
LABORATORY INVESTIGATION

Expression of β Defensins in Ocular Surface Tissue of Experimentally Developed Allergic Conjunctivitis Mouse Model

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Abstract

Purpose: To investigate the expression of β -defensin in the ocular surface of a mouse experimental allergic conjunctivitis (EAC) model.

Methods: Mouse β -defensin (mBD)-1 and -2 mRNA expressed in conjunctival tissue of both the EAC and the control group were analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. mBD-1 and mBD-2 mRNA expressed in cornea, limbus, and conjunctival epithelium of the EAC and the control group were also analyzed using a laser-assisted microdissection technique and the RT-PCR method.

Results: There was no significant difference in the amount of mBD-1 between the EAC and the control group. However, the EAC group had significantly less mBD-2 ($0.23 \pm 0.15 \times 10^4$ copy/ μ l) than the control group ($3.61 \pm 4.87 \times 10^4$ copy/ μ l) ($P < 0.01$, Mann-Whitney U test). mBD-1 mRNA was positive for all examined tissue in both groups. The expression of mBD-2 mRNA in the EAC group was definitely positive for the corneal and conjunctival epithelium and negative for the limbus.

Conclusion: mBD-2 mRNA was expressed significantly less in the EAC model than in the control. Analysis by site in the ocular surface tissue revealed that this reduction was caused by the diminished expression of mBD-2 in the limbus. **Jpn J Ophthalmol** 2006;50:1–6 © Japanese Ophthalmological Society 2006

Key Words: β -defensin, laser-assisted microdissection, mouse, ocular surface, real-time RT-PCR

Introduction

Defensins, a family of cationic peptides, are low-molecular-weight substances (2–6 kDa) that have antimicrobial activity against Gram-positive and -negative bacteria, fungi, and viruses. Defensins are classified as α -, β -, or θ -defensins, based on the position of the cysteine residue and disulfide

bonds. In humans, α - and β -defensins are expressed.^{1–4} α -Defensins are expressed in neutrophils and Paneth cells, and β -defensins are expressed in epithelial cells of the skin, urogenital tissues, and bronchial tract.^{5–9}

Regarding human β -defensin (human β -defensin; hBD), six subtypes, hBD-1 to hBD-6, have been identified.⁹ hBD-1, composed of 36 amino acids, is constitutively⁵ expressed in epithelial cells of the cornea, cultured corneal epithelial cells, lens capsule, conjunctiva, iris, ciliary body, lacrimal gland, and epithelium of the nasolacrimal duct.^{10–13} hBD-2 is composed of 41 amino acids, and its expression is upregulated by inflammatory reaction.⁶ In the ophthalmological field, McDermott et al.¹⁴ reported that the upregulation of hBD-2 occurs by coculture of human nonpigmented epithe-

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lium of the ciliary body or retinal pigment epithelium with interleukin (IL)-1 β ,¹⁴ and in the regeneration process of organ-cultured human corneal epithelial cells.¹⁵ Lipopolysaccharides (LPS) are reported to be one of the factors in hBD-2 upregulation in human cultured corneal or conjunctival epithelial cells.¹⁶ It has also been suggested that there is a correlation between hBD-2 upregulation and the presence of IL-1 in human cultured conjunctival epithelial cells.¹⁷ hBD-2 is not expressed in normal ocular tissues, but it is expressed in epithelial cells of the nasolacrimal duct in dacryocystitis¹² and in conjunctival epithelial cells in dry eye cases.¹⁷ We also reported that hBD-1 mRNA is expressed in the ocular surface constitutively, but that hBD-2 mRNA is expressed at a higher incidence in eyes of patients with inflammatory conditions such as thermal or chemical burn than in normal conjunctiva samples.¹⁸

In skin lesions, the expression of hBD-2 mRNA in patients with atopic dermatitis is lower than in patients with psoriasis.¹⁹ These results suggest that patients with atopic dermatitis have increased susceptibility to *Staphylococcus aureus*.¹⁹ It is occasionally observed that patients with allergic conjunctivitis are susceptible to infectious diseases such as herpetic keratitis and staphylococcal blepharoconjunctivitis. Therefore, it is considered possible that the expression of hBD-2 may decrease in ocular surface tissues in patients with allergic conjunctivitis. In this study, we investigated the expression of β -defensin in ocular surface tissues using a mouse experimental allergic conjunctivitis (EAC) model.

For mouse β -defensins (mBDs), 23 different genes have been recently identified on the VIIIth chromosome.²⁰ mBD-1 is a homolog of hBD-1 and has antibacterial effects on Gram-positive and -negative bacteria. Expression of mBD-1 has been detected in bronchial epithelial cells and in epithelium of collecting tubes of the kidney and female reproductive organs.^{21–24} The expression of mBD-2 on the epithelium of the respiratory tract was upregulated by LPS.²⁵ In ocular surface tissue, the status of mBD-1 and mBD-2 has not yet been reported. Therefore, the aim of our study was to investigate the expression of mBD-1 and mBD-2 mRNA in ocular surface tissue in mice with allergic conjunctivitis using a mouse EAC model.

Materials and Methods

Materials

We used 32 eyes of 18 specific pathogen-free BALB/c male mice, which were 7 weeks of age. They were divided into two groups, the EAC group and the untreated control group.

Mouse EAC Model

A mouse EAC model was developed as previously reported.²⁶ Sensitization was performed on day 1 and day 8

by intraperitoneal injection of 0.05 ml of a solution including 200 μ g/ml ovalbumin (OVA) (Sigma Chemical, St. Louis, MO, USA) and 20 mg/ml of aluminum hydroxide [$\text{Al}(\text{OH})_3$] (Cosmo Bio, Tokyo, Japan). Allergic conjunctivitis was induced by topical application of 0.05 ml OVA (5 mg/ml) on day 15. Ocular samples were obtained after euthanasia by cervical dislocation at 12 h after the topical eye drop treatment. In the control group, ocular tissue was obtained from anesthetized untreated mice.

Quantitative Analysis of mBD-1 and mBD-2 mRNA

The EAC group and the control group each comprised nine eyes of nine mice. The eye globe with its adnexa was enucleated, and the bulbar and palpebral conjunctiva, including the limbus, were dissected en bloc under a dissecting microscope. Immediately, the en bloc specimen was preserved in RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany). Then total RNA was extracted using an Rneasy Mini Kit (Qiagen) and preserved at -80°C . cDNA was synthesized using the Thermoscript reverse transcriptase-polymerase chain reaction (RT-PCR) system (Invitrogen Japan, Tokyo, Japan). Quantitative real-time RT-PCR analysis of mBD-1 and mBD-2 mRNA was performed using an ABI Prism 7000 (Applied Biosystems Japan, Tokyo, Japan). The RT-PCR analysis was performed using a 96-well tray with a 25- μ l reaction mixture comprising 2 $\times$ SYBR Green PCR Master Mix, 0.7 μ l each of forward and reverse primer, and 100 ng cDNA.

The sequences and product sizes of each primer are as follows: mBD-1²⁷ 5'-CACATCCTCTCTGCACTCTGGAC CC-3' (forward), 5'-CCATCGCTCGTCCTTTATGCTCA TTC-3' (reverse), 287 base pair (bp); mBD-2²⁷ 5'-CGAAGC AGAAGCTTGACCACT-3' (forward), 5'-TCGAACAGGG GTTCTTCTCT-3' (reverse), 113 bp.

The PCR conditions were 95°C for 10 min, and the 40 cycles of 95°C for 15 s and 60°C for 1 min. In this study, we used each of the RT-PCR products of mBD-1 and mBD-2 mRNA as standard templates to measure the starting quantity, amplified with the same primers described above. RT-PCR products of mBD-1 and mBD-2 were diluted with Tris-EDTA buffer and adjusted for the standard sample. Starting quantities (copies/ μ l) of mBD-1 mRNA and mBD-2 mRNA in the samples were measured with each standard curve. Statistical analysis was performed using the Mann-Whitney *U* test, and a *P* value of less than 5% was set as the significance level.

Expression of mBD-1 mRNA and mBD-2 mRNA by Site in Mouse Ocular Surface Tissue

Six eyes of six mice (EAC) and eight eyes of eight mice (control) were investigated. After enucleation of the eye globe with eyelid, the sample was immersed in Tissue-Tek (Sakura Finetechnical, Tokyo, Japan) without fixation and frozen in hexane/dry ice-acetone and preserved at -80°C

until use. Then the tissue was cryosectioned into 8- μ m-thick sections using a cryostat (HM505E; Meiwa Shoji, Osaka, Japan) and mounted on a membrane film-coated glass slide. The samples were then fixed in 100% methanol for 3 min and stained with toluidine blue. After air-drying for 1 h at room temperature, corneal, limbal, and conjunctival epithelial cells were microdissected separately using a laser-assisted microdissection instrument (LSI337; Cell Robotics, Albuquerque, NM, USA). Total RNA was extracted from the microdissected cells as previously reported.²⁸ The specimen was dissolved in RNA extraction buffer containing 200 μ l guanidinium thiocyanate buffer (pH 7.0) and 1.6 μ l β -mercaptoethanol. For purification, 20 μ l of 2M sodium acetate (pH 4.0), 220 μ l citrate-saturated phenol (pH 4.3), and 60 μ l chloroform-isoamyl alcohol were added, and the mixture was centrifuged for 30 min at 17400g. The upper aqueous layer was transferred into new tubes. Two hundred microliters isopropanol and 2 μ l glycogen were added, and the tubes were kept at -80°C for 1 h. They were recentrifuged for 30 min at 15100g, and the supernatant was removed. The pellet was washed with 70% ethanol, air-dried on ice, and preserved at -80°C . cDNA was synthesized using the ThermoScript RT-PCR system. The RT-PCR analysis was performed using AmpliTaq Gold (Applied Biosystems). Mouse β actin was used as an internal control. The PCR conditions were 95°C for 5 min and 50 cycles of 94°C for 30s, 60°C for 30s, and 92°C for 30s. mBD-1 and mBD-2 primer sequences and product sizes were the same as described above. The primer sequence and product size of mouse β actin, the internal control, were as follows: 5'-GATCTGGCACCACACCTTCT-3' (forward), 5'-GGGGTGTGAAGGTCTCAAA-3' (reverse), 138bp.

The RT-PCR products were analyzed by gel electrophoresis on 2% agarose gels stained with SYBR Green I.

Results

Quantitative Analysis of the Expression of mBD-1 mRNA and mBD-2 mRNA

The results of the real-time RT-PCR analysis are shown in Fig. 1. The quantity of mBD-1 mRNA was $34.2 \pm 29.1 \times 10^4$ copies/ μ l for the EAC group and $98.5 \pm 170 \times 10^4$ copies/ μ l for the control group. There was no significant difference between the two groups. The quantity of mBD-2 mRNA was $0.23 \pm 0.15 \times 10^4$ copies/ μ l for the EAC group and $3.61 \pm 4.87 \times 10^4$ copies/ μ l for the control group. The quantity of mBD-2 mRNA in the EAC group was significantly lower than that in the control group ($P < 0.01$).

Expression of mBD-1 mRNA and mBD-2 mRNA by Site in Mouse Ocular Surface Tissue

The results of the RT-PCR analysis are shown in Figs. 2 and 3. In the EAC group, mBD-1 mRNA was detected in all examined tissues. mBD-2 mRNA was detected in all corneal epithelium, six of six eyes and in conjunctival epithelium (4/6), but not in limbal epithelium (0/6). In the control group, mBD-1 mRNA was detected in all examined tissues. mBD-2 mRNA was detected in corneal (6/8), limbal (6/8), and conjunctival epithelium (7/8). Mouse β actin was detected in all specimens.

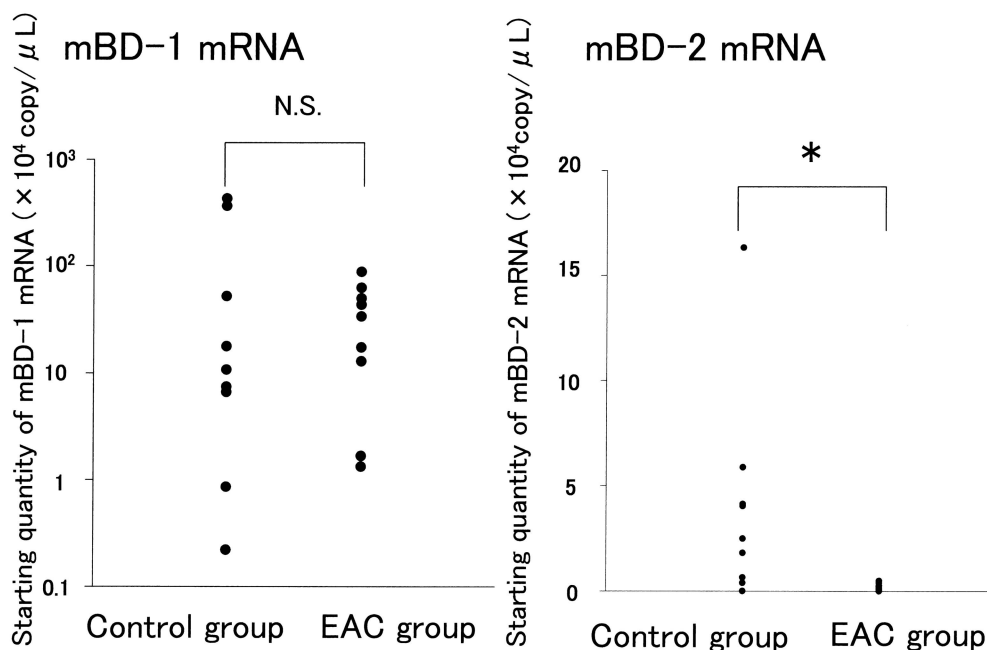


Figure 1. Results of the quantitative analysis of the expression of mouse β -defensin (mBD)-1 mRNA and mBD-2 mRNA. A scattergram of the starting quantities of mBD-1 mRNA and mBD-2 mRNA is shown. The quantity of mBD-1 mRNA was $34.2 \pm 29.1 \times 10^4$ copies/ μ l for the experimental allergic conjunctivitis (EAC) group and $98.5 \pm 170 \times 10^4$ copies/ μ l for the control group. There was no significant difference between the two groups. The quantity of mBD-2 mRNA was $0.23 \pm 0.15 \times 10^4$ copies/ μ l for the EAC group and $3.61 \pm 4.87 \times 10^4$ copies/ μ l for the control group. The quantity of mBD-2 mRNA in the EAC group was significantly lower than that in the control group ($P < 0.01$). * $P < 0.01$, Mann-Whitney U test.

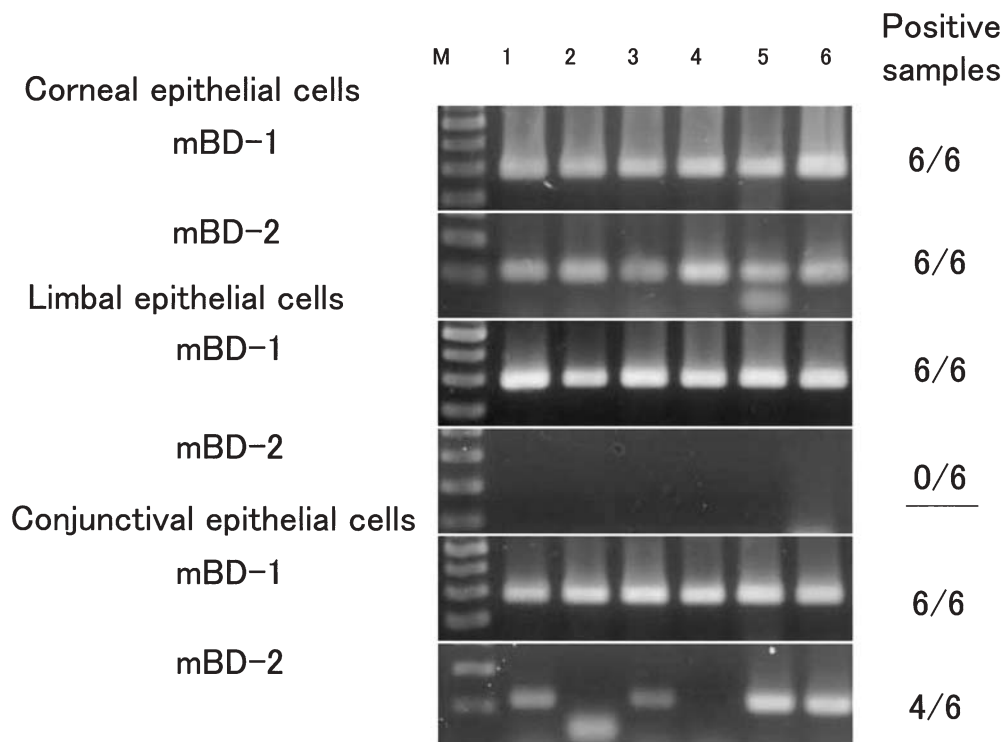


Figure 2. The expression of mBD-1 and mBD-2 mRNA by site in mouse ocular surface tissue in the EAC group. The *left lane* is the 100-bp marker. The expression of mBD-1 mRNA was positive in all examined tissues. The expression of mBD-2 mRNA was positive in all corneal epithelium, six of six eyes and in conjunctival epithelium (4/6), but negative in limbal epithelium (0/6).

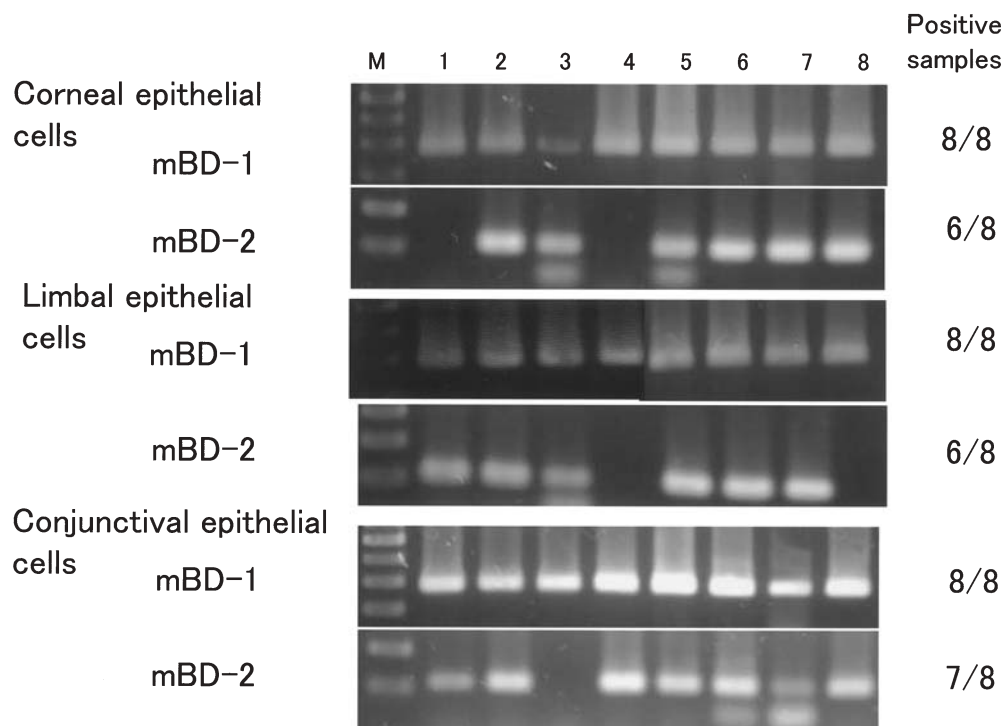


Figure 3. The expression of mBD-1 and mBD-2 mRNA by site in mouse ocular surface tissue in the control group. The *left lane* is the 100-bp marker. The expression of mBD-1 mRNA was positive in all examined tissues. The expression of mBD-2 mRNA was positive in corneal (6/8), limbal (6/8), and conjunctival (7/8) epithelium.

Discussion

In the mouse ocular surface tissues, mBD-1 mRNA was constitutively expressed regardless of the presence of allergic conjunctivitis in the mouse model. However, in the EAC group, the quantity of mBD-2 mRNA expression was significantly lower than that in the control group. The decrease of total mBD-2 mRNA expression in the ocular surface tissues of the EAC group may be related to the loss of mBD-2 mRNA expression in the limbal epithelium.

In this study, we investigated the quantity of mBD-1 and mBD-2 mRNA using a real-time RT-PCR assay. The real-time RT-PCR assay has high specificity and sensitivity in the study of cytokine gene expression compared with the gene array assay.²⁹ However, expression of housekeeping genes, such as β actin, β globin, or glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), changes quantitatively depending on the types of tissue or cells and various other factors.³⁰ For these reasons, we normalized the quantity of mBD-1 and mBD-2 expression not to the quantity of β actin mRNA but to the amount of cDNA, after the expression of β actin in each sample was confirmed.

Ong et al.¹⁹ reported that the expression of hBD-2 protein and mRNA were significantly lower in patients with atopic dermatitis than in patients with psoriasis. This study also showed that expression of mBD-2 mRNA was lower in the EAC group than in the control group. In general, the expression of hBD-2 is thought to be upregulated by inflammatory response. The upregulation of hBD-2 is suppressed by coculture with IL-4 alone or concomitantly with IL-4 and IL-13 in human keratinocytes.³¹ IL-4 and IL-13 are classified into the Th2 cytokine group, expression of which is increased in patients with allergic diseases. The suppression of hBD-2 expression in patients with atopic dermatitis may be caused by the same mechanism. Furthermore, TNF-α, IFN-γ, and IL-1β, which cause upregulation of hBD-2 expression, are decreased in patients with atopic dermatitis.³¹ It has been reported that Th2 cytokines act dominantly in allergic ocular surface diseases.^{32,33}

We also investigated the expression of IL-4 mRNA and IL-13 mRNA in conjunctival tissue and limbal epithelium tissue of both the EAC and control groups. There was no statistically significant difference between the EAC group and the control group (data not shown), and no correlation between the suppression of mBD-2 expression and IL-4 or IL-13 expression was found in the present study. Another possible cause of this suppression was the decrease in the number of epithelial cells due to cell damage by inflammatory reaction. However, that effect could be negligible, since the expression of mBD-1 mRNA did not differ between the EAC group and the control group. Therefore, we speculated that the cause of the decrease in mBD-2 mRNA expression was a suppressive mechanism of the allergic reaction. Although mBD-2 mRNA is expressed by inflammatory response in tracheal epithelium, it was expressed on the intact ocular surface of untreated mice in this study. The basic mechanism of hBD-2 mRNA expression in human ocular surface tissue should be investigated,

not only by qualitative methods but also by a quantitative analysis.

The expression of mBD-2 mRNA by site in mouse ocular surface tissue revealed that the decrease of mBD-2 mRNA expression was limited to the limbal epithelium, and was not observed in the corneal or conjunctival epithelium. These findings indicate that some suppression factor of mBD-2 mRNA expression is present mainly in the limbus but not in the cornea or conjunctiva. This phenomenon may be related to the strong clinical findings in allergic inflammatory diseases and the presence of immune response cells such as Langerhans cells and mast cells in the limbus. Further investigation is needed to resolve this question.

A patient with allergic ocular surface disease is easily susceptible to infectious diseases such as herpetic keratitis or staphylococcal blepharoconjunctivitis. Since hBD-2 has additive effects on *Staphylococcus aureus* with a similar antimicrobial peptide, cathelicidin, we considered that the decreased expression of hBD-2 might play some role in the susceptibility to staphylococcal blepharoconjunctivitis. The antimicrobial effect of hBD-2 on herpes simplex virus has not been elucidated.

Other subtypes of β-defensins should also be investigated in order to clarify the effects of β-defensins in the ocular surface, and we are continuing to study them. Furthermore, the investigation of the protein expression of β-defensins in addition to an mRNA expression analysis is necessary to determine protein localization.

In this study, we showed that decreased expression of mBD-2 mRNA in ocular surface tissue of the mouse EAC model is due mainly to its suppression in limbal epithelium. The suppression mechanism of BD-2 expression in limbal epithelium may be related to the increased susceptibility to infectious disease of patients with allergic ocular surface disease.

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