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J Invest Dermatol. Author manuscript; available in PMC 2009 January 29.

Published in final edited form as:
J Invest Dermatol. 2008 July ; 128(7): 1863–1866. doi:10.1038/sj.jid.5701235.

Sebocytes Express Functional Cathelicidin Antimicrobial Peptides and Can Act to Kill *Propionibacterium Acnes*

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TO THE EDITOR

Cathelicidins comprise an important family of antimicrobial peptides that are essential for protection of skin against bacterial infection in mice (Nizet *et al.*, 2001), and are relevant to infection in human skin diseases such as atopic dermatitis (Ong *et al.*, 2002). The human cathelicidin gene (*Camp*) is expressed as an inactive precursor protein (hCAP18) and is present in neutrophils, mast cells, keratinocytes, and eccrine glands (Braff *et al.*, 2005). hCAP18 consists of an N-terminal signal peptide, a cathelin domain, and a C-terminal region encoding the antimicrobial domain (Sorensen *et al.*, 2001). After proteolytic processing of hCAP18 to peptides such as LL-37, the peptide exerts antimicrobial activity that protects the host from infection. In this study we determined if cathelicidin is produced by sebocytes, thereby establishing its potential to add to defense of the skin by this cell.

The sebaceous gland has been suspected to provide protection to the external skin surface, but its functions were only recently understood (Zouboulis, 2003). Lipids produced by the sebocyte exhibit antibacterial activity (Georgel *et al.*, 2005) and peptides of the β-defensin family have been found in human pilosebaceous units (Chronnell *et al.*, 2001). Since cathelicidin has synergistic activity with β-defensins, it was of interest to determine the expression of cathelicidin in the sebaceous gland.

hCAP18 mRNA was detected by reverse transcriptase-PCR (RT-PCR) in isolated human sebaceous glands microdissected from normal scalp skin (Figure 1a). Cathelicidin protein was detected in sebaceous glands in normal human scalp tissue examined with anti-LL37 antibody (Murakami *et al.*, 2002; Figure 1b). Western blot analysis of extracts from isolated human sebaceous glands demonstrated a single band corresponding to LL-37 (Figure 1c). This finding is in contrast to cathelicidin isolated from neutrophils, where the predominant form seen was the hCAP18 precursor (Sorensen *et al.*, 2001).

hCAP18 mRNA was also detectable in SZ95 sebocytes (Zouboulis *et al.*, 1999) by quantitative real-time RT-PCR, and was induced by 1,25$(OH)_2$ vitamin D_3 (Figure 1d). The expression of

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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hCAP18 mRNA was also induced by *Propionibacterium acnes* (*P. acnes*) culture supernatant (Figure 1e). Cathelicidin protein was also detected by immunofluorescence and this was enhanced with 1,25$(OH)_2$ vitamin D_3 (100 nM) (Figure 1f). The concentrations of cathelicidin detected by ELISA were 0.17 μg per 10^6 SZ95 sebocytes (vehicle control) and 0.39 μg per 10^6 SZ95 sebocytes (100 nM 1,25$(OH)_2$ vitamin D_3) (Figure 1g).

To further define the response of SZ95 sebocytes to vitamin D, the expression of the vitamin D-dependent gene CYP24A1 (24-hydroxylase), and vitamin D receptor, was examined. CYP24A1 mRNA was induced 28-fold by 100 nM of 1,25$(OH)_2$ vitamin D_3 (Figure 1h). The vitamin D receptor was abundantly detected by immunofluorescence (Figure 1i). These results confirmed that SZ95 cells were vitamin D responsive.

To more precisely determine the mass and sequence of the cathelicidin peptide, SZ95 sebocytes were evaluated by surface-enhanced laser desorption/ionization–time of flight–mass spectrometry with anti-LL37 antibody capture. Surface-enhanced laser desorption/ionization–time of flight–mass spectrometry detected a single peak with a molecular mass of 4,496-Da (Figure 1j), identical to the expected mass of the LL-37 when processed in neutrophils.

To investigate the relevance of this finding to the antimicrobial function of sebocytes, we next determined if LL-37 could kill bacteria such as *Staphylococcus aureus* (*S. aureus*) and *P. acnes*, both potential pathogens of the human pilosebaceous unit. Under low-nutrient conditions, synthetic LL-37 (8 μM) killed 100% of *S. aureus* wild-type strain SA113 (ATCC 35556) (Figure 2a) and synthetic LL-37 (4 μM) killed 100% of *P. acnes* (Figure 2b). Acid-soluble protein extracts of SZ95 sebocytes also showed activity against *S. aureus* (Figure 2c) and *P. acnes* (Figure 2d). However, the amount of LL-37 in these extracts was insufficient to explain the antimicrobial activity: the concentration of LL-37 in SZ95 acid-soluble protein extracts as measured by ELISA was 0.17 μg ml^{-1} (0.038 μM).

Since other antimicrobials such as β-defensins (Chronnell *et al.*, 2001) and psoriasin (Glaser *et al.*, 2005) are expressed in sebocytes, and antimicrobial peptides have additive or synergistic functions when combinded (Nagaoka *et al.*, 2000), we next examined the potency of LL-37 against *P. acnes* when combinded with another antimicrobial peptide found in the sebocyte. Psoriasin alone (50 μg ml^{-1}) killed 80% of *S. aureus* (Figure 2e) and 95% of *P. acnes* (Figure 2f). When combined with psoriasin (10 μg ml^{-1}), LL-37 was much more effective against *P. acnes* (Figure 2g).

Taken together, these data show that cathelicidin is expressed in sebocytes and processed to the active peptide LL-37, where it may potentially aid in skin defense. Cathelicidin expression in sebocytes was both constitutive and inducible. This is reminiscent of the expression of cathelicidin in keratinocytes, where it is induced *in vivo* by infection, inflammation, or 1,25$(OH)_2$ vitamin D_3 (Frohm *et al.*, 1997; Weber *et al.*, 2005). We show that SZ95 sebocytes respond similarly to keratinocytes and increased cathelicidin when exposed to vitamin D_3 or extracts of *P. acnes*. This observation suggests a potential novel role for vitamin D in defense of the follicle. However, it remains to be determined what is the relative role of cathelicidin in sebocyte antimicrobial action. The form of cathelicidin produced by sebocytes (LL-37) can kill skin microflora *in vitro* such as *S. aureus* or *P. acnes*, but this is only likely to be relevant when it works together with other antimicrobial agents. The total antimicrobial activity in sebocytes is likely due to all antimicrobial peptides acting together, and *in vivo* is probably also aided by the antimicrobial action of fatty acids, lipids, and the hostile pH of the skin surface. Human β-defensin-2 mRNA can also be induced in SZ95 sebocytes by *P. acnes* (Nagy *et al.*, 2006), and psoriasin adds to the mixture. We show here that combining only two antimicrobials (LL-37 and psoriasin) produced a synergistic effect against *P. acnes* and greatly increased the potency of cathelicidin. The actual concentration of β-defensins, psoriasin, or

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other potential antimicrobial peptides in the sebaceous gland remains unknown, but the current findings support the conclusion that the sebaceous gland contributes to epithelial defense by the release of multiple antimicrobial molecules to the skin surface. LL-37 may contribute more to inflammation surrounding the sebaceous gland than in antimicrobial defense (Yamasaki *et al.*, 2007).

Abbreviations

P. acnes
: Propionibacterium acnes

RT-PCR
: *reverse transcriptase-PCR*

S. aureus
: Staphylococcus aureus

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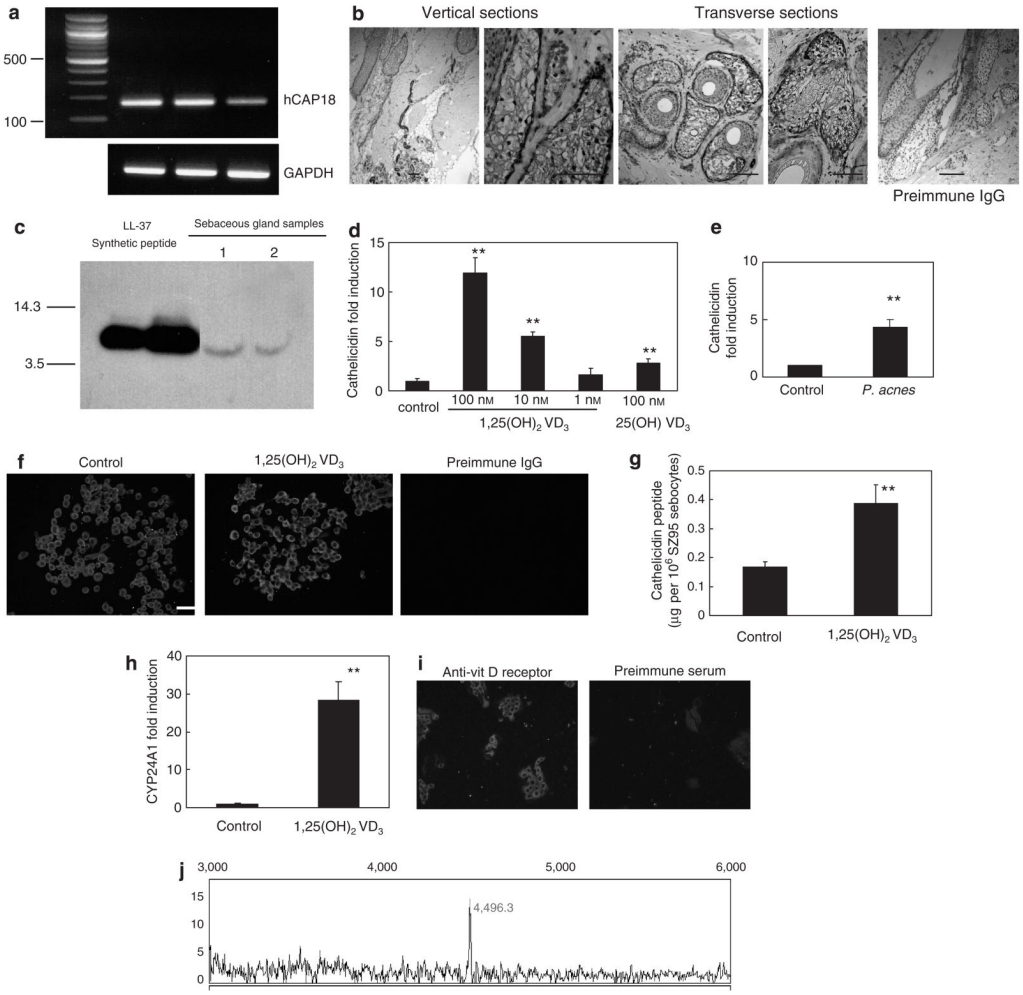

Figure 1. Cathelicidin is present in sebaceous glands isolated from normal scalp, in isolated sebaceous gland extracts, and in SZ95 sebocytes, where it is inducible by 1,25(OH)2 vitamin D3

(**a**) PCR amplification of hCAP18 mRNA from three independent isolates of normal scalp human sebaceous glands. (**b**) Histological sections of normal human scalp tissue examined for cathelicidin expression by immunohistochemistry with the anti-LL37 antibody. Human cathelicidin peptide was detected in both vertical and transverse scalp sections, and in eccrine structures, including glands and ducts. No staining was detected in the tissue sections stained with preimmune IgG. (**c**) Human sebaceous gland extracts evaluated by western blot with antibody to LL-37. Right two lanes show a single band at a size consistent with the expected 5-kDa mature form of LL-37 and similar to the LL-37 synthetic peptide in the left two lanes. (**d**) Real-time RT-PCR for hCAP18 SZ95 sebocytes treated with 1,25$(OH)_2$ vitamin D_3 or the vehicle for 24 hours. (**e**) Real-time RT-PCR for hCAP18 in SZ95 treated with *P. acnes* culture supernatant for 24 hours. (**f**) SZ95 sebocytes treated with the vehicle, or 100 nM 1,25$(OH)_2$ vitamin D_3 for 24 hours and stained with anti-LL-37 antibody or preimmune IgG. (**g**) Analysis of cathelicidin in SZ95 sebocyte extracts evaluated by ELISA. (**h**) Real-time RT-PCR for CYP24A1 in SZ95 treated with 1,25$(OH)_2$ vitamin D_3 (100 nM) or with the vehicle for 24 hours. (**i**) SZ95 sebocytes stained with anti-vitamin D receptor antibody. (**j**) Surface-enhanced laser desorption/ionization–time of flight–mass spectrometry analyses of cathelicidin isolated from SZ95 sebocyte extracts. A peak at 4,496-Da corresponding to the mature LL-37 peptide was detected in SZ95 sebocytes (**$P < 0.01$; Student's *t*-test, Bars = 20 μm). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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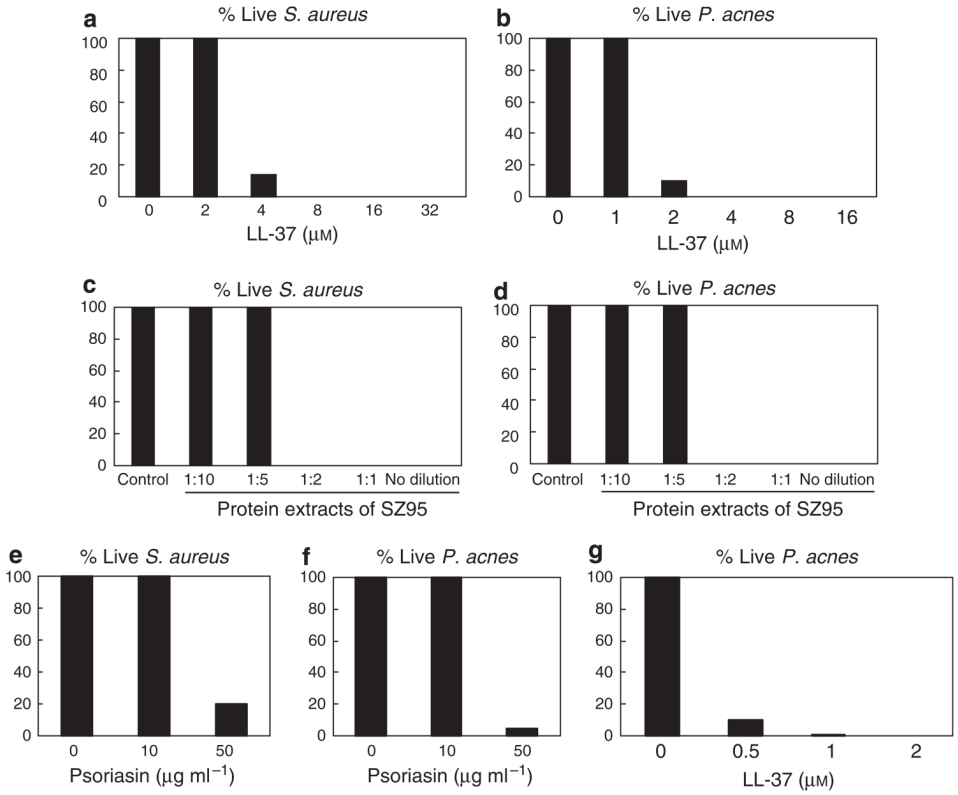

Figure 2. Synthetic LL-37 peptide, acid-soluble protein extracts of SZ95 sebocytes, and psoriasin kill S. aureus and P. acnes. LL-37 and psoriasin show a synergistic effect against P. acnes

The antimicrobial activity of synthetic LL-37 (**a, b**), acid-soluble protein extracts of SZ95 sebocytes (**c, d**), and psoriasin (**e, f**) was evaluated against *S. aureus* (**a, c, e**) and *P. acnes* (**b, d, f, g**) by solution killing assay. Bacteria were incubated for 3 hours at 37 °C in 10 mM sodium phosphate buffer (pH 6.5) supplemented with 100 mM NaCl. Recombinant psoriasin was a gift from A. Büchau and S. Kellermann, Dermatology, Heinrich-Heine University, Düsseldorf, Germany. Values are percentage of living bacteria compared with the control. Data shown are representative of triplicate determinations from three independent experiments.