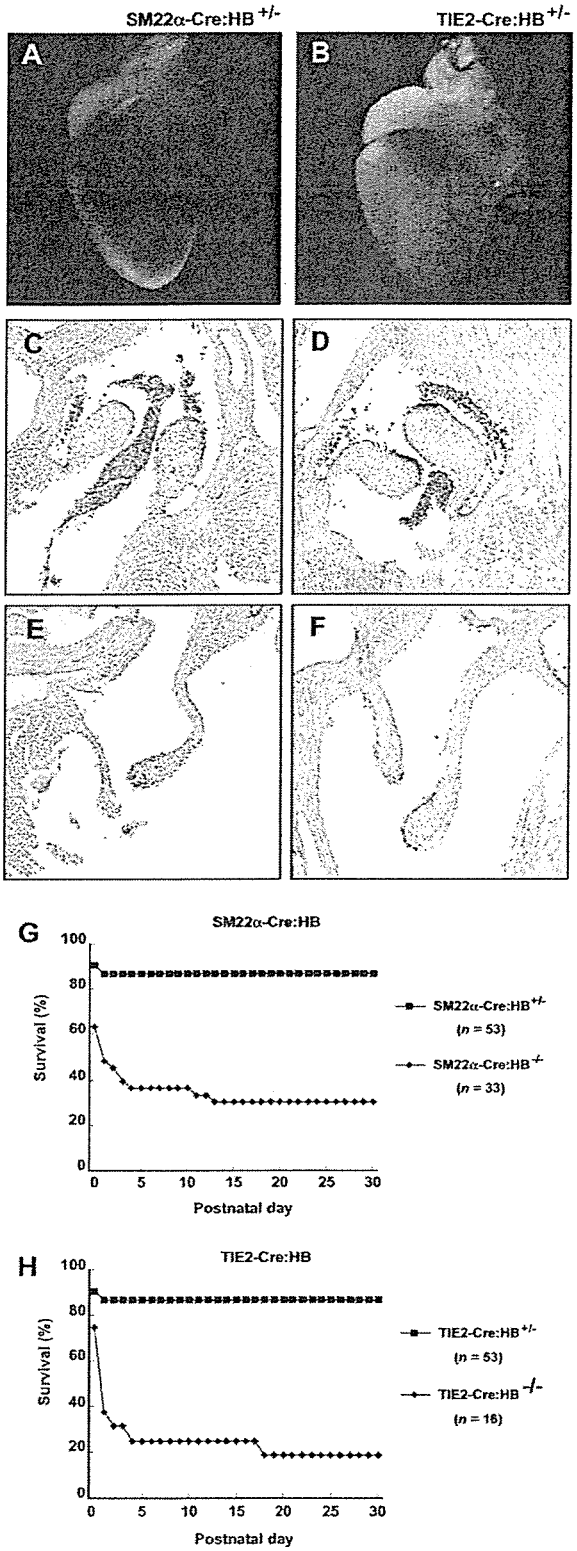

Histological analysis. Mouse tissues were fixed in 3.7% formaldehyde, dehydrated, and embedded in paraffin. Ten micrometer sections were stained with hematoxylin and eosin. For X-gal staining, after fixation with 2% formaldehyde and 0.2% glutaraldehyde, the tissues were stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). The stained tissues were fixed again with 3.7% formaldehyde, dehydrated, and embedded in paraffin. Ten micrometer sections were stained with eosin.

Results

Generation of HB-EGF conditional knockout mice

To generate cell-type specific HB-EGF knockout mice, mice carrying alleles with the *HB-EGF* gene flanked by *loxP* sites ($HB^{lox/lox}$; [6]) were crossed with SM22α-Cre [12] or TIE2-Cre [13] transgenic mice. The obtained mice carrying the wild-type and *loxP HB-EGF* genes, and Cre transgenes (SM22α-Cre:$HB^{WT/lox}$ or TIE2-Cre:$HB^{WT/lox}$, which we refer to as SM22α-Cre:$HB^{+/-}$ or TIE2-Cre:$HB^{+/-}$) were bred again with $HB^{lox/lox}$ mice to generate SM22α-Cre:$HB^{lox/lox}$ or TIE2-Cre:$HB^{lox/lox}$ mice, which we refer to as SM22α-Cre:$HB^{-/-}$ or TIE2-Cre:$HB^{-/-}$ mice. The genotype of mice was confirmed by PCR analysis (Fig. 1). No overt abnormalities were observed in $HB^{WT/lox}$ or $HB^{lox/lox}$ mice [6,23], and there was no evidence that strong expression of Cre recombinase induced abnormalities in wild-type mice.

HB-EGF expression in the heart of HB-EGF conditional knockout mice

The targeting vector used for the generation of $HB^{lox/lox}$ mice contains the *lacZ* reporter gene under the control of the native *HB-EGF* promoter, which is activated by Cre-mediated recombination [6]. Beta-gal staining of newborn (postnatal day 1; P1) hearts in SM22α-Cre:$HB^{+/-}$ and TIE2-Cre:$HB^{+/-}$ mice showed that HB-EGF was strongly expressed at the site where the great vessels and coronary arteries arise from the heart (Fig. 2A and B). Histological analysis of the heart revealed that β-gal positive cells were localized to the margins of all of the heart valves, including the semilunar (aortic and pulmonic) valves (Fig. 2C and D) and the atrioventricular (mitral and tricuspid) valves (Fig. 2E and F). These results indicate that HB-EGF expression was blocked in the endocardium of the heart valves and the coronary artery of SM22α-Cre:$HB^{-/-}$ and TIE2-Cre:$HB^{-/-}$ mice.

Fig. 2. The heart morphologies and the tissue sections of newborn (P1) SM22α-Cre:$HB^{+/-}$ and TIE2-Cre:$HB^{+/-}$ (corresponds to wild-type) mice and survival of conditional knockout mice. Whole mount β-gal staining revealed that HB-EGF was strongly expressed at sites at which the great vessels and coronary arteries arise from the heart in SM22α-Cre:$HB^{+/-}$ (A) and TIE2-Cre:$HB^{+/-}$ (B) mice. The longitudinal sections showed that HB-EGF was expressed at the margin of the semilunar (C; SM22α-Cre:$HB^{+/-}$ and D; TIE2-Cre:$HB^{+/-}$) and atrioventricular (E; SM22α-Cre:$HB^{+/-}$ and F; TIE2-Cre:$HB^{+/-}$) valves. Over half of the SM22α-Cre:$HB^{-/-}$ (G) and TIE2-Cre:$HB^{-/-}$ (H) mice died within the first day after birth. Approximately 70–80% of these knockout mice died with the first 2 weeks after birth.

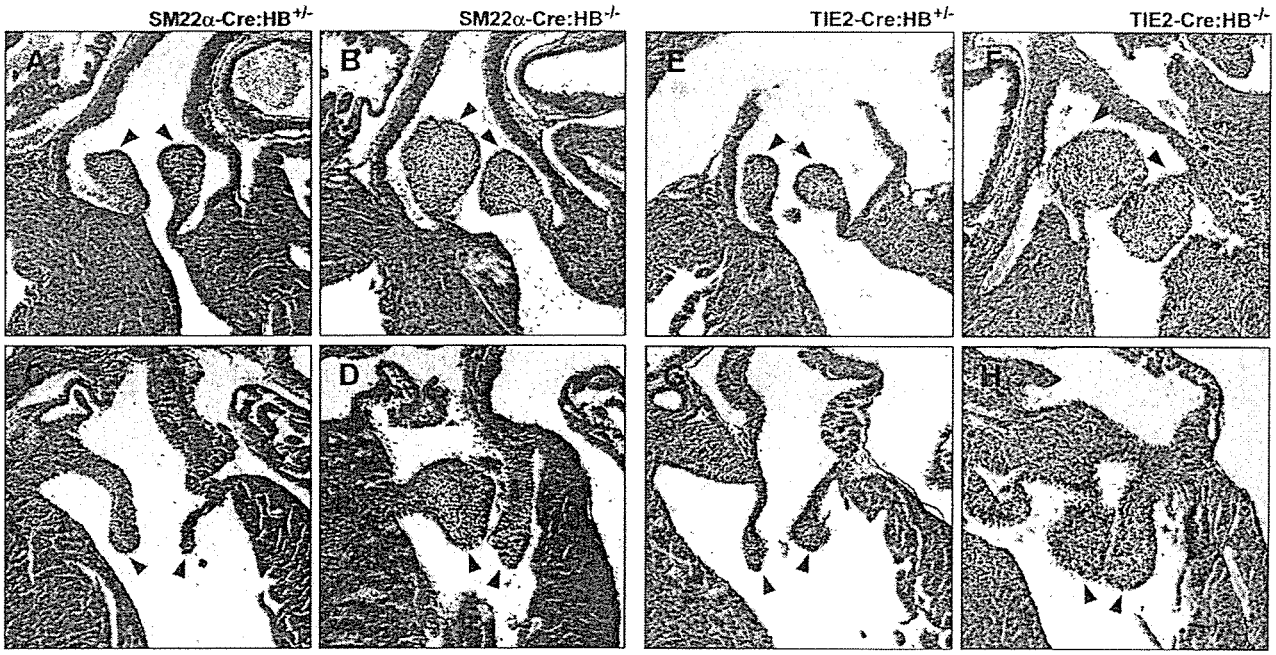

Fig. 3. Hematoxylin and eosin staining of the longitudinal sections of the newborn (P1) heart valves. Histological sections show semilunar (A, B, E and F) and atrioventricular (C, D, G and H) valves. In SM22α-Cre:$HB^{-/-}$ (B and D) and TIE2-Cre:$HB^{-/-}$ (F and H) mice, the valves were enlarged when compared with SM22α-Cre:$HB^{+/-}$ (A and C) and TIE2-Cre:$HB^{+/-}$ (E and G) mice. The valves are indicated with arrowheads.

Postnatal lethality of HB-EGF conditional knockout

Breeding of SM22α-Cre:$HB^{WT/lox}$ or TIE2-Cre:$HB^{WT/lox}$ with homozygous $HB^{lox/lox}$ mice yielded HB-EGF conditional knockout mice. Half of these mice died within one day after birth. Seventy percent of SM22α-Cre:$HB^{-/-}$ mice died within 13 days after birth (Fig. 2G), and 80% of TIE2-Cre:$HB^{-/-}$ mice died within 18 days after birth (Fig. 2H). SM22α-Cre:$HB^{-/-}$ and TIE2-Cre:$HB^{-/-}$ mice survivors displayed no obvious outward abnormalities and remained alive for at least several months after birth (data not shown).

Enlarged heart valves in HB-EGF conditional knockout mice

Histological analysis of newborn (P1) hearts revealed that SM22α-Cre:$HB^{-/-}$ and TIE2-Cre:$HB^{-/-}$ mice developed enlarged semilunar (Fig. 3A, B, E and F) and atrioventricular (Fig. 3C, D, G and H) valves when compared with SM22α-Cre:$HB^{+/-}$ and TIE2-Cre:$HB^{+/-}$ mice. This phenotype was consistent with that of HB-EGF-null mice [6,7]. The enlargement of neonatal heart in HB-EGF-deficient mice [6,7], however, was not observed in the P1 heart of these conditional knockout mice (data not shown).

Cardiac hypertrophy in HB-EGF conditional knockout mice

Although the survivors of SM22α-Cre:$HB^{-/-}$ and TIE2-Cre:$HB^{-/-}$ mice initially appeared normal, massive enlargement of the heart was apparent by 12 weeks of age when compared with control mice (Fig. 4A–D). Specifically, the mean heart-to-body wet weight ratio was $1.64 \pm 0.74\%$ for 12-week-old SM22α-Cre:$HB^{-/-}$ mice and was $0.65 \pm 0.16\%$ for 12-week-old SM22α-Cre:$HB^{+/-}$ mice (Fig. 4E). Further, the mean ratio of heart/body weight was $1.36 \pm 0.48\%$ for 12-week-old TIE2-Cre:$HB^{-/-}$ mice and was $0.76 \pm 0.063\%$ for 12-week-old TIE2-Cre:$HB^{+/-}$ mice (Fig. 4F).

Discussion

The present study demonstrated that loss of HB-EGF in smooth muscle or endothelial cell lineages resulted in heart valve malformations, postnatal lethality, and cardiac hypertrophy, which is a phenotype similar to that of HB-EGF-null mice [6,7]. The *HB-EGF* gene was deleted in endocardial cells of the heart valves in SM22α-Cre:$HB^{-/-}$ and TIE2-Cre:$HB^{-/-}$ mice. Data from the present study suggest that enlargement of the heart valves results from the loss of HB-EGF in the endocardial cells of heart valves, and the heart valve malformation is likely responsible for the postnatal lethality and cardiac hypertrophy of these *HB-EGF* conditional knockout mice.

Heart valves develop from endocardial cushions, which form when the endocardial cells undergo an endocardial-mesenchymal transition (EMT) and proliferate and invade the cardiac jelly, a basement membrane-like substance produced by the myocardial cells. The endocardial cushion area elongates and undergoes continuous remodeling to refine the primitive cushion into thin elongated valve leaflets.

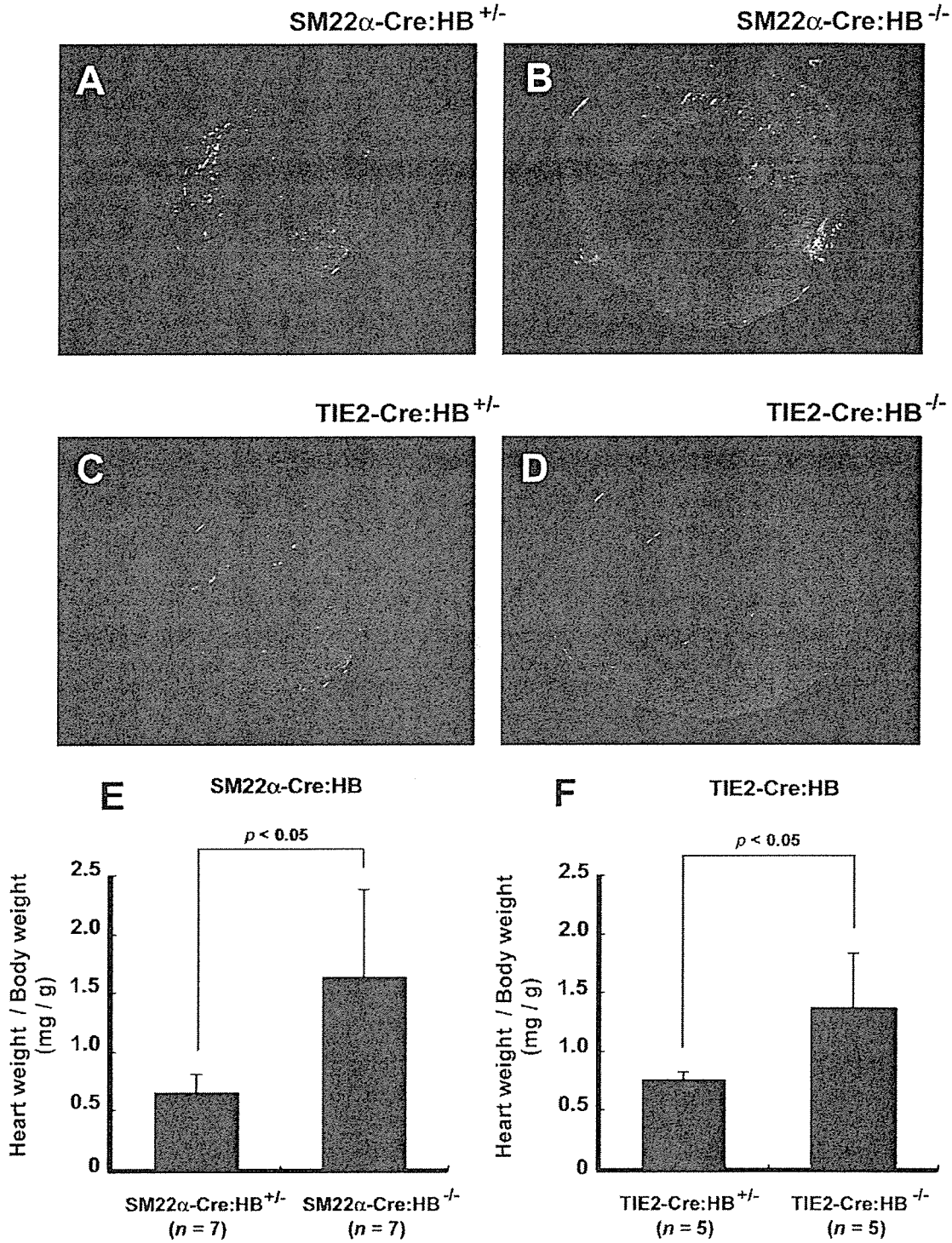

Fig. 4. Cardiac hypertrophy of the conditional knockout mice. Transverse sections of the hearts (A–D) and heart weight-to-body wet weight ratios (E and F) of 12-week-old mice. Values represent means ± SD. Massive enlargement of the heart was observed in SM22α-Cre:HB$^{-/-}$ and TIE2-Cre:HB$^{-/-}$ mice when compared with the control mice.

SM22α is expressed in cardiac muscle, smooth muscle, and skeletal muscle cells during embryogenesis, but becomes restricted to smooth muscle lineages at late embryonic stages and throughout adulthood [18]. Although HB-EGF is expressed in the heart [6,7], we could not detect obvious β-gal staining in myocardium of SM22α-Cre:HB$^{+/-}$ mice. *SM22α-lacZ* mice show the low expression of this transgene in myocardium during late development [24], which

suggests low *SM22α-Cre* activity and subsequent recombination efficiency with *loxP HB-EGF* in myocardium of SM22α-Cre:HB$^{+/-}$ mice. During early avian cardiac development, the endocardium-derived mesenchymal cells, which form endocardial cushions and subsequently form heart valves, have characteristics of smooth muscle-like myofibroblasts and express smooth muscle-specific alpha actin [25]. This finding suggests that the mesenchymal cells that had transformed from endocardial cells expressed the smooth muscle-specific *SM22α* gene, and this fact may account for *HB-EGF* gene deletion in the heart valves of SM22α-Cre:HB$^{-/-}$ mice.

TIE2 is expressed in endothelial cells throughout development [19,21,22]. Previous studies have reported that *TIE2*-Cre:R26R mice have β-galactosidase activity that was restricted to the endocardium and the mesenchyme of the endocardial cushions but was never observed in myocardium or epicardium of the developing heart [26]. These data indicate that the *HB-EGF* gene was eliminated in endocardial but not in myocardial cell lineages in the developing heart of TIE2-Cre:HB$^{-/-}$ mice. Therefore, the present study suggests that the loss of endocardial but not myocardial HB-EGF is at least responsible for the heart valve enlargement in HB-EGF-null mice.

Enlargement of heart valves has also been observed in mutant mice expressing an uncleavable form of proHB-EGF [27], in ADAM17-null mice [7], in EGFR-null mice with a CD1 background, and in mice expressing a mutant EGFR (*waved-2*) [8]. Together with data from the present study, these observations indicate that ectodomain shedding of proHB-EGF in endocardial cells of heart valves and subsequent EGFR activation are essential for remodeling of endocardial cushions. Although the mechanism of cushion remodeling is largely unknown, HB-EGF/EGFR signaling in the mesenchymal cells within the endocardial cushions may suppress cellular proliferation to refine the primitive cushion into thin elongated valve leaflets [7,28]. Recent studies have reported that the cytoplasmic domain of proHB-EGF and C-terminal fragment of proHB-EGF generated by ectodomain shedding have some functions [29–31]. These intracellular signaling might be involved in the heart valve formation.

In conclusion, the loss of *HB-EGF* gene expression in smooth muscle or endothelial cell lineages of the developing mouse results in heart valve malformations and cardiac hypertrophy. These data indicate the significance of endocardial HB-EGF for proper heart development and function.

Acknowledgments

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Review Article

Innate defences against methicillin-resistant *Staphylococcus aureus* (MRSA) infection

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Abstract

The innate immune system is the primary defence against bacterial infection. Among the factors involved in innate defence, anti-microbial peptides produced by humans have recently attracted attention due to their relevance to some diseases and also to the development of new chemotherapeutic agents. *Staphylococcus aureus* is one of the major human pathogens, causing a variety of infections from suppurative disease to food poisoning. Methicillin-resistant *S. aureus* (MRSA) is a clinical problem and with the recent emergence of a vancomycin-resistant strain, this will pose serious problems in the near future. In investigating the molecular biology of *S. aureus* infections to develop new chemotherapeutic agents against MRSA infections, knowledge of the interaction of innate anti-microbial peptides with *S. aureus* is important. *In vitro* and *in vivo* experiments demonstrate that exposure of *S. aureus* to host cells can induce the anti-microbial peptides β-defensin-2 (hBD2), hBD3, and LL37/CAP18. The induction level of these peptides differs among strains, as does the susceptibility of the strains, with MRSA strains exhibiting lower susceptibility. In summary, the susceptibility of *S. aureus* strains, including MRSA strains, to components of the innate immune system varies, with the MRSA strains showing more resistance to both innate immune factors and chemotherapeutic agents.

Keywords: ***Staphylococcus aureus*; MRSA; defensin; cathelicidin; innate immunity; keratinocytes**

Introduction

Environmentally, we are constantly exposed to many kinds of micro-organisms, including pathogens. The innate immune system, which monitors our environmental microbial flora, is the primary defence against microbial infection. Figure 1 summarizes the factors of the innate immune system that modulate bacterial infection. All act co-ordinately in host defence to eliminate the pathogens. Recently, anti-microbial peptides have been the focus of attention because low or high levels of the anti-microbial peptides *in situ* are linked to certain genetic diseases, where the peptides may be a potential alternative to long-term use of chemotherapeutic agents. The anti-microbial peptides kill micro-organisms directly and some act as chemokines. Crohn's disease [1–3], atopic dermatitis [3,4], Kostmann's disease [5], and cystic fibrosis [6,7] are linked to microbial infections due to decreased expression of anti-microbial peptides in these patients. By contrast, the anti-microbial peptides are highly expressed in the lesions of psoriasis, resulting in fewer microbial infections [3,8,9]. Furthermore, knockout mice lacking CRAMP or β-defensin-1 are highly susceptible to bacterial infections [10,11]. Therefore, the anti-microbial peptides are considered to be one of the central defence systems against microbial infections.

Staphylococcus aureus is a major human pathogen, causing suppurative diseases, toxic shock syndrome, pneumonia, food poisoning, staphylococcal scaled-skin syndrome (SSSS), and so on. Many virulence factors, such as enterotoxins, TSST-1, exfoliative toxins, serine proteases, haemolysins, leucocidins, and adhesins, have been identified in clinical isolates, demonstrating their association with clinical diseases *in vitro* and *in vivo* [12–15]. As shown in Figure 1, capsule or protease production resists some of the innate immune factors [16–19]. Chemotherapy is often used against *S. aureus* infections; however, the emergence of methicillin-resistant *S. aureus* (MRSA), which is resistant to β-lactam antibiotics and other chemotherapeutic agents, is a serious clinical problem [20,21]. Therefore, a new strategy is required for the development of new chemotherapeutic agents for the prevention of *S. aureus* infections. The anti-microbial peptides hold promise as chemotherapeutic agents due

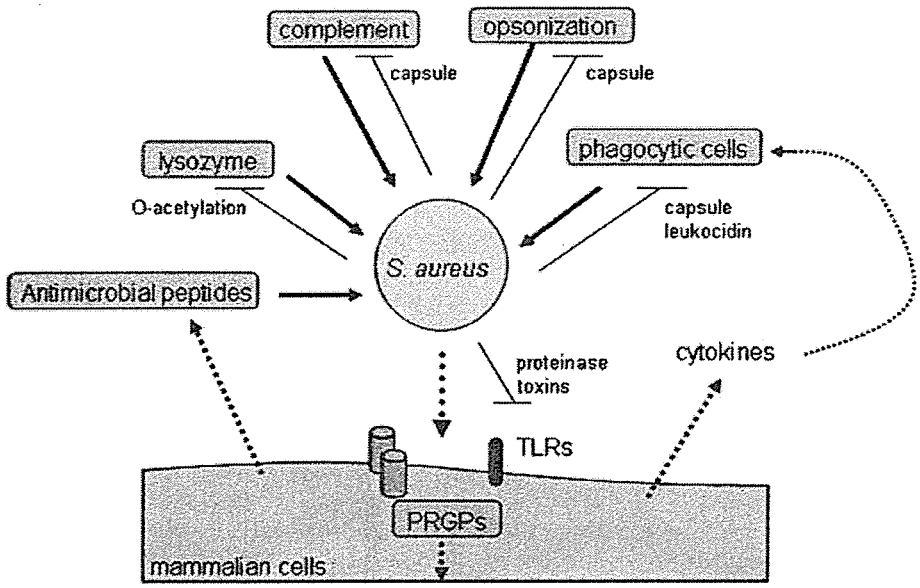

Figure 1. Model of the innate immune system against *S. aureus* infection. Several factors involved in the innate immune system attack invading bacteria. Also, mammalian cells respond to bacterial contact through several pattern recognition proteins, such as TLRs, and produce several factors, such as cytokines and anti-microbial peptides. Factors that confer resistance to the innate immune system in *S. aureus* are also indicated

to their antibacterial activity against *S. aureus* and their ability to activate the innate immune responses. However, the interaction of *S. aureus* with anti-microbial peptides is only partially understood.

In this review article, we briefly describe two major anti-microbial peptides, defensins and cathelicidin, and summarize the interaction of anti-microbial peptides with *S. aureus*, including MRSA, based on previous reports and our recent findings.

S. aureus develops resistance to chemotherapeutic agents; the difference between MRSA and MSSA

Chemotherapy is generally used against many bacterial infectious diseases including *S. aureus* infections. Chemotherapeutic agents (antibiotics) such as β-lactams, aminoglycosides, and quinolones are used against *S. aureus* infections; in particular, β-lactams have been used for many decades. The emergence of β-lactam-resistant strains makes it a problem to cure *S. aureus* infectious. Initially, *S. aureus* became resistant to β-lactams by acquiring the β-lactamase (penicillinase) gene by horizontal transfer [22–24]. To eliminate the resistant strains, modified β-lactams resistant to β-lactamase, such as methicillin and oxacillin, were developed. Subsequently, through the use of these antibiotics, MRSA strains have emerged [20,21]. The key molecule conferring this resistance against the modified β-lactams is a penicillin-binding-protein (PBP) 2′ (2A) [25,26]. *S. aureus* generally possesses four PBPs. In susceptible strains, they are inactivated in the presence of β-lactams, leading to cell death due to inhibition of cell wall biosynthesis. MRSA survives in the presence of β-lactams because an extra PBP2′ specific for MRSA has low affinity for β-lactams and the PBP2′ retains its activity in the presence of β-lactams, and continues cell wall synthesis. The glycopeptides vancomycin and teicoplanin are powerful agents with significant activity in MRSA infections. Vancomycin binds to the peptidoglycan precursor and then inhibits the incorporation of newly synthesized precursors in the cell wall peptidoglycan, thus causing inhibition of cell wall synthesis. Recently, vancomycin-intermediate susceptible *S. aureus* (VISA), or glycopeptide-intermediate susceptible *S. aureus* (GISA), emerged [27–31], followed in the USA by a vancomycin-resistant *S. aureus* (VRSA) [32,33]. The mechanism of vancomycin resistance in the VRSA is due to expression of the *vanA* gene, which modifies the structure of the peptidoglycan, causing a loss of affinity of vancomycin for the peptidoglycan precursor [34]. The mechanism of low susceptibility in VISA or GISA strains is reported to be due to the thickened cell wall that traps vancomycin [30], but the underlying genetic mechanism(s) responsible for this low susceptibility remains unknown. Therefore, because *S. aureus* adapts to new chemotherapeutics, curing MRSA infections will become difficult in the near future and new approaches to therapy will be required.

The difference between MRSA and methicillin-susceptible *S. aureus* (MSSA) is due to the presence or absence of the *mec* region including the *mecA* gene coding for PBP2′. The *mec* region is composed of mobile genetic elements that are called SCC*mec* cassettes. To date, five types of SCC*mec*s have been identified [35–38]. These cassettes have a variable size of about 20–70 kbp and contain *mecA*,

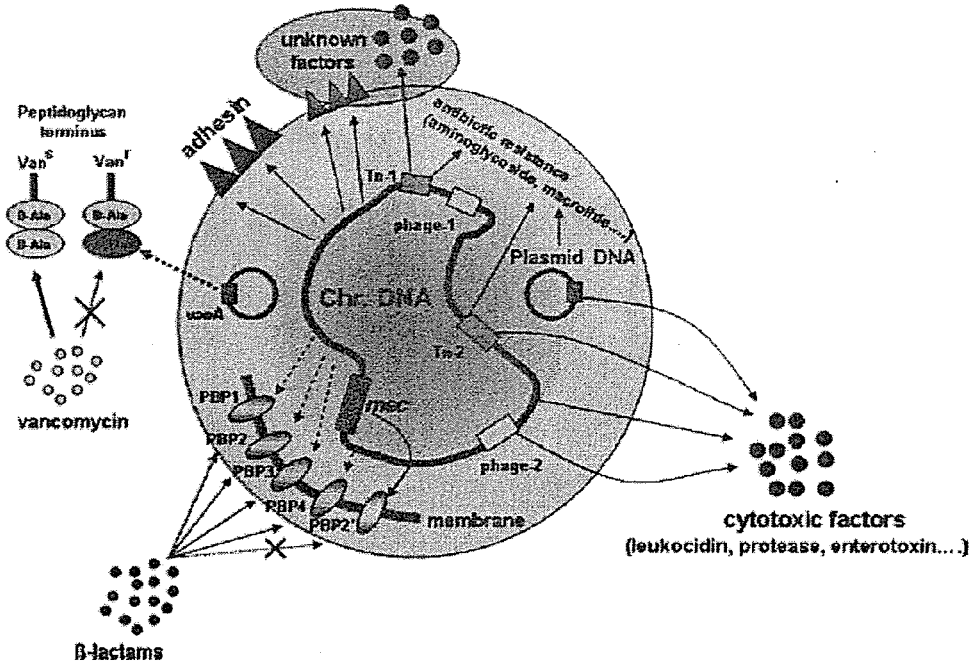

Figure 2. The evolution of *S. aureus* by acquisition of several factors by plasmid or phage. MRSA strains have emerged by acquiring the *mecA* gene within the *mec* region, which codes for PBP2′, a low affinity PBP to β-lactams. Also, other virulence factors, such as antibiotic resistance, cytotoxic factors, and adhesins, have been transferred by the mobile genetic elements in *S. aureus* including MSSA and MRSA strains. Van^s = vancomycin-sensitive; Van^r = vancomycin-resistant; Ala = alanine; Lac = lactate; Tn = transposon

ccrA (recombinase A), *ccrB* (recombinase B), and other *orf*s of unknown function. Except for methicillin resistance, clinical isolates of *S. aureus* including MRSA and MSSA strains show variable biological characteristics, ie variable production of virulence factors, the ability to infect humans, and resistance to antibiotics. This indicates that biological differences among strains are independent of the MRSA or MSSA mechanism. These variable biological characteristics in *S. aureus* strains contribute to the microbial intra-cellular and/or extra-cellular change. To possess the virulence factors and/or antibiotic resistance, *S. aureus* has acquired multiple extra-cellular genetic factors via phages or plasmids (Figure 2). Antibiotic resistance in MRSA has evolved to multiple drug resistances against chemotherapies. This implies that MRSA strains acquire extra factors at high frequency compared with the MSSA strains.

Human anti-microbial peptides

Mammalian cells, including human cells, produce several anti-microbial peptides, each of which plays a significant role in innate immunity. These peptides kill or inactivate bacteria, fungi, or viruses, although the spectrum of their activity varies. Recently, some of these peptides were shown to have potent chemokine activity. Many anti-microbial peptides have been demonstrated in various tissues and organs. In this review article, we focus on two major anti-microbial peptides, defensins and LL37/CAP18.

Defensins and LL37/CAP18

Defensins

Defensins are well-known cysteine-rich peptides and are classified into three types: α-(human neutrophil peptide, HNP), β-(hBD), and θ-defensins [39–43]. α- and β-defensins are found in humans, while θ-defensin is not, although a human orthologue of this defensin has been described [44]. The defensins are cysteine-rich peptides that have six cysteine residues with three disulphide bonds (Table 1). α- and β-defensins differ in their location, the length of amino acid residues, and the pairing of disulphide bonds. Recently, these peptides were found to have not only anti-microbial activity, but also chemokine-like activity [43,45]. The characteristics of both defensins are summarized below.

α-defensin To date, six HNPs have been identified [39,40,46–48]. The disulphide bonds of intramolecular cysteine residues in the HNPs are 1–6, 2–4, and 3–5 linked, and consist of an intricate tertiary structure. The way that the molecule is folded is associated with its bactericidal activity and protection against degradation by proteolysis during biosynthesis and from proteases in its micro-environment. HNPs 1–4 are largely stored in the granules of neutrophils, and HNPs 5 and 6 are stored in Paneth cells. When the cells are induced to degranulate, the peptides are released. HNPs demonstrate a broad spectrum of anti-microbial activity against Gram-positive and Gram-negative bacteria, fungi, and viruses [39,40,49]. The

Table 1. Human defensins and cathelicidin

α-defensins	
HNP1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP2	CYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP3	DCYCRIPACIAGERRYGTCIYQGRLWAFCC
HNP4	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRV
HNP5	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR
HNP6	FTCHCRR-SCYSTEYSYGTCTVMGINHRFCCL
	-C-C----C----------C----------CC
β-defensins	
hBD1	GLGHRSDHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK
hBD2	GIGDPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
hBD3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
hBD4	EFELDRICGYGTARC-RKKCRSQEYRIGRCP-NTYACCLRKWDESLLNRTKP
	-------C--------C----C---------C--------CC-----
Cathelicidin	
LL37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

anti-microbial activity of these peptides decreases in the presence of NaCl [39,40]. Recently, α-defensins were reported to have an anti-HIV effect by inhibiting HIV replication, suggesting their clinical use as preventive and therapeutic agents [41,50–52]. Other investigations for clinical use against microbial infection have been reported using animal models [53–55].

Besides the antibacterial activity of HNPs, other biological functions such as chemotactic activity to native resting T cells and dendritic cells, degranulation of mast cells, regulation of complement activation, and inhibition of glucocorticoid production have been implicated [45,47,56–59].

β-defensin Four β-defensins (hBD1–4) have been identified and found to be expressed in various epithelial tissues: the trachea, skin, and intestine; and in monocytes and dendritic cells [8,60–69]. Like α-defensins, hBDs have three disulphide moieties of cysteine linking residues 1–5, 2–4, and 3–6. hBD1 was first identified in haemodialysate fluid [70] and then from urogenital tissues, skin, intestine, and other tissues [61–69,71]. *In vitro* experiments show that hBD1 is constitutively expressed and that its production is not influenced by bacterial exposure, while other hBDs are inducible when exposed to bacteria [67,72–75]. In particular, hBD2, first identified from skin [8], is highly responsive to bacteria, pro-inflammatory stimuli (IL–1β, TNFα, LPS), and phorbol myristate acetate [63,72,74,76–79]. Previous reports show that hBD1 and hBD2 have strong antibacterial activity against Gram-negative bacteria and weak (or no) activity against Gram-positive bacteria, while hBD3 and hBD4 have strong activity against both Gram-positive and Gram-negative bacteria [8,41,50,61,66,80–83]. hBD1 is primarily found in differentiated keratinocytes, suggesting that the regulation of hBD1 is linked to cell differentiation [84,85]. Other hBDs are implicated in cell differentiation [72,85]. Compared with hBD1 and hBD2, hBD3 has strong activity against Gram-positive bacteria including *S. aureus* and Gram-negative bacteria, due to a strong net charge causing affinity for the bacteria [82].

As with the α-defensins, β-defensins have variable biological activities. They include being a chemotactic factor for CCR6 dendritic cells, degranulation of mast cells, and induction of the production of prostaglandin D2 [45,47,86,87].

Cathelicidin The cathelicidin family contains highly conserved regions known as cathelin (cathepsin L inhibitor) at the N-terminus, and in the variable region at the C-terminus, that code for the mature peptide, which has anti-microbial activity. Only one cathelicidin is found in humans: this was described in human bone marrow as FALL-39 [88] and was later termed LL37/hCAP18 [89,90]. LL37/hCAP18 is initially synthesized as an 18 kD protein; then during and after secretion, it is processed to the mature 5 kD peptide (37 amino acid residues) [90,91]. LL37/hCAP18 has been identified in neutrophils, monocytes, various epithelial cells, saliva, sweat glands, and other tissues [90–96]. The peptide has a linear form, resembling an alpha helical structure. LL37 has broad spectrum activity against Gram-positive and Gram-negative bacteria including antibiotic-resistant *Staphylococcus aureus, Pseudomonas aeruginosa*, and fungi [74,90,91,97,98]. In addition to bactericidal activity, LL37 is able to bind to LPS and neutralize its endotoxin activity [89,99,100], and acts as a chemotactic factor for neutrophils, monocytes, T cells, and mast cells [90,101,102].

Mechanism of action of anti-microbial peptides

Both defensins and LL37/CAP18 have broad anti-microbial activity against Gram-positive and Gram-negative bacteria, fungi, and viruses, although some

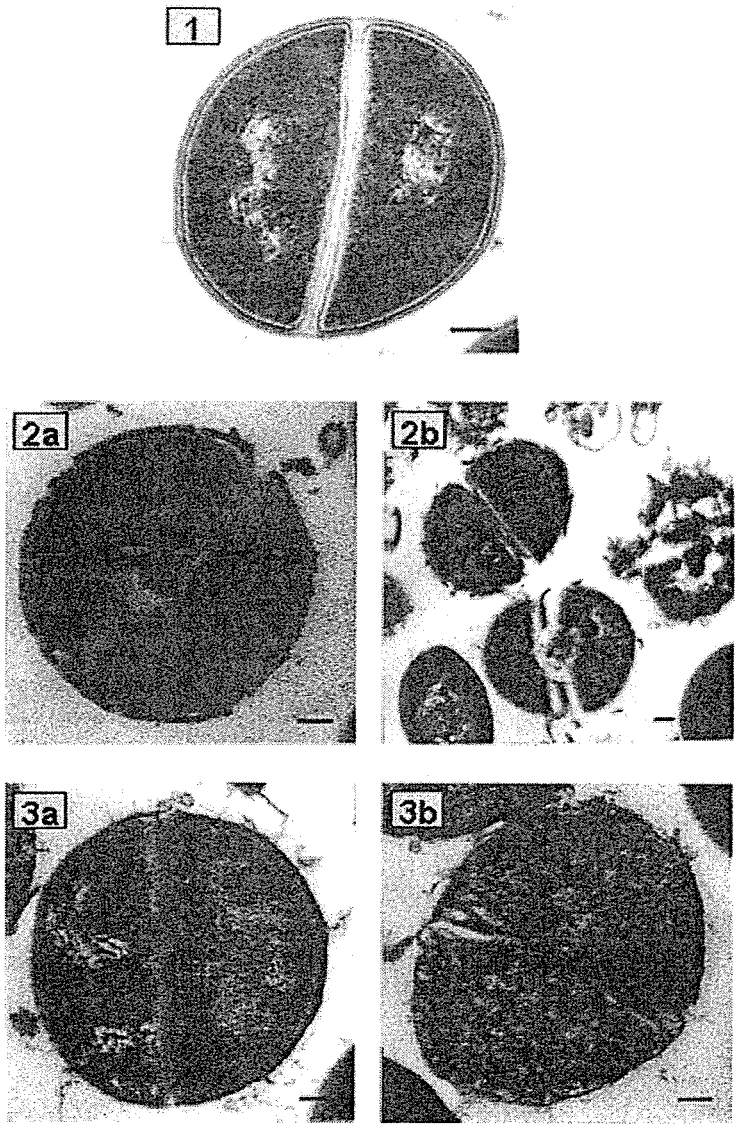

Figure 3. Thin sections of *S. aureus* 2PF-18 exposed to anti-microbial peptides. *S. aureus* cells were reacted without peptide (1) or with 200 μg/ml hBD3 (2a, 2b) or CAP18 (3a, 3b), respectively. Bars = 100 nm. Reprinted from Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, *et al. Staphylococcus aureus* susceptibility to innate antimicrobial peptides, β-defensins and CAP18, expressed by human keratinocytes. *Infect Immun* 2003;**71**:3730–3739, with permission from the American Society for Microbiology

of these peptides have weak activity against Gram-positive bacteria. The anti-microbial peptides are cationic polypeptides and they bind electrostatically to negatively charged bacterial surface molecules: LPS in Gram-negative bacteria, lipoteichoic acid and teichoic acid in Gram-positive bacteria, and the anionic cell membrane (phospholipids). After attracting the peptides to the bacterial cell surface, the membrane is made permeable. This involves pore or gap formation by aggregation of the peptides in the membrane, resulting in bacterial cell death [83]. Figure 3 shows a thin section of *S. aureus* cells treated with the synthetic peptides hBD3 or LL37/hCAP18. Treatment with the anti-microbial peptides causes perforation of the peripheral cell wall, leading to release of the cytoplasmic contents. Vesicle-like molecules are found near the cell surface of dead bacterial cells. In addition, some reports show that the induction of autolytic enzymes by anti-microbial peptides may be associated with bacterial cell death [103].

Regulation of anti-microbial peptide expression

Bacterial exposure induces β-defensin expression in several epithelial cell types. The bacteria or bacterial component, ie lipopolysaccharide and peptidoglycan, causes this induction. α-defensins are constitutively expressed, accumulating in granules [43,63,64,72]. Therefore, the α-defensins are not regulated at the transcriptional level but at the post-translational level when induction occurs to mobilize them within cells. Enteric α-defensin in Paneth cells is secreted by stimulation with microbial antigens [104]. NK cells secrete

HNP1–3 induced by bacterial antigens such as OmpA and flagellin [105]. In contrast to α-defensins, the expression of β-defensins is inducible at the transcriptional level in many tissues. In airway epithelia, hBD2 expression is up-regulated by infectious and inflammatory stimuli *in vitro* and *in vivo*. IL-1β, TNFα, LPS, and micro-organisms (Gram-positive and Gram-negative bacteria, and *Candida albicans*) can induce hBD2 expression. This up-regulation is mediated by NF-κB [72,106]. Furthermore, there is a predicted NF-κB binding site upstream of the *hBD2* gene. Therefore, the NF-κB pathway is important for hBD2 transcription [106]. In contrast, oral gingival keratinocytes exposed to the commensal bacterium *Fusobacterium nucleatum* in the oral cavity induced hBD2 expression not by NF-κB, but by using the MAP kinase pathway [107,108]. This suggests that the expression pathway leading to hBD2 production is variable among tissues.

For LL37/CAP18, the upstream region of the LL37/CAP18 gene contains binding sites for NF-κB, nuclear factor for IL 6, acute-phase response regulator, and CCAAT/enhancer binding protein [90], suggesting that several signals such as cytokines and bacterial stimuli are involved in *LL37/CAP18* gene expression.

In addition to bacterial components, several cytokines have been reported to induce anti-microbial peptides. In keratinocytes, IL-1β, IFNγ, or TNFα induced hBD2 and hBD3 expression [64,71,72,77]. Furthermore, it has been demonstrated that the expression of anti-microbial peptides influences cell differentiation [85,109].

Generally, Toll-like receptors (TLRs), as pattern recognition molecules, are considered to be the receptors that activate internal cell signalling pathways. Many TLRs (TLR1–11) have been identified and the molecules that bind each TLR are known, eg LPS against TLR4 and peptidoglycan against TLR2 [110–112]. In airway epithelia, TLR2 regulates hBD2 expression [113,114]. Lipoteichoic acid (LTA), a component of the cell wall in Gram-positive bacteria, induces TLR2-mediated hBD2 up-regulation through the NF-κB signalling pathway [106]. The intra-cellular pattern recognition protein (NOD2) binds to muramyl dipeptide, a component of peptidoglycan that signals expression of hBD2 through the NF-κB pathway [115,116]. Therefore, several pattern recognition proteins localized on the cell surface or intra-cellularly are associated with the expression of anti-microbial peptides.

Interaction of anti-microbial peptides with *S. aureus*

Expression of anti-microbial peptides in response to *S. aureus* infection

hBD2–4 and LL37/CAP18 are induced by bacteria, bacterial components (LPS and peptidoglycan), and some cytokines; however, there are few reports showing induction by *S. aureus* cells. Dinulos *et al* demonstrated that in keratinocytes from human neonatal skin, hBD2 expression is induced by bacterial contact including *S. aureus, E. coli*, and *P. aeruginosa*, while *S. pyogenes* induced expression poorly [73]. Harder *et al* reported that hBD3 is slightly induced in keratinocytes by *S. aureus* [67]. Because the total analysis of expression of anti-microbial peptides has not been performed in keratinocytes, we investigated the expression of hBD1–3 and LL37 in keratinocytes derived from skin exposed to *S. aureus* cells [74]. *S. aureus* cells (inactivated at 60 °C) were added to the cell culture at a final concentration of 10^8 per ml. The transcripts of hBD2, hBD3, and LL37 were induced at 4 h, and then gradually increasing to 12 h. hBD2 production was significantly increased after 12 h of bacterial exposure, where the transcription of hBD2 was more than ten-fold higher than shown in cells prior to bacterial contact, whereas hBD1 expression was not altered by *S. aureus* contact. The inflammatory cytokines IL-6, IL-1β, and TNFα were also produced in keratinocytes after 4 h of bacterial exposure. These cytokines can stimulate the expression of anti-microbial peptides; this indicates that direct contact with *S. aureus* cells and cytokines induces the expression of anti-microbial peptides in human keratinocytes derived from skin.

Figure 4 shows normal human epidermis immunostained with anti-sera against hBD1–3 or LL37. The peptides are expressed in the upper epidermis (the corneum and granulosa). LL37 is highly detectable in granulocytes during wound repair, in inflammatory skin disorders such as psoriasis, and nickel allergy epidermis [93]. hBD2 is found in chronic wounds of the skin [4,117]. The interaction of *S. aureus* with its host *in vivo* is supported by the presence of high concentrations of HNP1–3 and hBD2 in the nasal cavity of *S. aureus* carriers [118]. The concentration of HNP1–3 was 40-fold higher in the carrier than in non-carrier donors: hBD2 was almost absent in non-carriers, while hBD2 was easily observed in carriers. This suggests that *S. aureus* can induce the expression of some anti-microbial peptides *in vivo*.

Is there a difference in the expression levels of anti-microbial peptides between MSSA and MRSA?

Several clinical isolates of MSSA and MRSA strains were used to examine the expression of anti-microbial peptides in keratinocytes [74]. The expression levels of hBD2, hBD3, and LL37 were different among the strains, although all strains induced production (Figure 5). By contrast, hBD1 expression was not altered by *S. aureus* contact. All strains could induce significant production of hBD2 (>10-fold) and slight production of hBD3 (<10-fold). Although we found a difference in the expression of the peptides among strains, there was no difference between MRSA and MSSA strains. These results indicate that keratinocytes

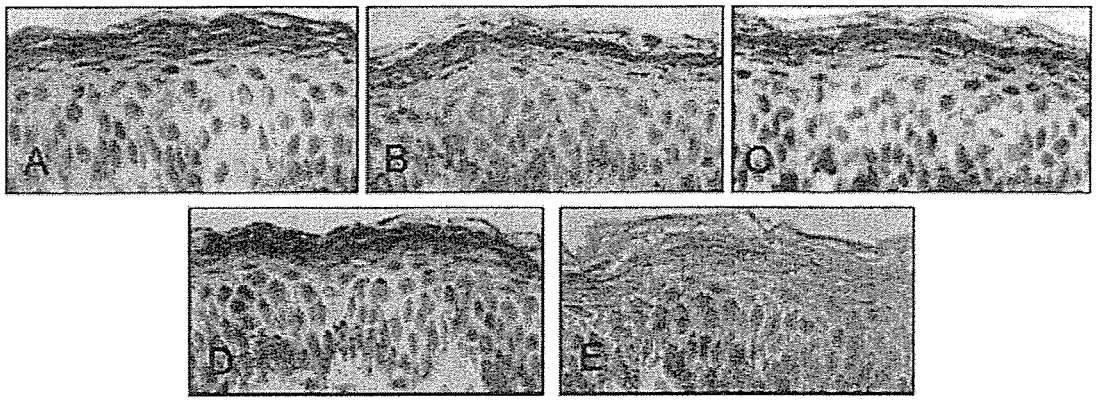

Figure 4. Expression of hBD1–3 and LL37 in normal human epidermis. Frozen sections of normal human skin were reacted with anti-hBD1 (A), anti-hBD2 (B), anti-hBD3 (C) anti-LL37 (D), and pre-immune rabbit serum (E). Anti-rabbit IgG conjugated with streptavidin–biotin–peroxidase was used as a secondary antibody and visualized with AEC. The nucleus was counterstained with haematoxylin. Reprinted from Sayama K, Komatsuzawa H, Yamasaki K, Shirakata Y, Hanakawa Y, Ouhara K, *et al.* New mechanisms of skin innate immunity: ASK1-mediated keratinocyte differentiation regulates the expression of beta-defensins, LL37, and TLR2. *Eur J Immunol* 2005;**35**:1886–1895, with permission from Wiley InterScience

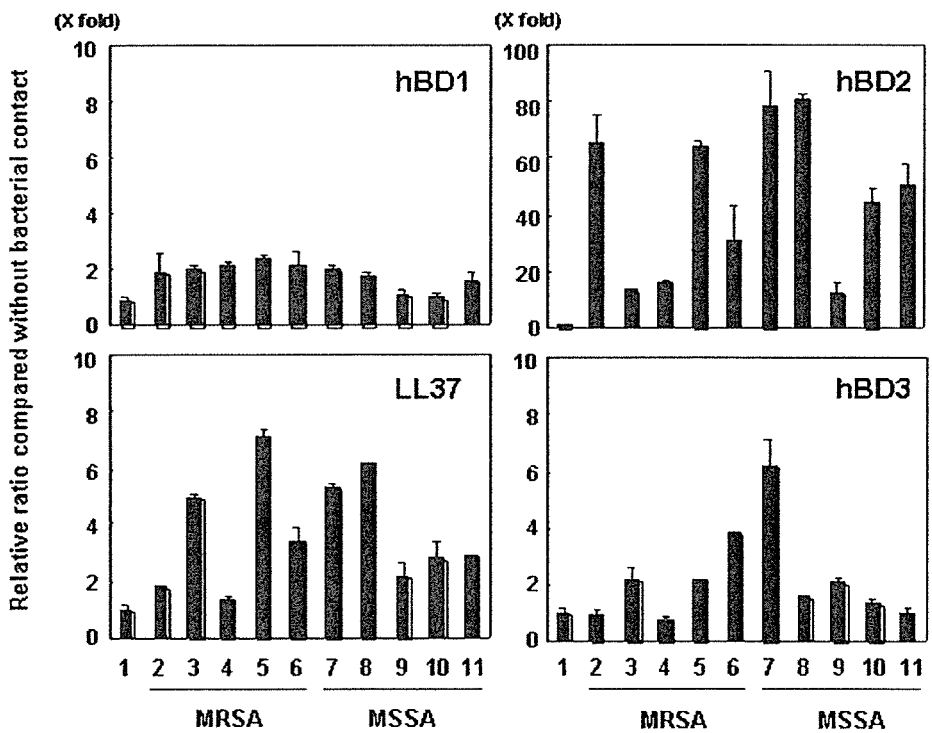

Figure 5. Expression of *hBD1-*, *hBD2-*, *hBD3-*, and *CAP18*-mRNA by human keratinocytes in contact with heat-inactivated, clinically isolated *S. aureus* cells. Total RNA was extracted from cells that had been in contact with heat-inactivated *S. aureus* for 8 h, and used for real-time PCR. Lane 1: no bacterial contact; lanes 2–6: MRSA strains; lanes 7–12: MSSA strains. Reprinted from Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, *et al. Staphylococcus aureus* susceptibility to innate antimicrobial peptides, β-defensins and CAP18, expressed by human keratinocytes. *Infect Immun* 2003;**71**:3730–3739, with permission from the American Society for Microbiology

induce the expression of anti-microbial peptides by any *S. aureus* strain. However, we need to know which bacterial molecules induce keratinocytes to produce anti-microbial peptides. Our study demonstrates that fibronectin-binding protein, a major adhesin in *S. aureus*, may play a central role in the expression of anti-microbial peptides (unpublished data). Additionally, high levels of capsule production reduced expression, possibly due to masking of fibronectin-binding proteins (unpublished data). Variable production of anti-microbial peptides among strains may be associated with these factors.

Anti-*S. aureus* activity of anti-microbial peptides

Since *S. aureus* induces the expression of some anti-microbial peptides; we need to know which peptides are active against *S. aureus*. Many reports show that these molecules produce anti-microbial activity, including the HNPs and hBDs. HNP1–3, hBD3, and LL37 have antibacterial activity against *S. aureus*, while hBD2 and hBD1 have no or weak activity against Gram-positive bacteria including *S. aureus* [39,119–123]. Since we had demonstrated the expression of hBD1–3 and LL37 in keratinocytes, we synthesized the four anti-microbial peptides in their mature forms, purified them, and then evaluated antibacterial activity against *S. aureus* [74]. Generally, two antibacterial assays were used for evaluation [67,74,102,119,124]. One was the minimum inhibitory concentrations (MICs) under growing conditions and the other was to measure the killing rate (%) under non-growing conditions using 10 mM phosphate buffer. We used the killing rate because the antibacterial effect was observed at low concentrations of the peptides (1–10 μg/ml). Because the concentrations expressed *in vivo* are from 1 to 100 μg/ml [61,71,77,125], the killing rate *in vitro* is relatively similar to the amount that is expressed *in vivo*. The antibacterial assay showed that all synthetic peptides of hBD1–3 and LL37 have dose-dependent antibacterial activity against *S. aureus*, although the effect was different among peptides. Thus, the antibacterial activity of hBD3 and LL37 was higher than that of the other two peptides, where the activity of hBD1 was weak. One microgram per ml of hBD3 and LL37 showed 70–80% killing of *S. aureus* cells. In the presence of NaCl, all of the peptides, especially hBD1 and hBD2, decreased in their activities.

Difference in susceptibility to anti-microbial peptides among *S. aureus* strains

We believe that anti-microbial peptides have a broad spectrum of activity against bacteria, fungi, and viruses, and may not induce the emergence of resistant bacteria. Since there are no reports showing the susceptibility of a large number of *S. aureus* strains to anti-microbial peptides, we tested the susceptibility to hBD3 (1 μg/ml) and LL37 (1 μg/ml) against 44 *S. aureus* clinical isolates including MRSA strains (Figure 6). The activity of these two peptides was variable among the strains, showing high, intermediate, and low susceptibility. However, the low-susceptibility strains were susceptible to high concentrations of the peptides (5 μg/ml), indicating that no *S. aureus* strains are resistant to the anti-microbial peptides. There was no correlation between susceptibilities to hBD3 and LL37 in the respective strains.

Interestingly, the proportion of strains that exhibited low susceptibility to anti-microbial peptides was higher for MRSA strains than for MSSA strains. Among low-susceptibility MRSA strains, most strains were highly resistant to methicillin (MIC of methicillin >512 μg/ml). We further investigated the activity of these two peptides against more than 500 clinical isolates and confirmed this tendency. Among the

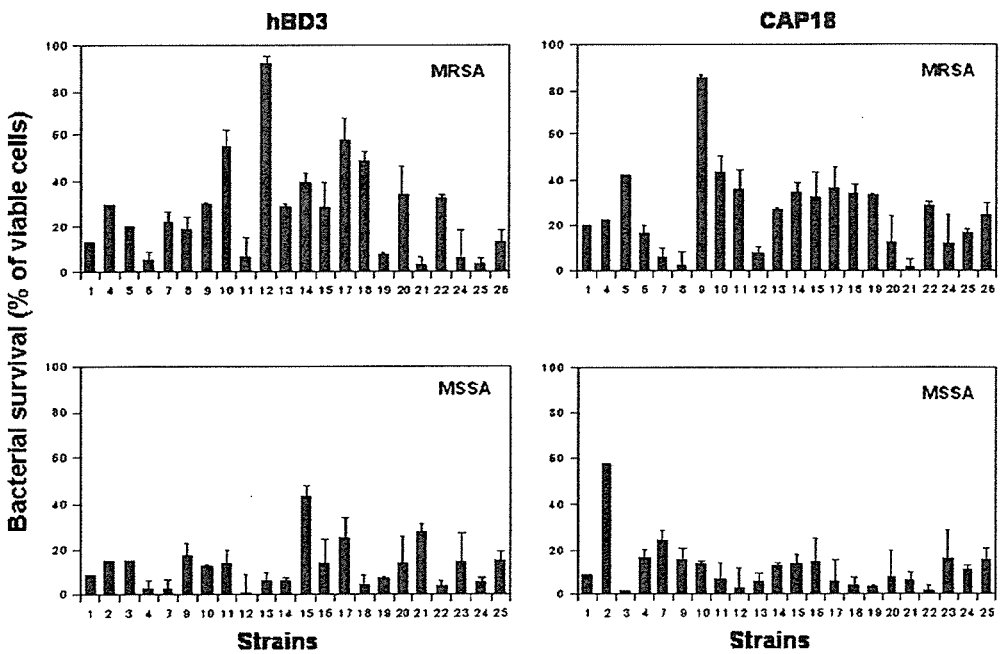

Figure 6. Antibacterial activity of hBD3 and CAP18 on *S. aureus* clinical isolates. The susceptibility of 22 MSSA and 22 MRSA strains to hBD3 (1 μg/ml) and CAP18 (0.5 μg/ml) was analysed using the method described by Midorikawa *et al.* [74]. The bacterial survival as % of viable cells is shown. The data are means ± SD from three independent experiments. Reprinted from Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, *et al. Staphylococcus aureus* susceptibility to innate antimicrobial peptides, β-defensins and CAP18, expressed by human keratinocytes. *Infect Immun* 2003;**71**:3730–3739, with permission from [publisher's name]

MRSA strains, especially highly methicillin-resistant strains, the proportions of strains that exhibited low susceptibility to anti-microbial peptides were higher than those of MSSA strains (unpublished data). These results imply that highly methicillin-resistant *S. aureus* is resistant not only to chemotherapy, but also to anti-microbial peptides.

The mechanism of susceptibility to these peptides was examined among strains. Three factors were identified to affect susceptibility to anti-microbial peptides. Inactivation of the gene *dlt*, which codes for the enzyme mediating the incorporation of D-alanine into teichoic acid, a major cell wall component, resulted in an increase in the negative charge of the cell surface, causing increased susceptibility to these peptides [126]. Inactivation of the *mprF/fmtC* gene that codes for the enzyme mediating the incorporation of lysine into phosphatidylglycerol, a major component of the bacterial membrane, gave a more negative charge to the cell membrane. Both mechanisms increased susceptibility to the peptides [124,127]. Staphylokinase inhibits the antibacterial activity of the α-defensins by reducing the binding affinity of these peptides [128]. Therefore, it is possible that up- or down-regulation of these factors in *S. aureus* may influence the susceptibilities. However, these factors are widely expressed among strains and are not particular to either MRSA or MSSA strains. Therefore, factors other than those described here may be involved.

Conclusion

Anti-microbial peptides involved in the innate immune system are functionally active against *S. aureus* infections as well as other micro-organisms. However, variable induction of activity and variable susceptibility among *S. aureus* strains imply that each *S. aureus* strain shows a variable response to the innate immune system. The ability of *S. aureus* to infect depends on many factors such as adhesion activity, production of cytotoxic factor(s), and defence against the immune system. When we compared MSSA with MRSA strains, the MRSA strains showed lower susceptibility to anti-microbial peptides, indicating that some MRSA strains are resistant not only to various antibiotics including methicillin, but also to some of the anti-microbial peptides. We do not know if the different susceptibilities to these peptides affect *in vivo* infection in the host because many factors are involved in host defence, including the innate immune system. Previous reports indicate the importance of these peptides for protection against microbial infections. Experiments using knockout mice lacking CRAMP or mouse β-defensin-1 revealed that these peptides were important for protection against bacterial infection. Therefore, this suggests that variable responses to anti-microbial peptides may affect the outcome of *S. aureus* infections in the host. Because there were no strains showing complete resistance to these peptides, and *in vivo* experiments using animal models against some pathogenic bacteria have shown promising results, we suggest that anti-microbial peptides could be potential candidates for further chemotherapeutic agents against *S. aureus* infection including MRSA.

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Enzyme-linked immunosorbent assay using bacterial recombinant proteins of human BP230 as a diagnostic tool for bullous pemphigoid

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Summary

Background: By immunoblot analyses of normal human epidermal extracts, the 230 kDa bullous pemphigoid antigen (BP230) is recognized by most bullous pemphigoid (BP) sera. We produced different recombinant glutathione-S-transferase-fusion proteins, which roughly presented N-terminal domain, central rod domain and C-terminal domain of human BP230.

Objective: In the present study, we developed an enzyme-linked immunosorbent assay (ELISA) using the recombinant proteins for detection of anti-BP230 IgG antibodies and assessed the usefulness of this assay in conjunction with an anti-BP180 ELISA to establish the diagnosis of BP.

Methods: Using the bacterial recombinant proteins of N-terminal and C-terminal domains, we developed an ELISA. A receiver-operating-characteristic (ROC) analysis was performed to determine a cut-off value for the BP230 ELISA.

Results: By this BP230 ELISA, 173 (72.4%) of 239 BP sera were positive, while only one (1.1%) of 94 sera from pemphigus vulgaris and pemphigus foliaceus patients was positive and all the 109 normal control sera were negative. Thus, the sensitivity and specificity of the BP230 ELISA were 72.4 and 99.5%, respectively. Interestingly, while 54 (84.4%) of 64 BP sera in active stage and 113 (64.6%) of 175 BP sera in remission

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were positive in BP180 ELISA, 37 (57.8%) of 64 BP sera in active stage and 136 (77.7%) of 175 BP sera in remission were positive in BP230 ELISA. These results indicate that the titer of anti-BP230 antibodies is not related with disease activity in some BP cases. Most significantly, by combining the results of BP230 ELISA and BP180 ELISA, 232 (97.1%) of 239 BP sera were positive.

Conclusion: The combination of BP230 ELISA and BP180 ELISA is the highly sensitive method for the diagnosis of BP.

1. Introduction

Bullous pemphigoid (BP) is an autoimmune subepidermal blistering disease characterized by the presence of IgG autoantibodies to the epidermal basement membrane zone in the sera. The circulating IgG antibodies in BP sera react with two major BP antigens present in hemidesmosomes [1–3], a 230 kDa intracellular protein termed BP230 (or BPAG1) that is localized to the inner plaque of hemidesmosomes [4–9], and a 180 kDa transmembrane protein termed BP180 (or BPAG2) that shows type II orientation with a long C-terminal extracellular component composed of 15 discontinuous collagenous domains separated by 16 non-collagenous domains [10–15].

BP230 is a member of plakin family protein [16,17], and is considered to play an important role in the stable structure of hemidesmosomes. N-terminal and C-terminal domains of BP230 are considered to be important in interactions with desmosomal transmembrane proteins and with keratin intermediate filaments, respectively, whereas the central alpha-helical coiled-coil domain apparently plays a role in its structural stability and the self-assembly [16].

It is now well known that circulating antibodies in BP sera react with epitopes that are tightly clustered in the 16th non-collagenous (NC16a) domain of BP180 extracellular domain, close to the cell membrane of the basal keratinocytes [12,13,18–21]. These antibodies were later shown to be pathogenic by a newborn mouse animal model [22,23].

In the previous studies, we developed an enzyme-linked immunosorbent assay (ELISA) using bacterial recombinant protein of BP180 NC16a domain, and evaluated its clinical benefit for diagnosis and monitoring disease activity [24,25]. The sensitivity of the ELISA was determined to be 84.4% and the specificity was 98.8%. The index values of the BP180 ELISA tended to fluctuate in parallel with the disease activity along the time course of BP patients, and reflected the disease activity much better than indirect immunofluorescence.

However, although BP230 was found as a BP antigen earlier, the role of anti-BP230 antibodies in the pathogenesis of BP is still unclear. This is mainly because BP230 is an intracytoplasmic protein and the autoantibodies are not considered to access to BP230 in the intact keratinocytes.

The earlier studies using various recombinant proteins and synthetic peptides suggested that BP sera react preferentially with C-terminal domains of BP230 [26–30]. A recent study using eukaryotic expression system showed that multiple epitopes in various domains of BP230 are recognized by BP sera [31]. However, the results of these previous studies are not convincing enough, because none of these studies examined the reactivity of BP sera with the entire molecule of BP230. In addition, the eukaryotic expression system can produce larger recombinant proteins, but can yield only a little amount of the proteins. Therefore, the background reactivity in immunoblotting using the lysates of transfected eukaryotic cells was usually very high, which made the results a little obscure.

In the previous study, as the first step to further characterize the role of anti-BP230 antibodies in the pathogenesis of BP, we prepared three bacterial recombinant fusion proteins that cover the entire molecule of BP230 [32]. These recombinant proteins roughly corresponded to N-terminal domain (BP230-N), middle rod domain (BP230-M) and C-terminal domain (BP230-C). We examined the reactivity of BP sera with the three recombinant proteins by immunoblotting, and found that BP sera reacted specifically with the BP230 recombinant proteins, particularly the C-terminal domain.

In the present study, using the recombinant proteins, we developed an ELISA for BP230, and showed that the ELISA is a very useful tool in diagnosis of BP.

2. Materials and methods

2.1. Sera

We collected 239 BP sera; 64 sera from 64 BP patients in active stage and 175 sera from 41 BP patients in remission. We also used the sera from 37 patients with pemphigus foliaceus, 57 sera from patients with pemphigus vulgaris and 109 normal

control sera. All the sera were stored at −30 °C or at 4 °C in the presence of 0.1% NaN_3. The diagnoses of BP, pemphigus vulgaris and pemphigus foliaceus were made by the typical clinical and histopathological features, as well as positive reactivity in both direct and indirect immunofluorescence.

All the BP patients showed typical clinical and histopathologic features and their sera exhibited IgG anti-epidermal basement membrane zone antibodies with titers ranging from 40 to 5120 by indirect immunofluorescence of normal human skin sections and/or 1 M NaCl split skin sections [33]. Pemphigus foliaceus and pemphigus vulgaris sera showed IgG anti-cell surface antibodies at titers ranging from 40 to 2560 by immunofluorescence of normal human skin sections. Normal control sera did not show any specific reactivity on immunofluorescence.

To confirm the position of recombinant fusion proteins with GST in immunoblotting, we used anti-GST mouse monoclonal antibody (MBL, Nagoya, Japan) and horse radish peroxidase-conjugated anti-mouse IgG polyclonal antibodies (MBL).

2.2. Preparation of GST-fusion proteins of BP230

The bacterial expression constructs of three different domains of human BP230 were generated as reported previously [32], except that His-tag was further introduced in the C-terminus of each construct. Preliminary experiments to generate GST, His-fusion proteins of human BP230 showed that N-terminal domain (BP230-N) was soluble in 2% Triton X-100 in phosphate-buffered saline (PBS), when the protein was induced at a lower temperature in the bacteria (*E. coli* strain, BL21 codon plus RIL). However, the middle rod domain (BP230-M) and C-terminal domain (BP230-C) were not soluble in this buffer.

Therefore, the three proteins were prepared by different methods. The bacteria transfected with plasmid of BP230-N were grown in LB medium at 37 °C, until OD600 reached to 0.6. Then, IPTG was added to a final concentration of 0.5 mM to induce expression of fusion protein. After an additional culture at 20 °C overnight, cells were harvested by centrifugation. Pellets were resuspended in 2% Triton X-100, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A in phosphate-buffered saline, mildly sonicated and clarified by centrifugation. Then, soluble BP230-N was first purified on a glutathione sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden). The eluted protein was further purified on a TALON column, washed with 10 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin A in PBS, and eluted with 500 mM imidazole, 10 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin A in PBS. Final eluate was dialyzed with 8 M urea in PBS.

The bacteria transfected with plasmids of BP230-M and BP230-C were grown in LB medium at 37 °C, until OD600 reached to 0.6. Then, IPTG was added to a final concentration of 1.0 mM to induce expression of fusion protein. After an additional culture at 37 °C for 2 h, cells were harvested by centrifugation. Pellets were resuspended in 2% Triton X-100, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A in PBS, mildly sonicated and clarified by centrifugation. Then, insoluble fraction was boiled in sample buffer and fractionated on SDS-PAGE. BP230-M and BP230-C were purified by eluting from the gels.

In the initial experiments, we developed separate ELISA plates for BP230-N, BP230-M and BP230-C. There are three reasons why we convert to a BP230 ELSIA that combined BP230-N and BP230-C. The first reason was that some of the BP180 negative sera showed a positive reactivity in either BP230-N ELISA or BP230-C ELISA. However, there were no BP180 negative sera which showed positive reactivity in BP230-M ELISA. Second reason was that we could obtain BP230-N with high purity through the double columns. The third reason was that the BP230-C was most frequently reacted by BP sera in our previous study [32]. From these reasons, we decided to purify the recombinant proteins of BP230-N and BP230-C, but not BP230-M.

2.3. SDS-PAGE and immunoblot analysis

Recombinant proteins, BP230-N and BP230-C, were separated by SDS-PAGE using 10% gel. The fractionated proteins were visualized by Coomassie-brilliant blue staining. For immunoblotting, the two recombinant proteins were transferred onto nitrocellulose membranes [34,35]. Blots were incubated with blocking solution BlockAce (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan) for 1 h at room temperature. The diluted anti-GST monoclonal antibody (1:5 dilution) was used as the first antibody. After three 5 min rinses with washing solution (0.05% Tween-20 in phosphate-buffered saline), blots were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (1:1000 dilution) as a secondary antibody for 1 h at room temperature and color was developed with ECL.

2.4. BP230 ELISA

The wells of a single ELISA plate were coated with both BP230-N and BP230-C at the concentration of