamidine, or SLIGKV. A proliferative assay was then performed with a cell-counting kit (Dojindo Laboratories, Kumamoto, Japan) after the cultures were incubated at 37°C for 42 hours. Ten microliters of a mixture of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolium]-1,3-benzene disulfonate, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid, and 1-methoxy-5-methylphenazinium methylsulfate was added to each well according to the attached protocols. The cells were then incubated at 37°C for 2 hours, and their absorbance was measured at a wavelength of 415 nm. Each experiment was performed in triplicate using fibroblasts from three different donors.

RT-PCR for PAR-2 mRNA

The conjunctival fibroblasts were cultured in six-well culture plates (2.0×10^5 cells per well) for 72 hours in DMEM/F12 supplemented with FCS. Then, the culture medium was replaced with FCS-free DMEM/F12, and the cells were cultured for 24 hours. The cells were then washed with PBS, and the total RNA was extracted (RNeasy Mini Kit; Qiagen, Valencia, CA). cDNA was obtained (Super Script II; Invitrogen, Carlsbad, CA). We performed reverse transcription followed by PCR amplification. Briefly, 1 ng of cDNA was added to a 24-μL reaction volume containing 1 μL of random primers and 23 μL of buffer (Platinum PCR Super Mix; Invitrogen). The amplification conditions were 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute for 38 cycles. Electrophoresis was conducted on 2% (wt/vol) analytical grade agarose gels that were subsequently stained with ethidium bromide. The sequences of the forward and reverse primers for PAR-2 and GAPDH are shown in Table 1.

Western Blot Analysis for PAR-2

The conjunctival fibroblasts were cultured in six-well culture plates (2.0×10^5 cells per well) for 72 hours in DMEM/F12 supplemented with FCS. Then, the culture medium was replaced with FCS-free DMEM/F12, and the cells were cultured for 24 hours. The cells were washed twice with ice-cooled phosphate-buffered saline and extracted with 100 μL of NP-40 (Sigma-Aldrich, St. Louis, MO) containing protease inhibitors. Protein extracts were obtained from the lysed tissues using a commercially available protein isolation solution (NP-40 with protein inhibitor cocktails). Ten microliters of protein extract and 10 μL of sample buffer with bromophenol blue were mixed and reacted at 100°C for 5 minutes, then electrophoresed (NuPAGE 10% BT; Invitrogen) and transferred onto a nitrocellulose membrane (Hybond-ECL; AP BioTech). The membrane was then blocked in a solution consisting of 5% nonfat dry milk and tris-buffered saline (TBS)-Tween 20 (pH 7.4). Immunoreactions were performed using an anti-PAR-2 monoclonal antibody (SAM-11) in 1% nonfat dry milk and TBS-Tween 20 (dilution 1: 500). This step was followed by the addition of a horseradish peroxidase–conjugated goat-anti mouse IgG1 (1:20,000; Upstate Biotechnology, Lake Placid, NY). The immune complexes were placed in chemiluminescent reagent (ECL Western Blot detection reagents; GE Healthcare, Piscataway, NJ) and exposed to film.

Immunohistochemistry

For immunohistochemistry, conjunctival fibroblasts from three different donors were seeded in four-well chamber slides at a density of 20,000 cells/well in DMEM/F12 containing 10% FCS. The cells were incubated at 37°C for 1 week. The cells were fixed with 4% formalin for 10 minutes at room temperature. Endogenous peroxidase was blocked by 3% H_2O_2 in methanol for 3 minutes, followed by incubation with normal donkey serum to block nonspecific staining for 10 minutes. After washing in TBS (0.1 M, pH 7.4), the cells were incubated with a mouse anti-PAR-2 monoclonal antibody (SAM-11; 10 μg/mL) for 1 hour at room temperature. For negative control experiments, the primary antibody was replaced with preimmune mouse IgG2a. After washing in TBS for 10 minutes, the samples were processed (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol (3,3′-diaminobenzidine [DAB]-peroxidase staining). The cells were then counterstained with hematoxylin and examined by light microscopy.

PAR-2 or tryptase immunoreactivity was also analyzed in surgically excised conjunctival tissue from three patients with VKC and three normal volunteers. Immediately after excision, the conjunctival specimens were frozen, embedded in OCT compound, and stored at −80°C until the assay. The specimens were cut with a cryostat, air dried, and fixed in acetone for 2 minutes at room temperature. After they were washed in TBS (0.1 M, pH 7.4), the sections were incubated with a mouse anti-PAR-2 monoclonal antibody (10 μg/mL), anti-human mast cell tryptase antibody (2 μg/mL), or preimmune mouse IgG1 and IgG2a for 1 hour at room temperature. Then, the samples were processed according to the manufacturer's protocol (Vectastain ABC Universal kit; Vector Laboratories) and counterstained with hematoxylin.

Statistical Analysis

In the cell proliferation study, the mean ± SD was calculated. The variation between the data sets was tested with ANOVA, and the significance was analyzed with an unpaired *t*-test and the Dunnett test. $P < 0.05$ were considered statistically significant.

RESULTS

Cell Proliferation by Tryptase Stimulation

We investigated the dose-dependent effect of tryptase on the proliferation of cultured conjunctival fibroblasts. Conjunctival fibroblasts were stimulated with 0.1, 1.0, 10, or 100 ng/mL or 1.0 μg/mL of tryptase for 48 hours. Each experiment was