J Periodont Res 2007; 42; 1–7

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2005.00857.x

Regulation of calprotectin expression by interleukin-1α and transforming growth factor-β in human gingival keratinocytes

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Hayashi N, Kido J, Suryono, Kido R, Wada C, Kataoka M, Shinohara Y, Nagata T. Regulation of calprotectin expression by interleukin-1α and transforming growth factor-β in human gingival keratinocytes. J Periodont Res 2007; 42: 1–7.

Background and Objective: Calprotectin, a heterodimer of S100A8 and S100A9 with antimicrobial properties, is expressed in gingival keratinocytes and plays an important role in innate immunity. Because calprotectin expression is localized in the spinous cell layer of the gingival epithelium, we hypothesized that the expression of calprotectin in keratinocytes is related to the differentiation stage. The aim of the present study was to investigate the relationship between calprotectin expression and keratinocyte differentiation using some factors that regulated its differentiation.

Material and Methods: Normal human gingival keratinocytes were isolated from gingival tissues obtained at the extraction of wisdom teeth, and were cultured in serum-free keratinocyte medium supplemented with interleukin-1α or calcium, which promote keratinocyte differentiation, and transforming frowth factor-β (TGF-β) or retinoic acid, which suppress its differentiation. The expression of S100A8/A9 mRNA and the production of calprotectin in normal human gingival keratinocytes were examined by northern blotting and enzyme-linked immunosorbent assay, respectively. The expression of cytokeratin 14, involucrin and filaggrin (marker proteins of keratinocyte differentiation) was investigated by immunohistochemical staining, and the DNA-binding activity of CCAAT/enhancer binding protein α (C/EBPα), a transcription factor, was examined by electrophoretic mobility shift assay.

Results: The expression of S100A8/A9 mRNA and the production of calprotectin were increased by interleukin-1α and calcium, but decreased by TGF-β. RA inhibited the expression of S100A8/A9 and keratinocyte differentiation, which were induced by interleukin-1α. C/EBPα DNA-binding activity in normal human gingival keratinocytes was enhanced by interleukin-1α and calcium, but suppressed by TGF-β.

Conclusion: The present study suggests that calprotectin expression is related to keratinocyte differentiation and that C/EBPα is a regulator of calprotectin expression in keratinocytes.

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Key words: CCAAT/enhancer binding protein α; calprotectin; differentiation; gingival keratinocyte

Accepted for publication September 22, 2005

Periodontal diseases occur as a result of the interaction of bacterial pathogens with the host defense system. The gingival epithelium functions as a mechanical and biological barrier against microbial challenges (1). To defend against microbial invasion, epithelial cells express several pro-inflammatory cytokines, chemokines and antimicrobial peptides, including calprotectin and β-defensin, which contribute to innate immunity (2–4). Calprotectin, a major cytosolic protein of granulocytes, is an antimicrobial protein (5); it is a heterogeneous complex composed of two small anionic proteins (S100A8 and S100A9) which belong to the S100 calcium-binding protein family (5). Calprotectin shows a broad spectrum of *in vitro* antimicrobial effects via zinc-chelating action and is constitutively expressed in squamous mucosal epithelia, such as the gingival epithelium and vaginal epithelium, and is also detected in certain inflammatory skin diseases (5,6).

The gingival epithelium is a cornified squamous epithelium consisting of four distinct morphological layers. It is maintained by the proliferation of stem cells in the basal cell layer, which differentiate into spinous cells, then into granular cells, which, in turn, differentiate into the cornified stratum corneum (7). In healthy gingival epithelium, calprotectin localizes in the spinous cell layers. During inflammation, calprotectin expression is enhanced and detected in the spinous and granular cell layers (8). Interleukin-1α, a cytokine synthesized by keratinocytes, promotes keratinocyte differentiation (9). Furthermore, Warner-Bartnicki *et al.* (10) reported that calprotectin expression in monocytes was related to cell differentiation. The results of these reports suggest that different levels of calprotectin expression in the gingival epithelium may be associated with the stage of keratinocyte differentiation, as well as inflammation.

In the present study, to examine the relationship between calprotectin expression and keratinocyte differentiation, we investigated the effects of interleukin-1α, calcium, transforming growth factor-β (TGF-β) and retinoic acid, regulators of keratinocyte differentiation, on calprotectin expression in human gingival keratinocytes. Furthermore, the DNA-binding activity of CCAAT/enhancer binding protein α (C/EBPα), a transcription factor that was shown to regulate S100A9 expression in monocytic cell lines (11), was examined.

Material and methods

Cell culture

Human gingival fragments were obtained from mandibular wisdom teeth extracted from patients at Tokushima University Hospital. Normal human gingival keratinocytes were isolated according to the method of Matsuyama *et al.* (12). Cells were cultured in Keratinocyte-SFM (Gibco, Invitrogen Co., Carlsbad, CA, USA) containing 0.2 ng/ml epidermal growth factor (Invitrogen) and 25 μg/ml bovine pituitary extract (Invitrogen). Cells of the third passage were used for each experiment. Cells ($12 \times 10^4/cm^2$) were seeded on collagen-coated dishes (Iwaki, Tokyo, Japan) and cultured for 3–4 d, then stimulated with 10 ng/ml interleukin-1α (Wako, Osaka, Japan), 10 ng/ml TGF-β1 (Wako), 1.0 mM $CaCl_2$ (calcium), 300 μg/ml retinoic acid (Wako) or 50 ng/ml phorbol 12-myristate 13-acetate (Wako) for 24, 36 or 48 h.

Immunohistochemical staining

Normal human gingival keratinocytes cultured on type I collagen-coated glass slides (IWAKI) were treated with 10 ng/ml interleukin-1α, 10 ng/ml TGF-β, or 1.0 mM $CaCl_2$ for 48 h, fixed in 4% paraformaldehyde in phosphate-buffered saline, and treated with acetone/ethanol (1 : 1, v/v). Cells were blocked with normal horse serum for 30 min and treated with antibodies to cytokeratin 14 (1 : 20 dilution; Cymbus Biotechnology Ltd, Hants, UK), involucrin (1 : 200 dilution; Neomarker, Fremont, CA, USA) and filaggrin (1 : 100 dilution; Biomedical Technologies Inc., Stoughton, MA, USA) at 4°C overnight. As a negative control, nonimmune mouse immunoglobulin G was used. After washing with phosphate-buffered saline, cells were treated with biotinylated horse anti-mouse immunoglobulin G for 30 min, with avidin-biotinylated glucose oxidase for 1 h (Vectastain ABC Kit; Vector Laboratories, Inc., Burlingame, CA, USA) and then reacted with Nitro Blue tetrazolium (NBT Substrate Kit; Vector Laboratories, Inc.) for 15 min and contrasted with Neutral Red (Wako).

Northern blotting analysis

The total RNA was isolated using RNeasy® (Qiagen Science, Valencia, CA, USA). Three micrograms of RNA was electrophoresed on a 6% formaldehyde/1% agarose gel, transferred to Hybond N+ (Amersham Bioscience, Bucks., UK) and fixed. S100A8, S100A9 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were synthesized by polymerase chain reaction (PCR) amplification using primers specific to human cDNA sequences for S100A8 (sense: 5′-GCTGGAGAAAGCCTTGAACT-C-3′; antisense: 5′-CCACGCCCATC-TTTATCACCA-3′), S100A9 (sense: 5′-TCGCAGCTGGAACGCAACAT-A-3′; antisense: 5′-AGCTCAGCTGC-TTGTCTGCAT-3′) and GAPDH (sense: 5′-TCCACCACCCTGTTGC-TGTA-3′; antisense: 5′-ACCACAGT-CCATGCCATCAC-3′). These probes were labeled with [α-^{32}P]dCPT using the BcaBest Labeling Kit (TaKaRa, Kyoto, Japan). Prehybridization was performed for 1 h at 42°C in solution comprising 50% formamide, 5 × saline sodium phosphate-EDTA (pH 7.4), 5 × Denhardt's solution, 0.5% sodium dodecyl sulfate and 200 μg/ml salmon sperm DNA, followed by incubation with ^{32}P-labeled probes, in the same solution, for 12 h at 42°C. Washing in 2 × saline sodium phosphate-EDTA (pH 7.4), 0.1% sodium dodecyl sulfate at 65°C three times and exposure to an imaging plate (Fuji Photofilm Co., Tokyo, Japan) was then performed. The density of the autoradiography band was assayed using a BAS 2000X Bio-Imaging analyzer (Fuji) and normalized to that of GAPDH. The data presented represent the

means ± standard deviation (SD) from six subjects.

Reverse transcription–polymerase chain reaction

cDNA was synthesized using Ready-To-Go reverse transcription–polymerase chain reaction (RT–PCR) beads (Amersham Biosciences). PCR was performed for 30 cycles, according to the following steps: 1 min at 95°C; 1 min at 65°C; and 1 min at 72°C. Human involucrin primers (sense: 5′-TGTTCCTCCTCCAGTCAATAC-CC-3′; antisense: 5′-ATTCCTCATG-CTGTTCCCAGTGC-3′) were used. The PCR products were analyzed by electrophoresis on a 2% agarose gel.

Enzyme-linked immunosorbent assay

Normal human gingival keratinocytes treated with interleukin-1α, TGF-β and calcium were collected, suspended in extraction buffer with protease inhibitor cocktail [1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml *N*-Tosyl-L-phenylalanine chloromethyl ketone, 1 μg/ml *N*-α-*P*-Tosyl-L-lysine chloromethyl ketone hydrochloride and 1 μg/ml pepstatin] and sonicated in ice water (cell fraction). The culture medium was mixed with the protease inhibitor cocktail (medium fraction). The calprotectin amount in the cell and medium fractions was determined by the Calprotectin enzyme-linked immunosorbent assay (ELISA) Kit (Calprest®; CalproAS, Oslo, Norway) according to the manufacturer's instructions. The absorbance of the substrate solution was measured at 405 nm using a microplate reader (BIO-RAD model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The calprotectin concentration was expressed as ng/μg of DNA. The DNA content was determined fluorometrically, according to the method of Labarca *et al*. (13).

Electrophoretic mobility shift assay

Nuclear extracts were isolated from normal human gingival keratinocytes incubated with the NucBuster™ Protein Extraction Kit (Novagen, Farmstadt, Germany), according to the manufacturer's instructions. Electrophoretic mobility shift assay was performed using the DIG Gel Shift Kit (Roche Molecular Biochemicals, Mannheim Germany). We used the double-stranded oligonucleotide 5′-GGTTTGCTGCTTAAGATGCCTG-3′ (containing the C/EBPα binding site) as probes. Six micrograms of nuclear extract was incubated with digoxigenin-labeled C/EBPα oligonucleotides (2.5 pmol) for 30 min. For competition assay, a 100-fold excess of unlabeled oligonucleotides was added. The C/EBPα binding complex was electrophoresed on a 5% native polyacrylamide gel in 0.4 × Tris-borate/EDTA buffer (pH 8.0), transferred to Immobilon™ (Millipore, Billerica, MA, USA), reacted with alkaline phosphatase conjugated anti-DIG antibody and CSPD® (Roche Molecular Biochemicals), and then developed on RU-X film (Fuji).

Results

Effects of interleukin-1α, TGF-β and calcium on differentiation of normal human gingival keratinocytes

In the control (Fig. 1A–D) and TGF-β-treated (Fig. 1I–L) cells, normal human gingival keratinocytes formed a monolayer, whereas normal human gingival keratinocytes cultured in

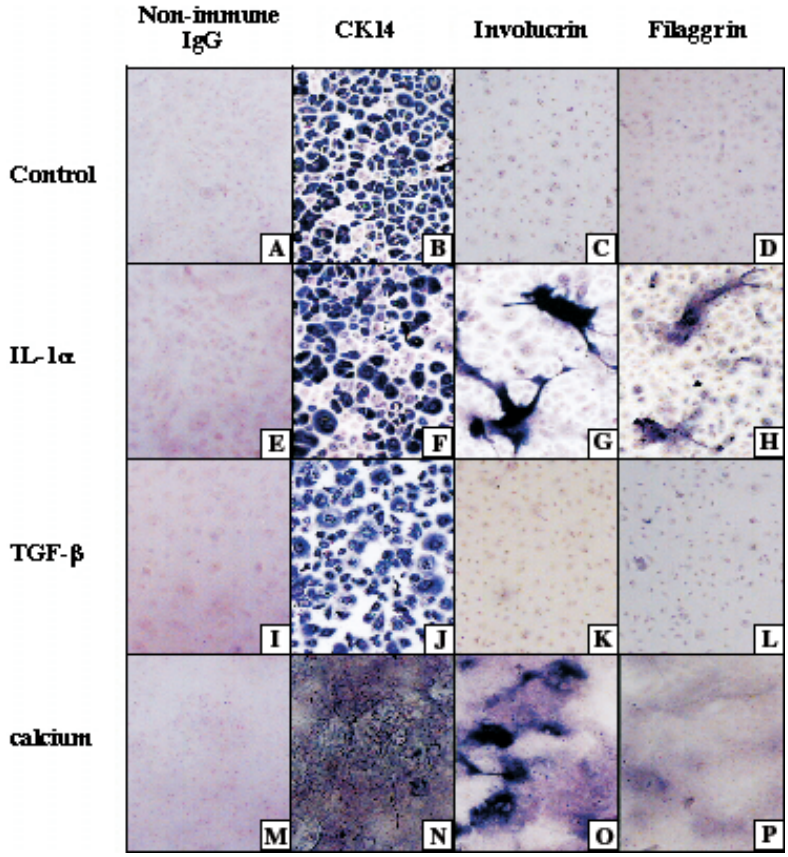

Fig. 1. Immunostaining of cytokeratin 14 (CK14), involucrin and filaggrin on the differentiation of normal human gingival keratinocytes. Normal human gingival keratinocytes were seeded on collagen-coated slides and cultured for 2 d. Cells were untreated (Control: Fig. 1A–D) or treated with 10 ng/ml interleukin-1α (Fig. 1E–H), 10 ng/ml transforming frowth factor-β (TGF-β) (Fig. 1I–L) or 1 mM $CaCl_2$ (Calcium: Fig. 1M–P) for 48 h. Immunostaining was performed with anti-CK14 (Fig. 1B, F, J, N), anti-involucrin (Fig. 1C,G,K,O), anti-filaggrin (Fig. 1D,H,L,P) or nonimmune mouse immunogloblin G (Fig. 1A,E,I,M) as described in the Material and methods. (Original magnifications, × 100).

calcium were multilayered (Fig. 1M–P). Normal human gingival keratinocytes cultured in interleukin-1α were partially multilayered (Fig. 1E–H). In the control, almost all normal human gingival keratinocytes expressed CK14, a marker of the basal cell layer, but expression of involucrin and filaggrin was not observed (Fig. 1B–D). CK14-positive cells were found after interleukin-1α treatment, but the expression of involucrin and filaggrin, which are markers of spinous and granular cells, was induced (Fig. 1F–H). The results of treatment with interleukin-1α were similar to those of calcium as a positive control (Fig. 1N–P). The cells treated with TGF-β expressed CK14, but expression of involucrin and filaggrin was not observed (Fig. 1J–L).

Effects of interleukin-1α, TGF-β, calcium and retinoic acid on the expression of S100A8, S100A9 and involucrin mRNAs in normal human gingival keratinocytes

The effects of interleukin-1α, calcium and TGF-β on the expression of S100A8/A9 mRNAs were investigated. Interleukin-1α and calcium up-regulated the expression of S100A8/A9 mRNAs in normal human gingival keratinocytes, whereas TGF-β suppressed them (Fig. 2A). S100A8 expression was significantly increased by interleukin-1α (8.7-fold) and calcium (9.9-fold). S100A9 expression was significantly increased by interleukin-1α (5.8-fold) and calcium (9.8-fold). TGF-β markedly suppressed the expression of S100A8 (0.3-fold) and S100A9 (0.1-fold) (Fig. 2B). To investigate whether S100A8/A9 expression is related to keratinocyte differentiation, retinoic acid, which is a factor inhibiting keratinocyte differentiation, was added to cultures. Retinoic acid suppressed the expression of S100A8/A9 and involucrin mRNA (Fig. 2C). Although interleukin-1α increased both S100A8/A9 and involucrin mRNA expression, as shown in Fig. 2(A), retinoic acid inhibited the expression of S100A8/A9 and involucrin induced by interleukin-1α (Fig. 2C).

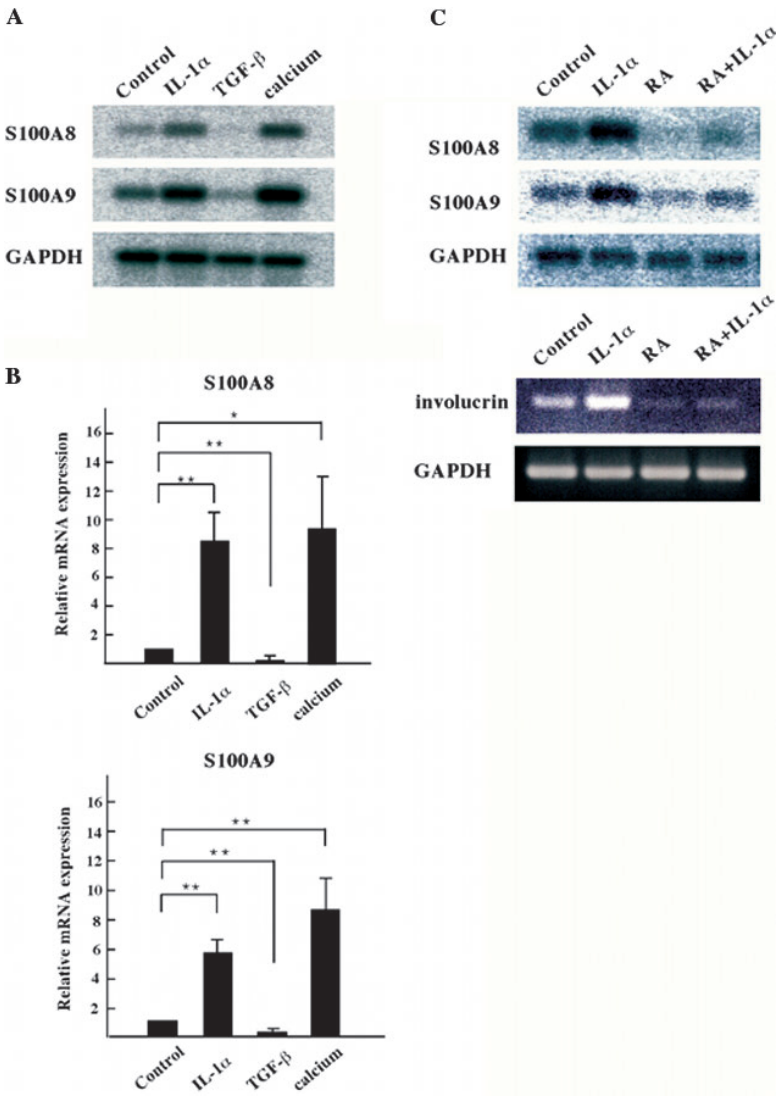

Fig. 2. Northern blotting analysis of S10A8/A9 mRNAs in normal human gingival keratinocytes. (Fig. 2A) Normal human gingival keratinocytes were cultured with 10 ng/ml interleukin-1α, 10 ng/ml transforming growth factor-β (TGF-β) or 1 mM $CaCl_2$ for 36 h. The expression of S100A8/A9 mRNAs was analyzed by Northern blot. (Fig. 2B) The relative ratios of S100A8/A9 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined as described in the Material and methods. Values are expressed as means ± standard deviation (SD) of samples from six separate examples. Significantly different from control (nontreated) (*$p < 0.05$, **$p < 0.01$). (Fig. 2C) Normal human gingival keratinocytes were cultured with 10 ng/ml interleukin-1α and 300 μg/ml retinoic acid for 36 h. The expression of S100A8/A9 mRNAs was analyzed by Northern blotting analysis, and involucrin mRNA expression was analyzed by reverse transcription–polymerase chain reaction (RT–PCR). The data are representative of five separate experiments.

Effects of interleukin-1α, TGF-β and calcium on calprotectin production in normal human gingival keratinocytes

The concentrations of calprotectin in the cell and medium fractions were determined when normal human gingival keratinocytes were cultured with interleukin-1α, TGF-β or calcium for 48 h (Fig. 3). Interleukin-1α and calcium caused an increase in the concentration of calprotectin in the cell fraction, but did not affect that

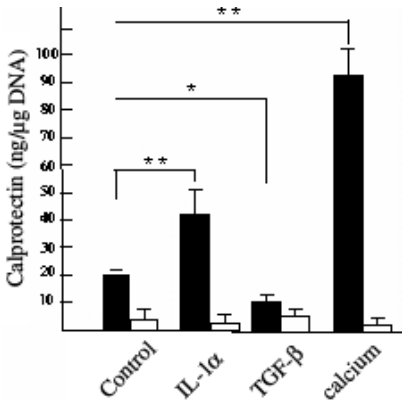

Fig. 3. Determination of the calprotectin concentration in normal human gingival keratinocytes. Normal human gingival keratinocytes were cultured with 10 ng/ml interleukin-1α, 10 ng/ml transforming growth factor-β (TGF-β) or 1 mM $CaCl_2$ for 48 h. The concentration of calprotectin in the cell (closed column) and medium (open column) fractions was determined by enzyme-linked immunosorbent assay (ELISA). Values represent the means ± standard deviation (SD) of five separate experiments. Significantly different from control (nontreated) (*$p < 0.05$, **$p < 0.01$).

amount in the medium fraction. In contrast, TGF-β markedly suppressed the calprotectin concentration in the cell fraction. Regarding the calprotectin concentration in the medium fraction, treatment with these factors for 0.5, 2, 4, 6, 12, 24 and 72 h had no significant effect (data not shown).

Electrophoretic mobility shift assay of DNA C/EBPα binding activity

To examine the mechanisms of interleukin-1α, calcium and TGF-β on calprotectin expression, the effects of these factors on C/EBPα DNA-binding activity were investigated (Fig. 4). The DNA–C/EBPα binding complex was expressed in nontreated (control), interleukin-1α- or calcium-treated cells. Interleukin-1α and calcium increased the binding activity compared with the control. TGF-β suppressed the binding activity. The DNA–C/EBPα binding complex was not observed in the competitive binding assay using excess unlabeled oligonucleotide.

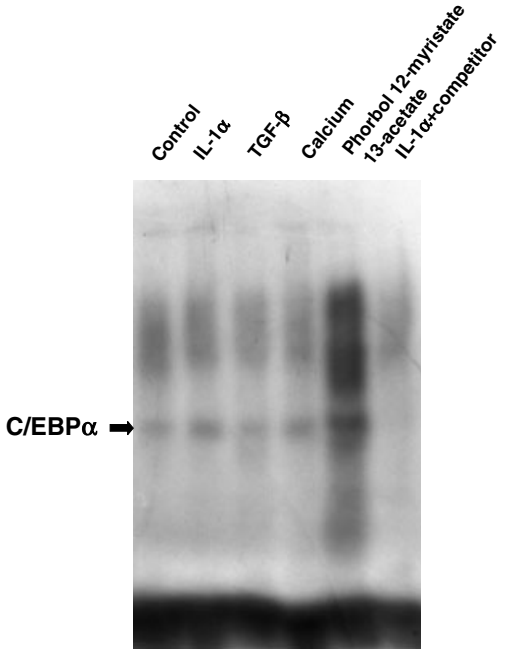

Fig. 4. Electrophoretic mobility shift assay of the DNA–C/EBPα binding activity. Nuclear extract (6 μg of protein) was isolated from normal human gingival keratinocytes incubated with 10 ng/ml interleukin-1α, 10 ng/ml transforming growth factor-β (TGF-β) or 1 mM $CaCl_2$ or 50 ng/ml phorbol 12-myristate 13-acetate for 24 h. Phorbol 12-myristate 13-acetate was used as a positive control. DNA–C/EBP-α binding activity was detected by electrophoretic mobility shift assay with DIG-labeled oligonucleotide. For competition assay, 100-fold excess of unlabeled oligonucleotide was added to the nuclear extract.

Discussion

Interleukin-1α promotes keratinocyte differentiation by the up-regulation of cellular retinoic acid-binding protein type II and of small, proline-rich protein type I, which have been shown to be associated with keratinocyte differentiation (9). In the present study, interleukin-1α stimulated the expression of involucrin and filaggrin (markers of spinous and granular cells of epithelial tissues) in normal human gingival keratinocytes (Fig. 1). These proteins were also increased by the presence of calcium, a well-known differentiation factor. Interleukin-1α and calcium formed a multilayer of normal human gingival keratinocytes, and this result was similar to the report of Liu *et al.*, using human epidermal keratinocytes (14). Interleukin-1α and calcium promote the differentiation of human gingival keratinocytes. TGF-β suppresses the proliferation and differentiation of keratinocytes (7,15). Our results showed that TGF-β inhibited the expression of involucrin and filaggrin. Furthermore, retinoic acid, a negative regulator of keratinocyte differentiation (7), suppressed interleukin-1α-induced involcurin expression. Normal human gingival keratinocyte differentiation is thought to be complexly up- and down-regulated by some factors, including interleukin-1α, calcium, TGF-β and retinoic acid.

There are few reports that describe the regulatory factor of S100A8/A9 expression in keratinocytes. Ross *et al.* (4) showed that phorbol 12-myristate 13-acetate stimulated the expression of S100A8/A9 mRNAs in human gingival keratinocytes, and Jansen *et al.* (16) reported that TNF-α increased their expression in human keratinocytes. We previously found that TNF-α, interleukin-1β and interleukin-6 stimulated their mRNA expression in normal human gingival keratinocytes (data not shown). Phorbol 12-myristate 13-acetate is known to promote keratinocyte differentiation (17). Interleukin-1α and calcium also promoted normal human gingival keratinocyte differentiation and increased the expression of S100A8/A9 mRNAs and protein (Figs 1 and 2). TGF-β is synthesized

by human keratinocytes and inhibits keratinocyte proliferation and also suppresses its differentiation (15,18). TGF-β markedly inhibited the expression of S100A8/A9 mRNA and calprotectin production. Retinoic acid, a well-known negative regulator of keratinocyte differentiation, decreased the expression of involucrin mRNA and inhibited the interleukin-1α-induced expression of S100A8/A9 mRNA. Thorey *et al*. (19) indicated that S100A8/A9 was highly expressed in the differentiated keratinocytes during cutaneous wound repair. Furthermore, S100A8/A9 genes are mapped to the epidermal differentiation complex region on chromosome 1q21. The genes encoding involucrin, loricrin and small, proline-rich protein, marker proteins of keratinocyte differentiation, are also localized in the epidermal differentiation complex region (20). These reports and results indicate that S100A8/A9 expression is regulated by the factors related to keratinocyte differentiation, as well as to inflammation-related factors. We believe that calprotectin expression is associated with keratinocyte differentiation.

Interleukin-1α and calcium significantly increased the concentration of calprotectin in the cell fraction of normal human gingival keratinocytes (Fig. 3). However, these factors had no effect on the concentration of calprotectin in the medium fraction (calprotectin release), and its level was much lower than that in the cell fraction. Although we examined the concentration of calprotectin in the medium fraction after normal human gingival keratinocytes were treated with interleukin-1α and calcium for short period of time (0.5, 2, 4, 6 and 12 h), the effect of two factors on calprotectin release was not observed. On the other hand, we showed that calprotectin release from human neutrophils and monocytes was increased by 30 min of treatment with lipopolysaccharide of *Porphyromonas gingivalis*, interleukin-1β or TNF-α (21,22). These results indicate that the released calprotectin may act on periodontopathic bacteria in the case of neutrophils and monocytes, but, in the case of keratinocytes, the intracellular calprotectin may act on those bacteria that invade gingival keratinocytes.

C/EBPα and -β are expressed in human and mouse keratinocytes (23,24). The expression of C/EBPα mRNA and protein were up-regulated in calcium-induced keratinocyte differentiation (23), and interleukin-1α increased the expression of C/EBPα and -β in mouse keratinocytes (25). Klempt *et al*. (11) found that C/EBPα was a transcription factor related to an increase of S100A9 expression in monocytic cells. In the present study, interleukin-1α and calcium, positive regulators of keratinocyte differentiation, promoted DNA–C/EBPα binding activity in normal human gingival keratinocytes (Fig. 4). These studies suggest that interleukin-1α and calcium induce calprotectin production by increases of C/EBPα expression and DNA–C/EBPα binding activity. It has been reported that TGF-β inhibited differentiation by suppression of C/EBPα in preadipocytes (26). However, the effect of TGF-β on C/EBP in keratinocytes was not known. Our results showed that TGF-β, a negative regulator of keratinocyte differentiation, inhibited DNA–C/EBPα binding activity and calprotectin expression in human gingival keratinocytes. Taken together, C/EBPα is believed to be one of the regulators of calprotectin expression in gingival keratinocytes.

In conclusion, we showed that calprotectin expression is related to the differentiation of gingival keratinocytes and that its expression may be regulated via the C/EBPα pathway.

Acknowledgements

This study was supported, in part, by Grants-in-Aid (#15592190) for Scientific Research from the Japan Society for the Promotion of Science.

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