Inserm *Propionibacterium acnes* CAMP factor 1 recognized Toll-Like Receptor-2

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ABSTRACT

Propionibacterium acnes (*P. acnes*) is a major skin-associated bacterium that was long considered commensal, until several studies revealed it to be an opportunistic pathogen involved in several invasive infections. *P. acnes* induced a strong immune response involving lipoteicoic acid and peptidoglycan but no *P. acnes* surface protein interacting with Toll-like receptors have been identified. *P. acnes* surface proteins were extracted and analyzed by Far Western blotting experiments and two proteins of 24.5- and 27.5-kDa were specifically recognized by TLR2 and subsequently characterized as CAMP factor 1 by LC-MS/MS. Purified CAMP factor 1 induce IL-8 mRNA and protein production by activating IL-8 gene promoter, and using antibodies against TLR2 significantly decrease the IL-8 response. The CAMP1-TLR2 binding intensity appears to be stronger in IB and II types strains associated with a capacity to produce large amount of IL-8, than strains typed IA1 and IA2 associated with low IL-8 production. Furthermore, CAMP factor 1 could be divided into two major distinct genetic groups corresponding with highly polymorphic sequence from strains with moderate and high CAMP1-TLR2 binding activities; and with low polymorphic sequences from strains presenting no, weak and moderate CAMP1-TLR2 binding intensity.

OBJECTIVES

The surface proteins of *P. acnes* involved in the activation of TLR2 on keratinocytes and monocytes and leading to inflammatory lesions in acne have yet to be identified. The aim of this study was to identify surface proteins of *P. acnes* recognized by TLR2 in several strains with different inflammatory profiles.

METHODS

Bacterial culture and protein extraction. 27 strains of *P. acnes* were used (from ATCC, the Dermatology Department of Nantes Hospital, and the Bacteriology Department of Cochin Hospital) (Table 1). *P. acnes* strains were grown under anaerobic conditions in liquid reinforced clostridial medium (RCM) at 37°C for five days. The total surface protein extract was obtained in presence of 1 M LiCl (LIE).

Cell culture and stimulation. The human keratinocyte HaCaT and the monocyte Thp1 cells lines were grown in DMEM and RPMI, respectively. Cells were incubated with a *P. acnes* suspension adjusted to the appropriate concentration (620 nm) or with LIE.

DNA extraction and phylotyping. DNA was extracted from bacterial strains with the E.Z.N.A.®Bacterial DNA kit. Phylotyping was performed by multiplex touchdown PCR [Barnard 2015].

Far-western blotting. *P. acnes* surface proteins were separated by LDS-PAGE in a NuPAGE Novex 4-12% Bis-Tris gel and transferred onto a nitrocellulose, saturated, and incubated with human recombinant TLR2. Membrane were then incubated with biotinylated human antibodies against TLR2. The surface protein-TLR2-biotinylated antibody complex was detected by incubation with horseradish peroxidase. Peroxidase activity was detected in a chemiluminescence assay.

Protein elution. Proteins were fractionated by 12% SDS-PAGE and excised protein bands were incubated for 18 h at 37°C in 1% SDS in PBS. Excess of SDS was removed by cold acetone precipitation Protein pellets were suspended in PBS and protein concentration was determined at 280 nm.

Cell transfection and luciferase reporter gene assay. HaCaT were transfected with reporter plasmids containing the CXCL8 promoter (-173 bp) or the NF-κB promoter (5 units) upstream from the firefly luciferase gene in JetPEI™. Transfection efficiency was normalized by co-transfecting the cells with 10 ng of pSra-*Renilla* luciferase expression vector. Cells were then stimulated with LIE or eluted proteins. Firefly and *Renilla* luciferase activities were measured on a MicroLumat Plus LB 96V Luminometer.

RNA isolation and RT-qPCR. HaCaT cells were stimulated with whole bacteria (MOI 10), LIE (50 µg/ml), or eluted proteins. Total RNA was isolated with the NucleoSpin® RNA II kit. RT-qPCR was performed with the iTaq Universal Sybr Green one-Step kit containing reverse transcriptase (RT) and Taq polymerase. Complementary DNA and standard PCR amplification were carried out on a LightCycler Nano. The amount of RNA in stimulated cells relative to control cells was calculated by the 2ΔCt method.

LC-MS/MS characterization of proteins of interest. Proteins were separated by 10% SDS-PAGE and proteins recognized by TLR2 were matched to stained bands, excised from the gel, reduced and alkylated. Trypsin digestion was performed and peptide extracts were enriched, separated and analyzed with a 6520 Accurate-Mass Q-TOF LC/MS machine equipped with an HPLC-chip cube interface. The fragmentation data were interpreted with Mass Hunter software (version B.03.01, Agilent Technologies). For protein identification, MS/MS peak lists were extracted, converted into mzdata.mxl format files and compared with the protein database, using the MASCOT Daemon (version 2.1.3; Matrix Science, London, UK) search engine.

CAMP-1 PCR amplification and sequencing. CAMP factor 1 nucleotide sequence from the 27 *P. acnes* strains was amplified by PCR [Valanne 2005]. Sequencing reactions were performed with ABI PRISM Ready Reaction Terminator cycle with the Big Dye V3.1 Terminator Kit and analyzed on an ABI PRISM 3100 DNA sequencer. DNA sequences were analyzed with SeqScape® v2.5 Software from Applied Biosystems.

Phylogenetic analysis. Analyses were carried out with the maximum likelihood method. Multiple sequence alignments were obtained with Mafft version 7 and exported into Seaview software, for input into JModelTest and ProtTest to identify an appropriate substitution model for phylogenetic analysis with the Bayesian information criterion. The phylogenetic analysis was performed with PhyML3.0, implemented in Seaview software. Sequences were randomized by 500 bootstrapping resamplings, and statistical analyses were performed on 100 datasets for each analysis.

Statistical analysis. The statistical significance of differences between data from experimental groups was analyzed in paired Student's t-tests. $P < 0.05$ was considered significant. Statistical significance is indicated by * ($P \leq 0.05$), ** ($P \leq 0.01$), *** ($P \leq 0.001$) and **** ($P \leq 0.0001$), respectively.

Table 2 : Comparison of *P. acnes* inflammatory capabilities with phylogroup and CAMP factor 1 gene nucleotide sequence

Strain ID	Type	Binding	IL-8 Production (pg/ml)	Nucleotide sequence		Amino acid sequence	
				Identity y (%)	No. of nucleotide difference	Identity (%)	No. of peptidic difference
75150	IA1	-	382 ± 48	100	0	100	0
16351	IA1	+/-	233 ± 23	100	0	100	0
17248	IA1	+/-	252 ± 43	100	0	100	0
53468	IA1	-	255 ± 26	100	0	100	0
41103	IA1	-	367 ± 24	100	0	100	0
A24	IA1	-	232 ± 14	100	0	100	0
A26	IA1	-	309 ± 12	100	0	100	0
6919	IA1	-	318 ± 58	100	0	100	0
78910	IA2	+/-	151 ± 45	100	0	100	0
A44	IA1	-	242 ± 12	99	1 (G361.T)	99	1 (V121.L)
A30	IA1	+	290 ± 27	99	1 (A31.G)	99	1 (I11.V)
38862	IA1	+	366 ± 6	99	2 (A31.G; A319.G)	99	2 (I11.V; I107.V)
RON	IA1	+/-	413 ± 46	99	2 (A31.G; A319.G)	99	2 (I11.V; I107.V)
TRI	IA1	+/-	242 ± 58	99	2 (A31.G; A319.G)	99	2 (I11.V; I107.V)
22197	IA1	+	291 ± 47	99	2 (A31.G; A319.G)	99	2 (I11.V; I107.V)
CHR	IA2	++	590 ± 74	99	2 (A31.G; G221.A)	99	2 (I11.V; E74.K)
14230	IB	+++	93 ± 5	99	2 (A31.G; G220.A)	99	2 (I11.V; E74.K)
12513	IB	+++	591 ± 15	99	2 (A31.G; G220.A)	99	2 (I11.V; E74.K)
22795	IB	+/-	457 ± 12	99	2 (A31.G; G220.A)	99	2 (I11.V; E74.K)
47474	IB	+++	314 ± 53	99	2 (A31.G; G220.A)	99	2 (I11.V; E74.K)
27387	IB	+++	259 ± 8	99	2 (A31.G; G220.A)	99	2 (I11.V; E74.K)
25236	IB	+++	267 ± 30	99	2 (A31.G; G220.A)	99	2 (I11.V; E74.K)
A9	IB	+++	310 ± 24	99	2 (A31.G; G220.A)	99	2 (I11.V; E74.K)
GUE	IB	++	262 ± 75	99	2 (A31.G; G220.A)	99	2 (I11.V; E74.K)
PIE	IB	++	447 ± 55	99	2 (A31.G; G220.A)	99	2 (I11.V; E74.K)
<div>27647 II ++ 603 ± 26 96 36</div> <div>(A31.G; A102.G; C163.T; A222.G; G231.A; C241.A; T242.C; T249.C; C258.T; G267.C; G278.A; G304.A; G306.T; C309.T; A319.G; T324.C; T330.G; C384.T; C387.T; T400.C; C450.T; A465.G; A472.G; C556.A; A561.G; T572.G; A592.G; T601.G; T606.C; C642.T; A679.T; T705.C; T813.C; T826.C; C835.G; T836.C)</div> <div>(I11.V; I34.M; L82.T; R93.K; V102.I; I107.V; T158.A; L186.I; V191.G; T198.A; S201.A; M226.L; S276.A; L279.A)</div>							
<div>A11 II +++ 569 ± 41 96 36</div> <div>(A31.G; A102.G; C163.T; A222.G; G231.A; C241.A; T242.C; T249.C; C258.T; G267.C; G278.A; G304.A; G306.T; C309.T; A319.G; T324.C; T330.G; C384.T; C387.T; T400.C; C450.T; A465.G; A472.G; C556.A; A561.G; T572.G; A592.G; T601.G; T606.C; C642.T; A679.T; T705.C; T813.C; T826.C; C835.G; T836.C)</div> <div>(I11.V; I34.M; L82.T; R93.K; V102.I; I107.V; T158.A; L186.I; V191.G; T198.A; S201.A; M226.L; S276.A; L279.A)</div>							

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RESULTS

Figure 1 : Induction of CXCL8 production in keratinocytes by *P. acnes* surface proteins. HaCaT cells were incubated for 18 h with lithium extracts of bacterial proteins at concentrations of 50 µg/ml (A) and 3.12, 12.5, and 50 µg/ml (B). Total RNA was extracted and CXCL8 mRNA levels were determined by real-time RT-PCR. CXCL8 production was assessed by ELISA.

Figure 2 : TLR2 binding to *P. acnes* surface proteins. *P. acnes* surface proteins were separated in 4-12% NuPAGE LDS BisTris gels (50 µg/ml), transferred onto nitrocellulose membranes and incubated with recombinant TLR2 (0.1 µg/ml). TLR binding activity was detected with specific biotinylated antibodies against TLR2. Lane 1 contains molecular mass markers. Lanes 2 to 22 contain proteins from strains 75150, 22197, 14230, 78910, 16351, 17248, 12513, 22795, 53468, 36862, 41103, 47474, 27647, 27387, 25236, A9, A11, A24, A26, A30, and A44, respectively. Arrows indicate the positions of the 24.5- and the 27.5-kDa bands of interest.

Keratinocytes were stimulated with LIE for 18 h and representative results are shown for the evaluation of the CXCL8 production from the 27 *P. acnes* strains used in this study (Fig 1, Table 1). LIE induce CXCL8 mRNA and protein production in a dose-dependent manner with a variation level from the different strains tested (Fig 1A, B). ***P. acnes* surface proteins extract are a suitable starting material for identification of the surface protein recognized by TLR2.** Far-western blotting was performed on total LIE, and two protein bands with apparent molecular masses of 24.5- and 27.5-kDa were recognized by TLR2 (Fig 2). Eluted 24.5- and 27.5-kDa proteins were able to activate the NF-κB and CXCL8 promoters (Fig. 3A), to induce 10- to 100-fold induction of CXCL8 transcription (Fig. 3B) as well as CXCL8 protein production to 10 pg/ml (Fig 3C). **As the 24.5-kDa and 27.5-kDa proteins appeared to be recognized by TLR2 in 48% of the strains tested, we choose to focus on the characterization of these two proteins.**

Figure 3 : *P. acnes* surface proteins recognized by TLR2 have proinflammatory activity *in vitro*. Surface protein extracts were separated in 10% SDS-PAGE and proteins of interest were excised from the gel. (A) HaCaT cells were transfected with CXCL8 (-173 bp) (light gray bar) and NF-κB (dark gray bar). Cells were stimulated by incubation with the eluted proteins (10 µg) for 24 h at 37°C. Relative NF-κB and CXCL8 promoter activities were determined as the ratio of firefly and *Renilla* luciferase activities. HaCaT cells were stimulated with the eluted proteins (20 µg) for 24 h at 37°C and CXCL8 mRNA levels were evaluated by RT-qPCR (B), and CXCL8 protein levels were assessed by ELISA (C).

Figure 4 : Comparison of the sequences of the 27.5 kDa *P. acnes* surface protein and *P. acnes* CAMP factors. The peptide sequence of the 27.5 kDa protein was obtained after LC-MS/MS analysis corresponding to 66% coverage (in bold). Differences between sequences are highlighted in gray.

Proteins of interest were characterized by LC-MS/MS and protein sequence database searches identified the two proteins of interest as products of gene encoding a hypothetical 285-amino acid protein from the **CAMP factor superfamily** (Accession number gi|488487765), covering 66% of the amino-acid sequence for the **27.5-kDa** protein and 61% of the sequence for the **24.5-kDa** protein. The sequence of the 27.5-kDa was 99%, 38%, 33%, 50% and 51% identical to the *P. acnes* CAMP factors 1, 2, 3, 4, and 5, respectively (Fig 5), and both proteins corresponded to the same *P. acnes* surface protein. The stimulation of keratinocytes with eluted CAMP-1 protein after their prior treatment with anti-TLR2 antibodies resulted in significantly lower levels of NF-κB ($P = 0.008$) and CXCL8 ($P = 0.0012$) promoter activation (Fig 5A, B), and, consequently, significantly lower levels of CXCL8 mRNA ($P = 0.00047$) (Fig 5C) and CXCL8 protein production in keratinocytes ($P = 0.0024$) (Fig 5D). No such effect was observed when this experiment was performed with the IgG isotype control. Thus, CAMP factor 1 isolated from *P. acnes* interacts with TLR2, triggering a downstream signaling pathway leading to CXCL8 production.

Figure 6 : *P. acnes* CAMP factor 1 polymorphism and phylogenetic analysis. Nucleotide (dark bold) and peptide (light gray) changes identified in *P. acnes* isolates relative to the reference sequence of the *P. acnes* NTCT 737 strain (GenBank accession number AY527218.1) are indicated by vertical bars. (A), (B) and (C), (D) and (E), (F) correspond to CAMP1 with no, 1, 2, and several mutations, respectively. Phylogenetic analysis illustrating the relationship between CAMP factor 1 and 19 protein reference sequences from *P. acnes*, CAMP 5 factor Protein and sequences of the 27 *P. acnes* strains used in this study. Phylogenetic trees were constructed by the maximum likelihood method and rooted on the CAMP factor 5 protein sequence. Bootstrapping was applied to the trees (500 datasets) and bootstrap values are shown at nodes. The bar indicates the number of substitutions per site. Sequences were classified into four groups (A, B1 to B3). Squares correspond to phylogroup IA₁ (red), IA₂ (yellow), IB (green), II (purple), and to CAMP1-TLR2 binding intensity with no binding as - (light gray), weak binding as +/- and + (dark gray), strong binding as ++ and +++ (black).

We identified four different phenotypes: IA₁ (15 strains), IA₂ (2 strains), IB (9 strains) and II (2 strains) (Table 2) with most of the strains of types IB and II having strong TLR2 binding activity and producing high level of CXCL8 (Table 2). CAMP factor 1 DNA sequences were then classified into 4 groups according to the number of nucleotide polymorphisms. Group A corresponded to CAMP factor 1 gene with no nucleotide polymorphism. Groups B&C corresponded to CAMP factor 1 genes with 1 nucleotide polymorphism. Groups D&E consisted of CAMP factor 1 genes displaying two single nucleotide polymorphisms. Group F corresponded to CAMP factor 1 genes with sequences containing 36 single nucleotide polymorphisms (Fig 6, Table 2). Strains with weaker TLR2 binding activity were found to have CAMP factor 1 genes with no nucleotide polymorphism or sequences with 1 or 2 nucleotide polymorphisms, corresponding to I11>V, I107>V and V121>L. Conversely, *P. acnes* strains with strong CAMP1-TLR2 binding activity had CAMP1 sequences with nucleotide polymorphisms resulting in I11>V and E74>K substitutions or had large numbers of nucleotide polymorphisms, resulting in 14 amino-acid changes (Table 2). Phylogenetic analysis of the CAMP factor 1 sequences revealed the existence of two distinct genetic groups supported by high bootstrap values, and corresponding to strains phylogrouped II with CAMP factor 1 containing 36 nucleotide polymorphisms (group A), and to the strains phylogrouped IA₁, IA₂, IB with CAMP factor 1 containing no nucleotide polymorphism, one nucleotide polymorphism, and 2 nucleotide polymorphisms (Group B) (Fig 6; Table 2). Group B contained three genetically related clusters (B1, B2, B3) corresponding to CAMP1 very weakly (B1), strongly (B2) and not recognized (B3) by TLR2.

CONCLUSIONS

Our findings indicate that CAMP factor 1 may contribute to *P. acnes* virulence, by amplifying the inflammation reaction through direct interaction with TLR2.