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Heat-killed *Propionibacterium acnes* is capable of inducing inflammatory responses in skin

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Abstract: The etiology of acne is a complex process, and acne is one of the most common skin disorders affecting millions of people. The pathogenesis of acne is closely associated with the bacterium, *Propionibacterium acnes* which was previously known as *Corynebacterium parvum*. Both viable and non-viable *P. acnes/C. parvum* have been shown to induce an immunostimulatory effect *in vivo*, suggesting that even dead bacteria continue to activate an inflammatory response. Acne treatments with lasers or devices, induce a bactericidal effect through heat generation which may not address the immunogenic activity of *P. acnes* and the resulting acne inflammation. Therefore, we sought to determine whether killed *P. acnes* is capable of inducing an inflammatory response and therefore could be a contributing factor in acne. Direct heat treatment of *P. acnes* cultures with temperatures ranging from 50°C to 80°C reduced *P. acnes* viability. Both viable and heat-killed *P. acnes* activated the p38 MAP kinase and its downstream substrate Hsp27. Stimulating keratinocytes with normal and heat-inactivated *P. acnes* resulted in an induction of proinflammatory nitric oxide and IL-8 production. Thus killed *P. acnes* is capable of inducing inflammation in skin suggesting that therapies that have both bactericidal and anti-inflammatory effects may result in a more effective treatment of patients with acne than treatments that are bactericidal alone.

Key words: acne – cytokine – inflammation – keratinocyte – P38 – *Propionibacterium acnes*

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Background

Acne vulgaris is a chronic disorder of the pilosebaceous follicles of human skin whose pathogenesis is not yet well understood (1,2). The inflammatory nature of acne has been associated with the proliferation of *Propionibacterium acnes*, a normal human skin bacterium, in the obstructed follicles. The overgrowth of *P. acnes* in the anaerobic and sebum-rich environment of the follicle can induce the secretion of proinflammatory cytokines including interleukin(IL)-1α, IL-8, and tumor necrosis factor (TNF)-α from keratinocytes and monocytes (3,4). Laser or device treatments for acne, including intense pulsed light and photodynamic therapies can combine light and heat treatments to induce a bactericidal process (5). However, these treatments may not be sufficient in eliminating the appearance of acne as not only the presence of *P. acnes* but also the ability to promote inflammation could be a contributing factor in acne (6). Indeed studies on *Corynebacterium parvum*, the previous classification for *P. acnes*, have shown that viable and non-viable *C. parvum* can trigger an immunostimulatory effect in mice (7,8). In addition, studies of other bacteria have demonstrated that cellular membrane components of dead bacteria can still trigger an inflammatory reaction (9).

Abbreviations: IL-8, interleukin-8; Hsp27, heat shock protein27; NO, nitric oxide.

Questions addressed

In this study, we sought to determine whether heat killing *P. acnes* was sufficient to prevent *P. acnes* from inducing an inflammatory response.

Experimental design

Normal human epidermal keratinocytes were obtained from Cascade Biologics (Portland, OR, USA) and maintained in serum-free Epilife medium. *Propionibacterium acnes* was obtained from ATCC (Strain 11828 and 6919; Manassas, VA, USA). *Propionibacterium acnes* cultures were heat-killed by subjecting to temperatures ranging from 50°C to 80°C for 20 min and viability determined by measuring mitochondrial activity according to manufacturer's instructions (Alamar Biosciences Inc., Sacramenta, CA, USA). Partially confluent keratinocytes were inoculated with *P. acnes* at a ratio of 3×10^6 *P. acnes* per 30 000

keratinocytes. Nitric oxide production from keratinocytes was measured using the Greiss assay system (Promega Corporation, Madison, WI, USA). IL-8 production from keratinocytes was measured using the Luminex kit (10). Student's *t*-test was used for comparisons between treatment groups with a value of $P < 0.05$ considered significant. Western blotting (10) was used to study the activation of p38 MAP kinase and Hsp27.

Results

Direct heat treatment of *P. acnes* with temperatures ranging from 50°C to 80°C was found to produce a temperature-dependent loss of viability. The viability of room temperature, 50°C heat-treated, 70°C heat-treated and 80°C heat-treated *P. acnes* was 74%, 31%, 3.37% and 0.09% respectively (Fig. 1a). We next examined whether heat-killed *P. acnes* was capable of inducing inflammatory signalling. We found that both viable and heat-killed *P. acnes* activated p38 MAP kinase and its downstream substrate Hsp27 (Fig. 1b). Blots were reprobed with p38 and Hsp27 antibodies to account for equal protein loading. We next examined the potential of heat-killed *P. acnes* to induce the release of proinflammatory mediators from keratinocytes. Stimulating keratinocytes with viable and heat-killed (50°C and 70°C) *P. acnes* resulted in a significant induction of nitric oxide production (Fig. 1c). Similarly, stimulating keratinocytes with viable and heat-killed *P. acnes* resulted in a significant proinflammatory IL-8 release (Fig. 1d), which was only reduced by 0.63% and 26% in the 50°C and 70°C heat-killed *P. acnes* respectively compared with viable bacteria. Thus, heat-killed *P. acnes* induces significant inflammatory responses similar to viable *P. acnes*.

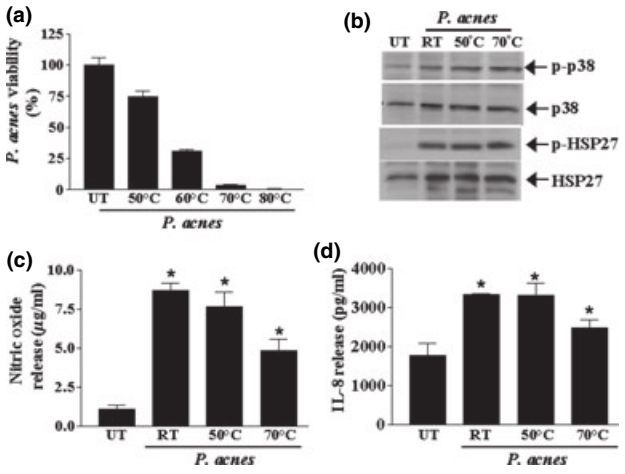

Figure 1. (a) *Propionibacterium acnes* was either left untreated or treated with heat at the indicated temperatures. Cell viability was determined by the alamar blue assay. (b) Keratinocytes were serum starved for 24 h followed by treatment with *P. acnes* left at room temperature (RT) or heated at 50°C or 70°C for 15 min. Whole cell extracts (20 μg of protein) were subjected to western blotting and probed with phospho-p38, phospho-Hsp27, p38 and Hsp27 antibodies. Untreated (UT) lane did not get any *P. acnes* treatment. (c) Nitric oxide production or (d) IL-8 production was measured from keratinocytes UT or treated with viable (RT) or heat inactivated (50°C and 70°C) *P. acnes* for 24 h. Data are representative of one of three separate experiments. $^*P < 0.05$ compared with keratinocytes treated with viable *P. acnes*.

Conclusions

Our results demonstrate that viable and heat-killed *P. acnes* are capable of inducing inflammatory responses in keratinocytes. In the previous work, non-viable *P. acnes* has been used on mice (11–13) and humans (14) and has successfully induced immunostimulatory responses and killed *P. acnes* adjuvant has been used as an immunological stimulant in veterinary medicine (15). Recent reports have suggested that *P. acnes* may induce inflammation through activation of the toll-like receptors (TLRs), especially TLR2 and TLR4, which are expressed on keratinocytes (16). Activation of TLR2 and TLR4 has been shown to stimulate the release of proinflammatory cytokines such as IL-1α, IL-6, IL-8 and TNF-α (16). Interleukin-8 is a potent neutrophil chemotactic factor and this *P. acnes*-induced IL-8 secretion may be a primary factor in the inflammatory response seen in some forms of acne (17). Although the antigenic component of *P. acnes* has not been fully identified, a peptidoglycan-polysaccharide found in the bacterial cell wall of *P. acnes* was found to induce TLR2 and TLR4 expression in human keratinocytes (4). Indeed lipopolysaccharide from *Escherichia coli* has been found to activate TLR4 on keratinocytes (9). Thus bacterial protein fragments, independent of the presence of viable bacteria can activate TLRs and initiate inflammation. Our findings contrast with an earlier study that showed that non-viable *P. acnes* did not induce cytokine production from keratinocytes (18); however, in those studies formaldehyde was used to kill the bacteria, which may have denatured the surface epitopes of *P. acnes* leading to inactivity of the killed bacteria. Taken together these results are consistent with our observations that killed *P. acnes*, or possibly membrane components of *P. acnes*, are capable of inducing inflammatory responses.

Several recent reports have revealed the existence of different phylogenetic groups amongst *P. acnes* demonstrating their heterogeneity (19–21). Adding to the complexity, isolates belonging to different phylogenetic groups can induce distinct gene expression profiles (17,22,23). To confirm that the effect we observed was not limited to a single strain of *P. acnes*, we performed similar experiments with another strain of *P. acnes* (ATCC 6919) and obtained identical results in terms of stimulation of IL-8 release from keratinocytes (data not shown).

Many therapeutic options are available to clinicians for the treatment of patients with acne including: oral and topical antibiotics such as clindamycin, retinoids, lasers and devices such as narrow band blue light. Interestingly many of these agents have anti-inflammatory effects independent of the anti-microbial activity. Indeed clindamycin, which has been shown to be an effective topical anti-acne agent (24) also has direct anti-inflammatory activity reducing cytokine release from bacterial stimulated macrophages (25). The anti-inflammatory activity of tetracycline has been shown to reduce the release of neutrophil chemotactic factor from human neutrophils exposed to *P. acnes* at sub-Minimum Inhibition Concentration (MIC) concentrations of the antibiotic (26). In addition, other antibiotics have also been shown to possess anti-inflammatory/immunomodulatory properties such as decreasing the release of proinflammatory cytokines (TNF-α, IL-1, IL-6) and increasing the secretion of anti-inflammatory cytokines (IL-10) (6). Similarly blue light (405–420 nm) which is bactericidal for *P. acnes* (27) has been shown to be effective in the treatment of mild to moderate inflammatory acne lesions (28) and also has direct anti-inflammatory activity independent of the bactericidal effects (29). Thus the anti-acne benefits from many therapeutic agents may be due to the direct anti-inflammatory activity in addition to or independent of the anti-microbial activity.

Acne is characterized by the hyperkeratinization of follicular keratinocytes in the pilosebaceous ducts. Obstruction of the canal and the overgrowth of the sebaceous follicle with *P. acnes* are believed to be the major causative factors for acne vulgaris. A central role of inflammation in the pathogenesis of acne has previously been reported (1,2,30). Interestingly inflammation may have a role in the hyperproliferation of follicular keratinocytes and thus may be an initiating event in the development of acne (2).

Hyperproliferation of follicular keratinocytes has been shown to be triggered by exposure to proinflammatory cytokines in the human pilosebaceous infundibulum *in vitro*, similar to that seen in comedones (31). Furthermore, treating sebocytes with proinflammatory cytokines increased expression of melanocortin receptors, notably MC-1R (32), and MC-1R expression was found to be increased in bioposies of acne lesions (33) primarily in the ductus seboglandularis, a region susceptible to hyperproliferation which can lead to obstruction of the canal. Thus while the proinflammatory activity of killed *P. acnes* does not make the bacterium key in the development of acne, it is interesting to speculate that the induction of proinflammatory cytokines by *P.acnes* may effect the physiology of the skin and create a microenvironment susceptible to the development of acne.

Taken together these results suggest that targeting the inflammatory component of acne is important to therapeutic efficacy in the treatment of acne. Treatments that have both bactericidal and anti-inflammatory effects may therefore contribute to the efficacy of the drug in the treatment of patients with acne greater than treatments that are bactericidal alone.

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