A Novel Mouse β-Defensin, mBD-6, Predominantly Expressed in Skeletal Muscle*  

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Yasuhiro Yamaguchi‡†, Shigetomo Fukuhara‡, Takahide Nagase‡, Tetsuji Tomita‡, Shigemi Hitomi‡, Satoshi Kimura‡, Hiroki Kurihara‡§, and Yasuyoshi Ouchi‡†  

From the ‡Departments of Geriatric Medicine and Infectious Control and Prevention, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan, the §Division of Integrative Cell Biology, Department of Embryogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto 860-0811, Japan, and the ¶Department of Infectious Diseases, Institute of Clinical Medicine, University of Tsukuba, Tsukuba 305-8575, Japan

Defensins are a family of cationic antimicrobial peptides that is characterized by the conserved 6 cysteine residues. They are expressed in the epithelial cells of various organs and are identified as key elements in the host defense system at the mucosal surface. We isolated a novel mouse β-defensin gene from the bacterial artificial chromosome DNA containing the mouse β-defensin-3 gene. The full-length cDNA was cloned from skeletal muscle cDNA and called mouse β-defensin-6 (mBD-6). The predicted peptide conserved the 6-cysteine motif and had 59% amino acid sequence identity with mouse β-defensin-3 and 59% identity with mouse β-defensin-4. We demonstrated the expression of mBD-6 in skeletal muscle in addition to the esophagus, tongue, and trachea. In animal models of endotoxemia, mBD-6 expression was also induced in the lung. mBD-6 showed potent antimicrobial activity against Escherichia coli and would play an important role in host defense in the esophagus, airways, and skeletal muscle. mBD-6 is the first reported β-defensin predominantly expressed in skeletal muscle. This unique tissue specificity suggests some novel physiological roles of this peptide family.

Defensins are cationic antimicrobial peptides that include six specific cysteine residues and can be divided into the α- and β-defensin subfamilies. The human α-defensin family comprises six members: human neutrophil peptide-1, -2, -3, and -4 (HNP-1, -2, -3, and -4) in the neutrophilic granules and human defensin-5 and -6 in the intestinal Paneth’s cells (1–4). HNP-1 and HNP-2 showed microbicidal activity against Gram-negative bacteria and Cryptococcus neoformans and directly inactivated herpes simplex virus type 1 (1).

The first mammalian β-defensin was discovered from bovine respiratory tract and was named tracheal antimicrobial peptide (5). Subsequently, lingual antimicrobial peptide was isolated from bovine tongue (6).

Three human β-defensins have been identified to date: human β-defensin-1, -2, and -3 (hBD-1, -2, and -3) (7–10). hBD-1 and hBD-2 show a salt-sensitive microbicidal activity predominantly against Gram-negative bacteria (8, 11–13), whereas hBD-3 exhibits a microbicidal activity against both Gram-negative and Gram-positive bacteria in a salt-insensitive manner (9). In addition, β-defensins could act as a chemoattractant for immature dendritic cells and memory T cells by activating CCR6 chemokine receptors (14).

hBD-1 is mainly expressed in urogenital tracts, but its expression was also detected in the pancreas, lung, skin, and intestine (11, 12, 15–17). hBD-2 was first isolated from psoriatic scale extracts and is expressed in human skin, lung, and trachea (8, 13). The inducible expression of hBD-2 was also detected in intestinal epithelium (18). The tissue distribution of hBD-3 is more unique. Its expression was detected in placental membrane, heart, and skeletal muscle in addition to esophagus, trachea, and cultured gingival keratinocytes, although the analysis was not quantitative enough (9, 10).

In animal models, five mouse β-defensin genes and two rat β-defensin genes have been cloned so far: mouse β-defensin-1, -2, -3, -4, and -5 (mBD-1, -2, -3, -4, and -5) and rat β-defensin-1 and -2 (19–24). They are expressed in the epithelial cells of various organs, such as lung, trachea, and kidney.

Recently, the novel antimicrobial peptide Bin1b was identified in the rat epididymis, and its putative amino acid sequence includes the conserved 6-cysteine motif (25). Interestingly, the expression of bin1b was confined to the caput region of the rat epididymis.

These findings indicate the widespread distribution of the β-defensin family. Many β-defensin isoforms are expressed in multiple tissues redundantly. However, in some organs, the specific β-defensin serves as an important factor of the host defense, such as bin1b in the caput region of the epididymis. Because microbial infection can occur in any organ, more novel defensin isoforms are expected in unique tissues.

The four mouse β-defensin genes encoding mBD-1, -2, -3, and -4 are all located within a contiguous region in chromosome 8, although the chromosomal location of mBD-5 has not been reported yet, suggesting the existence of a large β-defensin gene cluster. This observation prompted us to hypothesize that unidentified β-defensin genes would still exist in this gene cluster.
Here, we report a novel isoform of mouse β-defensin called mBD-6, which is located in physical proximity to mBD-3 gene. Unlike other defensins, mBD-6 is expressed predominantly in skeletal muscle in addition to esophagus, tongue, and trachea.

EXPERIMENTAL PROCEDURES

Screening of a Bacterial Artificial Chromosome (BAC) Library—A mouse genomic BAC library was screened with probes containing mBD-3 cDNA for the mBD-3 gene-specific clones at Incyte Genomics. The insert sizes of these clones were determined using pulse-field electrophoresis after the digestion of BAC DNAs with NotI enzyme.

Cloning of the mBD-6 Gene—To screen additional H9252-defensin-related genes in the region adjacent to mBD-3, we designed two primers from the homologous regions of mBD-3 and mBD-4 (forward primer: 5' GCTTCAGTCGATAGGATGATCCATTACCTG-3'; reverse primer: 5' GATTAGATCCATTACCTG-3'). PCR was performed on 5-ng BAC DNAs using the Advantage-HF2 PCR kit (CLONTECH). The PCR conditions were 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 10 min carried out for 35 cycles.

The partial sequence of the 5'-flanking region was identified by direct sequence analysis of the BAC clone using a specific antisense oligonucleotide from the putative intron of mBD-6 gene. Based on the putative exon sequences, we further designed two specific intron-spanning primers for RT-PCR to isolate mBD-6 cDNA (forward primer: 5'-TCATGAGATGATCCATTACCTG-3'; reverse primer: 5'-TGTGATTTCTAGCAAGAGAAAG-3'). Reverse transcription was performed on total RNA from adult mouse gastrocnemius muscle using Superscript II. The PCR conditions were 94 °C for 15 s and 68 °C for 3 min carried out for 35 cycles. Then the full-length cDNA sequence was determined using 5'-RACE and 3'-RACE kits (Life Technologies, Inc.).

Synthesis of Mature mBD-6 Peptide—We inferred the amino acid sequence of mature mBD-6 peptide from the sequence of mature hBD-2 and mBD-3 peptide (8, 13, 22). The putative mature peptide was chemically synthesized and air-oxidized for three disulfide bonds at the Peptide Institute (Minoh, Japan). The material, eluted in a single peak on reverse phase-high pressure liquid chromatography and confirmed by mass spectroscopy, was lyophilized and dissolved in 0.01% acetic acid.

Analysis of Antimicrobial Activity—We followed the colony count assay described by Harwig et al. (26) with some modification. Midlogarithmic-phase Escherichia coli (ATCC 25922 strain) was suspended in 10 mM sodium phosphate buffer to adjust the density to 5 × 10^7 colony-forming units/ml, and this suspension was mixed with mBD-6 solution. The final sodium concentration of this mixture was 15 mM, and the mBD-6 concentration was adjusted to 2 μg/ml, 20 μg/ml, or 200 μg/ml. As a control, the mixture without mBD-6 was also incubated. After a 2-h incubation of these mixtures at 37 °C, the 10-fold serial dilutions were spread over trypticase soy agar plates and incubated at 37 °C for 48 h. After counting the numbers of colonies on the plates, we...
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FIG. 3. Antimicrobial effect of mBD-6. Mid-logarithmic-phase E. coli was incubated with mBD-6 or HNP-1 solution at the indicated concentration. The survival ratio is the ratio of the number of survived colonies to that of control colonies. The means and the standard errors of the log_{10} survival ratio are depicted. A shows that the synthetic mBD-6 peptide had antimicrobial activity at the concentration of 20 μg/ml. The bactericidal effect of mBD-6 was significantly more potent than HNP-1 (Student’s t test, p < 0.01). Then, we adjusted the sodium concentration to 15 mM, 50 mM, 100 mM, and 140 mM at the mBD-6 concentration of 20 μg/ml. B shows that the antimicrobial activity was significantly reduced at the sodium concentration of 50 mM (Student’s t test, p < 0.01).

FIG. 4. Tissue distribution of mBD-6 mRNA. A, Northern blot analysis of mBD-6 expression was carried out. 2 μg of full-length poly(A)⁺ RNA from each organ was applied (MTN Blot; CLONTECH). The most intense signal can be detected in skeletal muscle. This analysis failed to detect mBD-6 expression in heart, brain, spleen, liver, kidney, and testes. B, detection of mBD-3, mBD-4, and mBD-6 expression in various tissues by RT-PCR. All three β-defensins are expressed in esophagus, tongue, and trachea. In skeletal muscle, mBD-3 expression was not detected, consistent with a published report (22), and mBD-4 and mBD-6 expression was detected by RT-PCR although the signal intensity of mBD-4 was very low. After 25 and 30 cycles of PCR, mBD-6 transcripts were almost comparable among the esophagus, tongue, and skeletal muscle. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair.

FIG. 5. Inducible expression of mBD-6 mRNA in lung. Female ICR adult mice were injected intraperitoneally with 200 μg of LPS from P. aeruginosa Serotype 10. Total RNA of the lung was isolated before the treatment and 2 and 12 h after the treatment. The amplified mBD-6 could be visualized only 12 h after the treatment. G3PDH, glyceraldehyde-3-phosphate dehydrogenase; bp, base pair.

Animal Procedures—We used an animal model of endotoxemia to study the regulation of mBD-6 expression. Female ICR adult mice were injected intraperitoneally with 200 μg of lipopolysaccharide (LPS) from Pseudomonas aeruginosa Serotype 10 in PBS(−) solution (NaCl 137 mM, NaHPO₄ 8.10 mM, KCl 2.68 mM, and KH₂PO₄ 1.47 mM). The mice were sacrificed 2 and 12 h after the treatment. Total RNA was isolated from the lung of each mouse, and RT-PCR was performed as described above. The PCR was carried out for 30 cycles to be quantitative.

RESULTS AND DISCUSSION

Identification of a Novel Mouse β-Defensin Isoform—We first obtained three BAC clones containing the mBD-3 gene: 123,
D11, and K02. PCR with primers common to mBD-4 and mBD-3 on BAC D11 revealed a novel β-defensin-related sequence. Subcloning and characterization of a 2.7-kb genomic fragment containing this sequence then showed two putative exons separated by a 2.4-kb intron. This exon-intron structure and the existence of a corresponding transcript were confirmed by RT-PCR on mouse skeletal muscle cDNA and subsequent 5' and 3'-RACE using primers deduced from the putative exon sequence (Fig. 1). A sequence comparison analysis using a BLAST search at the NCBI identified no homologous nucleotide sequences other than mBD-3, mBD-4, mBD-5, and rat β-defensin-2.

This novel gene, called mBD-6, is located within 240 kb from mBD-3 on chromosome 8 as inferred from the insert size of BAC D11. The mBD-6 genomic sequence also revealed a partial 5'-flanking region including a TATA box and an NF-κB site. It is noteworthy that the NF-κB site and its adjacent region in the mBD-6 gene are very similar to that of the mBD-3 gene, whereas the NF-κB site is lacking in the mBD-4 gene (22, 23).

The predicted amino acid sequence of mBD-6 is 59, 59, and 51% identical with mBD-3, mBD-4, and mBD-5, respectively, with a lower homology to mBD-1 and mBD-2 (Fig. 2). The 6 cysteine residues characteristic of the β-defensin family are completely conserved in this novel peptide. As is true of other β-defensins, mBD-6 has a sequence matching a signal peptide, suggesting that mBD-6 would also be a secretory peptide.

Antimicrobial Activity of Synthetic mBD-6 Peptide—To confirm the antimicrobial activity of mBD-6, we synthesized a putative mature peptide spanning 41 COOH-terminal amino acids of mBD-6 precursor as shown in Fig. 1, assuming that mBD-6 peptide would be cleaved at the analogous position of hBD-2 and mBD-3 peptide. The synthesized material showed significantly more potent bactericidal activity against E. coli at the concentration of 20 μg/ml than synthetic hNP-1 (Student’s t test, p < 0.01) (Fig. 3A), whereas hNP-1 showed bactericidal activity at the concentration of 200 μg/ml. The antimicrobial effect of mBD-6 was also comparable with other β-defensins because the 90% lethal dose (the dose that achieves 90% reduction of colony-forming units) of the isolated hBD-2 was 10 μg/ml against Gram-negative bacteria, and the minimal inhibitory concentration of recombinant mBD-3 was 16 μg/ml against E. coli (8, 22). The antimicrobial potency of mBD-6 was significantly reduced at high concentrations of NaCl, similar to hBD-1, hBD-2, and mBD-1 (Student’s t test, p < 0.01) (Fig. 3B) (8, 11, 13, 20).

mBD-6 Tissue Distribution and Its Inducibility—Our evaluation of the mBD-6 expression revealed the unique tissue specificity. The Northern blot analysis detected mBD-6 mRNA in skeletal muscle and whole lung (i.e. lung and trachea). Interestingly, the hybridization signal was much more intense in skeletal muscle than that in whole lung (Fig. 4A). RT-PCR also confirmed that mBD-6 expression was comparable among the esophagus, tongue, and skeletal muscle, whereas its expression was low in the trachea and undetectable in the lung. This expression pattern is similar to that of mBD-3 and mBD-4 except for the distinctively high expression of mBD-6 in skeletal muscle (Fig. 4B). In lung tissues, no expression of mBD-6 was detected by RT-PCR, indicating that the hybridization signal of mBD-6 in whole lung by Northern blot analysis is mainly attributable to mRNA in trachea, which is similar to mBD-4 tissue distribution (23).

The abundant expression in skeletal muscle had not been reported for any other β-defensins. The previous reports had revealed much lower expression of mBD-1 and mBD-3 in skeletal muscle than in other tissues (19, 20, 22). As for human β-defensins, hBD-3 expression was detected in skeletal muscle by RT-PCR analysis, but the signal intensity was not so high as in esophagus, trachea, and placental membrane (10).

To determine the induction of mBD-6 expression, we challenged the mice intraperitoneally with 200 μg of LPS in phosphate-buffered saline solution, which is about one-fifth of the lethal dose (27). RT-PCR revealed that the mBD-6 expression was induced in the lung 12 h after the LPS challenge (Fig. 5). This result suggests that mBD-6 expression in the lung is inducible rather than constitutive. In contrast, in our animal model of endotoxemia, the increase of mBD-6 expression in skeletal muscle was not detected by Northern blot analysis or RT-PCR (data not shown).

The β-defensin family has been divided into two groups in terms of the inducibility of expression. mBD-1 and mBD-4 are expressed only constitutively; mBD-2 and mBD-3 can be induced by inflammatory stimuli such as LPS or bacterial infection (19–23). The previous reports showed that tracheal antimicrobial peptide and hBD-2 are induced by LPS through CD14-dependent NF-κB activation in the tracheobronchial epithelial cells (28, 29). The inducibility of gene expression appears to be correlated to the presence or absence of an NF-κB site in the mBD-3, mBD-4, and mBD-6 5' flanking region. However, it might be possible that the regulatory mechanisms of β-defensin expression are different among tissues and organs. Although this issue should be further investigated, the discovery of this widespread distribution of the β-defensin family also suggests that the regulation of β-defensin expression is more complicated.

The present study adds a new member of the defensin family involved in innate immunity in the mouse airways and esophagus, in synergy with mBD-3 and mBD-4. More interestingly, mBD-6 is highly expressed in skeletal muscle, which is not explicitly involved in the host defense mechanism. Skeletal muscle is known to be sterile and relatively resistant to the formation of infectious foci. Thus, mBD-6 may play a role in the regional defense mechanism in skeletal muscle against bacterial infection. Furthermore, it should be noteworthy that skeletal muscle occupies a great mass of the total mammalian body. Considering the large mass of its producing tissue, mBD-6 may also contribute to the systemic defense against bacterial infection.

Recently, the novel defensin-like peptide Bin1b was identified in the rat epithidymis (25). HEβ21 is also expressed in human epididymis and is homologous with the amino acid sequence of Bin1b, including the conserved 6-cysteine motif, although the antimicrobial activity has not been evaluated (10, 30). These findings intensify the significance of tissue specificity among variable isoforms of the β-defensin family. Because the microbial infection can occur in any organ, β-defensins might have evolved to acquire functional adaptation to the microenvironment and invading microorganisms in specific organs (31). Thus, tissue-specific regional defense mechanisms involving defensins may afford resistance against infection in concert with the systemic immune system.

In summary, we describe a novel mouse β-defensin, mBD-6, which shows unique tissue specificity and antimicrobial activity. In particular, its expression in skeletal muscle as well as in esophagus and trachea suggests a novel role of defensins in the regional and/or systemic defense.
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