

Modulation of integrins and filaggrin expression by *Propionibacterium acnes* extracts on keratinocytes

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Received: 2 March 2007 / Revised: 28 June 2007 / Accepted: 20 July 2007 / Published online: 8 August 2007
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Abstract *Propionibacterium acnes* plays an important role in the pathogenesis of acne and it is established that this bacteria is involved in the induction and maintenance of the inflammatory phase of acne. The aim of our work was to determine if *P. acnes* extracts could modulate integrins and filaggrin in vitro expression by keratinocytes. Integrins and filaggrin expression was examined using immunohistochemistry technique both on Normal Human Epidermal Keratinocytes (NHEK) and on deep-frozen sections of normal human skin explants incubated with three different *P. acnes* extracts. In addition, the expression of filaggrin was investigated on biopsies of acne lesions and by western-blot associated with its precursor profilaggrin. We demonstrated that *P. acnes* extracts induced $\beta 1$ integrin expression significantly on both proliferating keratinocytes and differentiated keratinocytes. In addition, *P. acnes* induced $\alpha 3$, $\alpha 6$ s and $\alpha V\beta 6$ integrin expression and filaggrin expression on differentiated keratinocytes. Finally

P. acnes extracts increased filaggrin expression by suprabasal layer of epidermis of explants. Western-blot confirmed that total amount of filaggrin was increased. These results indicate that *P. acnes* extracts are directly able to modulate the differentiation of keratinocytes suggesting that this bacteria play a role not only in the development of inflammatory acne lesions but also in the formation of the microcomedo.

Keywords Acne · Filaggrin · Integrins · *Propionibacterium acnes*

Introduction

Acne is a skin disorder of the pilosebaceous unit resulting from multiple factors. Microcomedo is considered as the early step of the formation of acne lesions before closed or opened comedones and inflammatory lesions. The mechanisms inducing the formation of comedones are still partially known, but they are believed to involve hyperkeratinization of the follicle secondary to both hyperproliferation of keratinocytes and abnormalities of their differentiation and decreasing desquamation [3, 6].

Bacterial hypercolonization of the sebaceous follicle, especially *P. acnes*, has been considered as a major causative factor for the development of the inflammatory reaction in acne vulgaris. Indeed, it is widely accepted that inflammation in acne may be mainly mediated by different factors secreted by *P. acnes*, such as lipase which induce the liberation of free fatty acids from sebum that are pro-inflammatory [17]. Recent studies have also shown that *P. acnes* triggers anti-microbial peptide and cytokine secretion of keratinocytes in vitro [11, 12]. *P. acnes*, itself is able to induce cytokines-like such as TNF- α , IL-1 α and IL-

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8 [5]. The genome sequence of *P. acnes* encodes many factors that may have immunomodulatory potential [2]. However, *P. acnes* has also been identified at a high concentration in microcomedones with 10^5 – 10^6 organisms recovered from these preclinical lesions [10]. *P. acnes* is also able to induce the proliferation of keratinocytes in vitro. Confirmed both by MTT viability test and Ki67 immunolabelling [8, 11]. Thus, the precise role that *P. acnes* could play in the formation of comedone in addition of its action in the development of the inflammatory reaction remains unclear.

Integrins play an important role in the modulation of both differentiation and proliferation of keratinocytes in epidermis [14, 15]. Integrins are heterodimeric transmembrane receptors consisting of one α and one β subunit. $\beta 1$ integrins play a crucial role in regulating the balance between epidermal proliferation and terminal differentiation. Keratinocytes with high levels of $\beta 1$ integrins are less motile than keratinocytes with lower level [16]. $\alpha 3\beta 1$ and $\alpha 6\beta 4$ (laminin receptor) expression is constitutive whereas $\alpha V\beta 6$ (receptor for fibronectin and tenascin) is induced in NHEK culture, on wounding and pathological conditions. The conclusion from analysis of mice lacking $\beta 1$ or $\alpha 6\beta 4$ integrins is that $\beta 1$ integrins are indeed important for normal epidermal proliferation, whereas the role of $\alpha 6\beta 4$ is primarily one of anchorage. Thus abnormal keratinocytes differentiation induce alterations of the expression of $\beta 1$, $\alpha 3$, $\alpha 6$ integrins sub-units. $\alpha V\beta 6$ was used as keratinocytes suffering marker. Interestingly, on biopsies of acne lesions, it has been demonstrated that the alteration of keratinocytes differentiation is associated with abnormal integrins expression [7].

Filaggrin is a cationic (basic) protein named for its ability to aggregate filaments of keratin into fibers or macrofibrils. Human filaggrin (37,000 apparent molecular weight), is synthesized in the granular cells of the epidermis as a large, highly phosphorylated precursor, profilaggrin, which accumulates in the keratohyalin granules [4]. A number of keratinization disorders are associated with dysregulation in the formation of keratohyalin granules and in the processing of profilaggrin [13]. Kurukawa et al. [9] have shown that seborrhoeic and acne skin revealed considerable amounts of filaggrin in the intermediate layers of the sebaceous duct and the infundibulum indicating a premature terminal keratinization process in these areas. Furthermore they observed by electron microscopic studies an increased number of keratohyaline granules in acne skin.

Thus, the aim of this work was to determine if *P. acnes* is directly able to modulate integrins and filaggrin expression by keratinocytes which will be new arguments to think that this bacteria could play a role not only in the formation of inflammatory lesions but also of retentional lesions in acne.

Materials and methods

Materials

Keratinocytes culture

Normal Human Epidermal Keratinocytes (NHEK) from healthy prepuces were obtained from pediatric surgery department of Nantes Hospital and were grown at 37°C in a humid 5% CO₂ atmosphere, in serum free keratinocyte growth medium KSFM (Invitrogen, Cergy-Pontoise, France) supplemented with 25 µg/ml bovine pituitary extract (BPE), 0.5 ng/ml of recombinant epidermal growth factor (EGF), 2.5 µg/ml fungizone (Bristol-Myers Squibb, Paris, France) and Penicilline 100 UI/ml-Streptomycine 100 µg/ml. Cells were used after a limited number ($n = 2$) of subcultures. NHEK were seeded at the density of 20,000 per well in four-wells chamber slides (Dutscher, Brumath, France) in low calcium (CaCl₂ 0.09 mM) defined medium KBM (keratinocyte basal medium) (Promocell, Heidelberg, Germany) with HC (hydrocortisone) supplemented with 0.4% of BPE, EGF 0.125 ng/ml, insulin 5 µg/ml, transferrine 10 µg/ml, epinephrine 0.1 µg/ml. The medium was replaced by KBM without HC 24 h before starting the experiment and NHEK were used at 80% of confluence.

Skin explants

Explants were prepared from skin abdominal plasties received after plastic surgery. After fat removing, explants (4 mm diameter) were incubated at 37°C in presence of *P. acnes* extracts in order to cause stimulation or in control medium (KBM without HC). The explants were submerged and *P. acnes* extracts added to the culture medium. After incubation, explants were removed from the culture medium and frozen in liquid nitrogen before storage at –80°C.

Inflammatory acne lesions

Five frozen biopsies of inflammatory acne lesions (papulopustules) were obtained from Immunodermatology Laboratory. They were kept in liquid nitrogen before storage at –80°C.

Bacterial extracts

We used three extracts of *Propionibacterium acnes* (IP 53113) provided by Pierre Fabre Laboratories (Toulouse, France). This reference strain was first described in 1968.

Supernatant A (SA) and pellet C were obtained after bacterial culture centrifugation, *freeze/thawing of the cell pellet* and a last centrifugation at 4,000 rpm during 15 min.

Supernatant B (SB) was obtained after a second centrifugation (4,000 rpm, 15 min) of pellet C. The membrane fraction (FM) extract was obtained after reconstitution of pellet resuspended in KBM without HC.

The membrane fraction (FM) contained peptidoglycan and lipoteic acid. Supernatant A (SA) contained cytosolic proteins and supernatant B (SB) was rich in membrane proteins.

Preliminary experiments were carried out in our laboratory to determine the effect of different dilutions of *P. acnes* extracts on keratinocytes viability (MTT test). Following extracts dilutions: FM diluted at 1/2, SA and SB diluted at 1/5 were considered to be the most appropriated [8].

NHEK and cutaneous explants incubation with P. acnes extracts

The different *P. acnes* extract (FM 1/2, SA 1/5 and SB 1/5) were deposited on cutaneous explants for 3 (Immunohistochemistry and Western immunoblotting) or 24 h (Western immunoblotting).

Antibodies used for immunohistochemistry

They are listed in table 1.

Methods

Labelling of smears and biopsies (Immunohistochemistry)

Sections, 5 µm thick, were obtained from skin explants frozen in liquid nitrogen and fixed in acetone for 10 min. Immunostaining was performed using an immunoperoxidase technique using phosphate-buffered saline (PBS)/bovine serum albumin (BSA) 0.1% for dilution of antibodies and Tris-buffered saline (TBS)/Tween 20 0.05% (w/v)/BSA 0.1% w/v for washes.

Explants sections and chamber slides were incubated with the primary monoclonal antibodies at room temperature and in darkness for 30 min. The slides were then washed in TBS/BSA 0.1% and incubated with a second biotinylated antibody for 30 min, and then washed again and further incubated with streptavidine/peroxidase (DAKO) for 30 min. After a final wash, reactions products

were revealed using hydrogen peroxide/aminoethylcarbazole (AEC) (DAKO) peroxidase substrate. The reaction was stopped with distilled water (10 min) and counter-staining was done with Mayer haemalun (VWR International, Strasbourg, France) for about 1 min. The slides were rinsed with distilled water, and mounted in an aqueous medium and observed with a Leitz microscope (20× magnification).

Negative controls included irrelevant monoclonal antibody of the same isotype as the primary antibody. The intensity of the labelling was quantified according to a semi-quantitative scale: none (0), weak (1), moderate (2) and strong (3). Three slides from a total of three donors were examined. For each slide, the intensity of the labelling was determined using three fields and two different examiners read the slides.

Western immunoblotting

Explants cultured in control medium or with *P. acnes* explants for 3 h were lysed using Sample grinding Kit (Amersham Biosciences, Freiburg, Germany). Samples extracts mixed with an equal volume of 2× Laemmli Sample Buffer (Biorad-Rad, Marnes la Coquette, France) containing 50 mM 2β-mercaptoethanol were boiled for 5 min. Equal amount of protein from each explants lysate were electrophoresed on 8% SDS-polyacrylamide gel and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were blocked overnight using blocking reagent (BM chemiluminescence kit, Roche Diagnostic GmbH, Indianapolis, IN) and then incubated for 1 h with anti-human filaggrin primary antibody: AHF2 or AHF7 (kindly provided by M.Simon, Toulouse [14]) diluted at 1/2,500. After four washes in TBS containing 0.05% Tween 20, membranes were incubated with a horseradish peroxidase-conjugated secondary mix anti-mouse/anti-rabbit antibody dilute at 1/5,000 (BM chemiluminescence kit). Blots were developed using a classic chemiluminescence detection system (BM chemiluminescence kit).

Statistical analysis

Results were expressed as mean ± SEM. Statistical analyses were performed using the paired non parametric Wilco-

Table 1 Antibodies used for immunohistochemistry and their different concentrations

Specificity	Clonality, isotype	Origin	Supplier	Concentration
Filaggrin	Monoclonal, IgG1	Mouse	HarborBio-product, Norwood, USA	1/500°
Integrin α3	Monoclonal, IgG1	Mouse	Chemicon, Temecula, USA	2 µg/ml
Integrin α6	Monoclonal, IgG2a	Mouse	Chemicon, Temecula, USA	10 µg/ml
Integrin αVβ6	Monoclonal, IgG1	Mouse	Chemicon, Temecula, USA	10 µg/ml
Integrin β1	Monoclonal, IgG2a	Mouse	Chemicon, Temecula, USA	2 µg/ml

xon test. A P value less than 0.05 were considered to be significant.

Results

Effects of *P. acnes* extracts on integrin expression by NHEK [Ca^{2+} 0.09 mM] and by skin explants

Concerning NHEK (proliferating keratinocytes)

The expression of $\beta 1$ integrins was increased at the limit of significativity ($P = 0.06$) on keratinocytes incubated with SB extract (3 ± 0 versus 2.25 ± 0.5 in control) (Fig. 1).

The expression of $\alpha 3$ integrins was unchanged in presence of the three *P. acnes* extracts. The expression of $\alpha 6$ integrins was not significantly increased by SA and SB *P. acnes* extracts (1.5 ± 0.57). The expression of $\alpha V\beta 6$ integrins was not modified in presence of *P. acnes* extracts.

Concerning skin explants (ex vivo model with epidermis)

We observed that the expression of $\beta 1$ integrins was significantly increased with FM (2.5 ± 0.5), SA (2.33 ± 0.58) and SB extract (2 ± 1), and extracts compared to control

(2 ± 0) (Fig. 2). The expressions of $\alpha 3$ integrins, $\alpha 6$ integrins were not significantly increased in presence of *P. acnes* extracts. Concerning, $\alpha V\beta 6$ integrins the expression was not significantly induced with SB (1 ± 1.17).

Effects of *P. acnes* extracts on filaggrin expression by NHEK [Ca^{2+} 0.09 mM] and by skin explants

Concerning NHEK (proliferating keratinocytes)

We observed that the three types of *P. acnes* extracts did not induced an expression of filaggrin by in vitro proliferating keratinocytes (Fig. 3).

Concerning skin explants (ex vivo model with epidermis)

Immunohistochemistry using commercial anti-filaggrin (Fig. 4) We noted that the three types of *P. acnes* extracts induced a filaggrin expression on both suprabasal layer (granular and spinous) of epidermis (the strongest effect was observed with SA and SB extracts) (Fig. 4c, d, e).

On in vivo biopsies from of a retentional acne lesion we confirmed that the expression of filaggrin was extended to the lower part of epidermis (Fig. 4b) in a similar manner than we obtained with our skin explants incubated with *P. acnes* extracts.

Fig. 1 Expression of $\beta 1$, $\alpha 3$, $\alpha 6$, $\alpha V\beta 6$ integrins by NHEK incubated with *P. acnes* extracts (FM membrane fraction, SA supernatant A, SB supernatant B). Histograms represent the average intensity of immunolabelling: none (0), weak (1), moderate (2) and strong (3). The results represent the main of three experiments. * $P < 0.05$ (Student test)

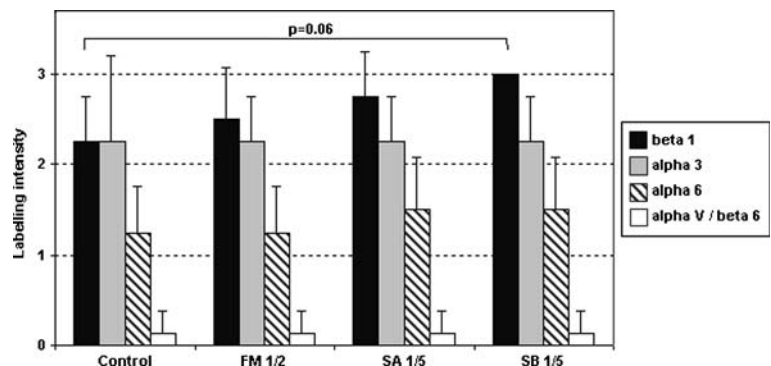


Fig. 2 Expression of $\beta 1$, $\alpha 3$, $\alpha 6$, $\alpha V\beta 6$ integrins on cutaneous explants incubated with *P. acnes* extracts. Histograms represent the average intensity of immunolabelling: none (0), weak (1), moderate (2) and strong (3). The results represent the main of three experiments

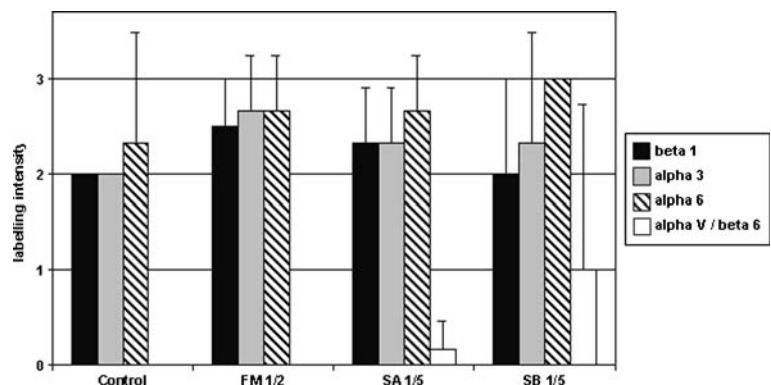
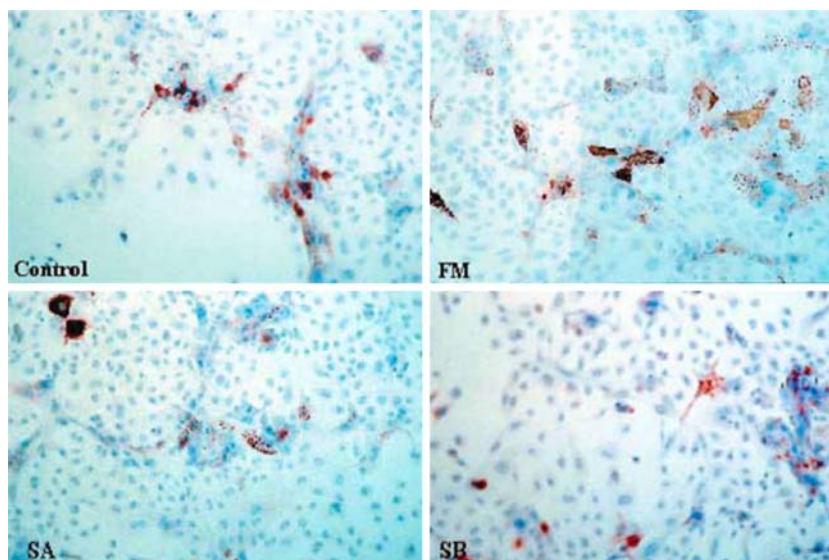


Fig. 3 Expression of filaggrin (commercial antibody) on NHEK incubated with *P. acnes* extracts compared to control (KGM) (magnification $\times 20$). This figure is representative slide from one donor. Note that Filaggrin expression in NHEK was not modified in presence of *P. acnes* extracts



Western-blot using non-commercial anti-filaggrin AHF2 and AHF7 (Fig. 5) Western-blot confirmed that total amount of filaggrin was increased after 3 or 24 h of incubation with *P. acnes* compared to control.

Discussion

We show for the first time that *P. acnes* extracts modulate the expression of $\beta 1$ integrin and filaggrin using two in vitro models. Explants have the advantage to be a model very closed to in vivo conditions. Thus it is more specifically of interest to study modulations of differentiation of keratinocytes. In addition, the model of NHEK has the advantage to study keratinocytes alone eliminating interactions with other cells of the skin. We do not use whole *P. acnes* bacteria, but the three extracts represent the different components of *P. acnes* which in vivo can be directly in contact with the keratinocytes (protein wall) or indirectly by secretion.

P. acnes induces an increase (but not significant) of $\beta 1$ integrins expression by proliferating keratinocytes (NHEK [0.09 mM]) and a significant expression of filaggrin on a differentiated epidermis. Moreover, an induction of the expression of integrins ($\beta 1$, $\alpha 3$, $\alpha 6$ and $\alpha V\beta 6$) is also noted on differentiated epidermis (explants) but not at a significant level.

Loss of $\beta 1$ integrin by keratinocytes has been shown to decrease the proliferation of basal keratinocytes by about 70% [1]. Thus, the induction of $\beta 1$ integrin by *P. acnes*, by modulating the proliferation of keratinocytes can play a role in the formation of micro-comedon.

Concerning $\alpha 3$ and $\alpha 6$ integrins, an in vivo study focusing on follicles of acne patients [7] has shown an aberrant $\alpha 2$ and $\alpha 3$ integrins expression around comedones and uninvolved pilosebaceous follicles from acne lesions. Our results suggest that *P. acnes* which is present in the sebaceous follicle could play a role in the modulation of these integrins, specifically $\alpha 3$ and $\alpha 6$.

Fig. 4 Expression of filaggrin on cutaneous (commercial antibody) explants incubated with *P. acnes* extracts: FM (c), SA (d) and SB (e), compared to normal skin (a) and acne lesion (b). All sample were treated in same conditions excepted acne's biopsy. This figure is representative slide from one donor. Note that *P. acnes* extracts induce filaggrin expression by keratinocytes of suprabasal (granular and spinous) on explants skin model

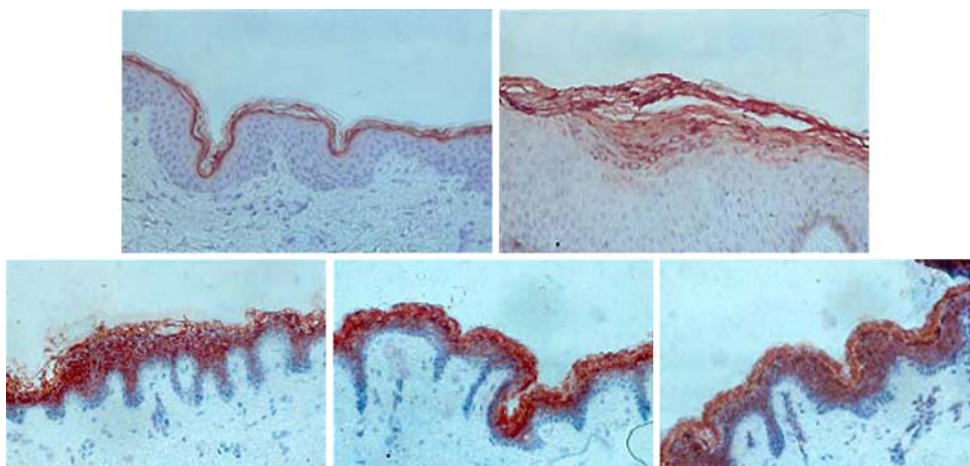
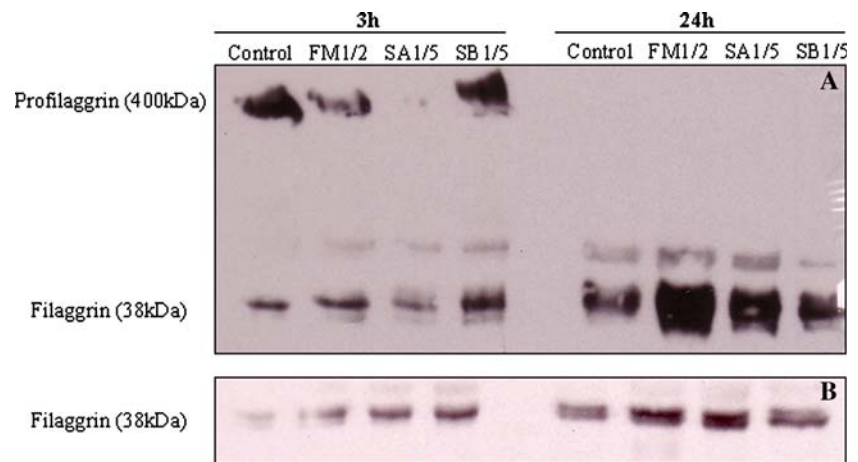


Fig. 5 Filaggrin (non-commercial antibody) immunoblot on cutaneous explants incubated with *P. acnes* extracts during 3 and 24 h. The blots show results obtained with AHF2 antibody, which interact both with profilaggrin and filaggrin (a) and AHF7 antibody, which interact only with filaggrin (b). This figure represents results obtained with 3 donors. Note that total amount of filaggrin protein was increased in explants incubated with *P. acnes* extracts compared to control



Our work also demonstrates that *P. acnes* extracts induce filaggrin expression on epidermis (suprabasal) of explants skin model whereas no induction of filaggrin expression was observed in NHEK model where keratinocytes were not differentiated. Concerning this part of our study, one of weaknesses is that we cannot directly transfer our in vitro results in vivo even if explants are closed to in vivo conditions. The other one, is the large error bars in Fig. 1 and 2, which can be explained by the variability between donors.

By western-blot, we confirmed that total amount of filaggrin was increased in explants incubated with *P. acnes* extracts compared to control. Moreover, western-blot, show that only filaggrin is increased in the presence of *P. acnes* extracts, whereas profilaggrin is totally processed in filaggrin. Thus, these results suggest that *P. acnes* modulates the terminal phase of differentiation of keratinocytes. Interestingly, this effect was observed with the three extracts of *P. acnes*. FM extract contains peptidoglycan and lipoteichoic acid, SA extract contain cytosolic proteins and SB is rich in membrane proteins. Thus, *P. acnes* modulates filaggrin expression on keratinocytes both via proteins localized on cell wall and cytoplasmic components excreted. Our results are in agreement with those obtained in vivo by Kurokawa et al, on pilosebaceous follicle in acne patients. Investigating the distribution of cytokeratins and filaggrin in human pilosebaceous unit in specimens obtained from normal, seborrheic, and acne skin [9], they show that both seborrheic and acne lesions displayed considerable amounts of filaggrin in the intermediate layers of the sebaceous duct and the infundibulum, indicating a premature terminal keratinization process in these areas. Furthermore they observed by electron microscopic studies an increased number of keratohyaline granules in acne skin. Our results argue for a role of *P. acnes* in the induction of filaggrin in the infundibulum.

Finally in a previous paper, we described that FM extract of *P. acnes* increased the proliferation of keratinocytes con-

firmed both by MTT viability test and by Ki67 immunolabelling [8]. Our hypothesis is that subsequently to sebum increase, *P. acnes* localized in pilosebaceous follicle proliferates which could be associated with two steps. At an early step *P. acnes* secretes inflammatory factors inducing the formation of inflammatory infiltrates surrounding follicles as demonstrated by Jeremy et al. [7]. Then, in a second step, the interaction between *P. acnes* and keratinocytes induce modulation of both proliferation and differentiation of keratinocytes as shown in our study, resulting in the formation of comedo.

In conclusion, our results suggest that *P. acnes* is implicated in acne lesion as soon as the step of formation of the microcomedo: Thus, they confirm that *P. acnes* is a key component in acne physiopathology.

Acknowledgments Financial support and *P. acnes* extracts were provided by Pierre Fabre Laboratories (Toulouse, France). No conflicts of interest exist. We thank surgeons from the plastic surgery Department of Nantes Hospital for abdominal skin samples and the pediatric surgery Department of Nantes Hospital for foreskin samples. The authors thank Simon M. (Toulouse) for antibodies AHF2 and AHF7 gift.

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