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Apoptosis of oral epithelial cells in oral lichen planus caused by upregulation of BMP-4

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BACKGROUND: Bone morphogenic protein (BMP-4) is a member of transforming growth factor (TGF-β) family and involved in various functions including apoptosis during neural ectoderm development. The objective of this study is to determine whether BMP-4 is involved in apoptosis, one characteristic, of human oral lichen planus (OLP).
METHODS: Immunohistochemistry and *in situ* hybridization for BMP-4 were carried out in OLP ($n = 21$) and normal human oral mucosa (NOM, $n = 31$). Five tissue samples from NOM and OLP were underwent reverse transcriptase-polymerase chain reaction (RT-PCR). *In vitro* organ culture of oral mucosa was carried out with beads soaked with various concentration of BMP-4 (0.1, 1, and 10 μg/ml). The samples from *in vitro* organ culture were undergone haematoxylin and eosin staining, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling technique (TUNEL) assay, and immunohistochemical study with p53, matrix metalloproteinases (MMP)-1, and MMP-3. Involucrin expression was determined by western blot analysis after treatment with BMP-4 or TGF-β1 on human oral keratinocytes.
RESULTS: In immunohistochemical analysis, expression of BMP-4 was higher in OLP than NOM. BMP-4 mRNA expression was also detected in epithelial cells of both NOM and OLP together with underlying T-lymphocytes by *in situ* hybridization and RT-PCR. In oral mucosa organ culture, BMP-4 soaked beads induced apoptosis of epithelial cells. Acantolysis combined with apoptosis in oral epithelium was observed at 1 μg/ml of BMP-4 beads and it was due in part to the induction of p53 and MMP-1. Even MMP-3 induction was found in lower concentration of BMP-4 (0.1 and 1 μg/ml). Moreover, the expression of MMP-1 and MMP-3 was also observed in OLP. Recombinant BMP-4 or TGF-β1 increased involucrin expression in human oral keratinocytes cell line.
CONCLUSIONS: Expression of BMP-4 of epithelial cells was higher in OLP than NOM. High concentration of BMP-4 caused an apoptosis of oral epithelial cells in oral mucosa organ culture. Therefore, over-expression of BMP-4 is one causing factor for apoptosis of oral epithelial cells through upregulation of p53, MMP1 and MMP3 in OLP.
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Keywords: apoptosis; BMP-4; oral lichen planus

Introduction

Lichen planus is common disease in skin or oral mucosa (1). The difference of lichen planus between skin and oral lesion is their clinical outcome. Skin lesion is usually self-limited and it resolves spontaneously (1). Oral lichen planus (OLP), however, usually goes to chronic and most of them are inflammatory rather than neoplastic conditions (2, 3). The characteristics of OLP are apoptosis, hyperkeratosis, and the infiltration of inflammatory cells in the subepithelial layer and most of them are T-lymphocytes (2– 4). Therefore, OLP has been known as autoimmune disease (4). However, the exact causing factor and pathogenesis are still unknown (5).

In our recent study, bone morphogenic protein (BMP)-4 was expressed in oral epithelium (6). The expression of BMP-4 in the gingival epithelium is explained in the view of the regeneration of the alveolar bone (7). Considering it's various expression sites and functions including morphogenesis, cell differentiation, and apoptosis (8–10), it is plausible to hypothesize that abnormal expression of BMP-4 may be involved in the pathogenesis of OLP. The aim of this study was to examine the expression level of BMP-4 between normal oral mucosa (NOM) and OLP, and to determine whether expression of BMP-4 in OLP is related with apoptosis of oral epithelial cells.

Materials and methods

Materials

Twenty-one samples were taken from the portion of the biopsy specimens in OLP patients including adjacent normal portion (female: 12, male: 9, average age:

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51.43 ± 10.45). Ten samples were taken from the portion of the marginal trimming during tooth extraction and they showed mild inflammation (female: 7, male: 3, average age: 21.20 ± 8.90). Normal portion of OLP patients and additional 10 samples were used as control ($n = 31$). All samples were taken under the regulation of University of Hallym IRB committee with patient's consent. Tissue samples were fixed with 5% paraformaldehyde and embedded in paraffin. Five fresh samples in OLP (male: 2, female: 3, average age: 50.00 ± 12.90) were also taken and NOM was separately taken from resection margin. Samples were stored in liquid nitrogen and undergone reverse transcriptase polymerase chain reaction.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The primer designs were performed based on mRNA sequence of BMP-4 and GAPDH (Table 1). The total RNA was extracted from the homogenized tissue (0.1 g) using TRI REAGENT^{R} under the conditions recommended by the manufacturer (Molecular Research Center, Inc. Cincinnati, OH, USA). The subsequent procedure was carried out according to the manufacturer's protocol. The concentration of total RNA was measured using spectrophotometer (Ultrospec 2000 UV/Visible Spectrophotometer; Pharmacia Biotech, Piscataway, NJ, USA). The 5 μg of the total RNA was used for first strand cDNA synthesis using M-MuLV Reverse Transcriptase (50 U/μl; Stratagene, La Jolla, CA, USA). The reactions were primed with oligo (dT). Aliquots of these reactions were used as templates in the PCR using Taq DNA polymerase (5 U/μl). After denaturing the total DNA at 95°C for 10 mim, the temperature was cycled at 94°C for 30 s, at 56°C for 30 s, at 58°C for 30 s, with 30 cycles for each specimen. The PCR products were run on 1.5% agarose gel for the confirmation.

Immunohistochemistry and the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling technique (TUNEL assay)

Antibodies (goat polyclonal antibody of BMP-4 and monoclonal antibody of p53, raised against a peptide at the amino terminus of p53 of human, transforming growth factor (TGF)-β1, and involucrin anti-human monoclonal antibody) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Matrix metalloproteinases (MMP)-1 and MMP-3 antibodies were goat polyclonal antibody raised against a peptide at the carboxy terminus of human protein and purchased from Santa Cruz Biotechnology Inc. The dilution ratio was 1:20 for BMP-4 and 1:50 for MMP-1, MMP-3, and p53. Sections of 4 μm thickness were prepared for immunohistochemistry as described in our previous report (11). For the detection of MMP-1 and MMP-3, the slide was treated with trypsin for antigen retrieval. We detected BMP-4 and p53 expression without antigen retrieval procedure. Immunohistochemical staining was carried out with Universal LSAB®+ Kits (Dako, Glostrup, Denmark). The positive control for BMP-4 was the adenocarcinoma of the prostate and the immunostaining without primary antibody was used as a negative control. The positive control for MMP-1, MMP-3, and p53 was the squamous cell carcinoma of the oral cavity of which expression had been confirmed previously via RT-PCR, and the immunostaining without primary antibody was used as a negative control.

The TUNEL assay was carried out according to the manufacturer's protocol with Apoptag® Peroxidase kits (Intergen, Purchase, NY, USA). The sections were counterstained with Mayer haematoxylin. For the positive control, specimens were treated for 10 min in DN buffer (30 mM Tris-HCl buffer, pH 7.2, 4 mM $MgCl_2$, 0.1 mM DTT) and DNase (bovine pancreas, 0.1 μg/ml; Amresco Inc., Solon, OH, USA). As negative controls, isotype-matched antibodies were used at the same concentrations as the primary antibodies.

In situ hybridization

The total RNA was extracted from the homogenized tissue using TRI REAGENT® under the conditions recommended by the manufacturer (Molecular Research Center Inc.). Poly(A)$^{+}$ RNA (0.1 μg) was converted to cDNA by reverse transcription using 100 units Molony murine leukaemia virus reverse transcriptase (Pharmacia Biotech). The primers used for making BMP-4 mRNA probe were aattgtttctgggctcatta (sense) and taaagatgcctcacctgact (antisense) and the sequence of mRNA for BMP-4 was acquired from web site (http://www.ncbi.nlm.nih.gov). The product size was 411 bps. Sense and anti-sense digoxigenin-labelled single-stranded RNA probes were synthesized by *in vitro* transcription of BMP-4 cDNAs with DIG RNA Labelling Kit (Roche, Mannheim, Germany). *In situ* hybridization was carried out as described by our previous report (11). Positive controls were the samples of the odontogenic keratocysts and negative controls were a sense probe for BMP-4 was used.

Organ culture of oral mucosa

In vitro organ culture of oral mucosa was carried out as described by Zhang et al. (12) and Thomas et al. (13). BMP-4 beads were prepared using various BMP-4 (R&D Systems, Minneapolis, MN, USA) concentrations from 0.1 to 10 μg/ml and Bio-Gel A (bead size: 150–300 μm, Bio-Rad Inc., Hercules, CA, USA) after washing with phosphate-buffered saline (PBS) before incubating with BMP-4 protein. Control beads were soaked with PBS without BMP-4 protein. All protein-soaked beads were stored at 4°C and used within 1 week.

Table 1 The design of primers

	Primer design	*Product size*
GAPDH	F: AGG ACT CAT GAC CAC AGT CCA T R: TGT TGC TGT AGC CAA ATT CGT T	430 bp
BMP-4	F: GCT AGC CAT TGA GGT GAC TCA C R: TCA TCC AGG TAC AGC ATG GAG A	465 bp

The NOM was taken from human ($5 \times 5 \times 5$ mm size) and they were stored in PBS with horse serum on 4°C for 1 h. Beads were injected in the connective tissue layer and they simulated the infiltrated T-lymphocyte excreting BMP-4. After injecting the control beads or protein-soaked beads to the connective tissue layer in the extracted tissue, they were placed on Millipore filters (pore size, 0.2 μm) supported by metal grids. The tissue was oriented with epithelium side facing down. All tissues were cultured in Dulbecco's minimal essential medium (DMEM) with 10% foetal calf serum (FCS) at 37°C for 24 h in CO_2 incubator. Samples were fixed with 5% paraformaldehyde and processed for immunohistochemistry and TUNEL assay.

Cell cultures, western blotting, and DNA fragmentation assay

Immortalized human oral keratinocytes were kindly gifted from Dr Park NH (UCLA, Los Angeles, CA, USA). It was grown to confluence in Ham's F12/DMEM (Gibco, BRL, Gaithersburg, MD, USA) containing 1% penicillin/streptomycin, FGF-2 (100 μg/ml), and 10% FCS. BMP-4 (Novocastra, Newcastle, UK) was given as 50 and 100 ng/ml. TGF-β1 (R&D systems, Minneapolis, MN, USA) was given 1, 5 and 10 ng/ml. The reaction was stopped with 0.01% trypsin-0.5 mM ethylenediaminetetraacetic acid (EDTA).

Cells were washed with PBS twice, centrifuged at 1800 ***g*** for 5 min and the pellet was mixed with sodium dodecyl sulphate (SDS) sample buffer (0.1 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.01% bromophenol blue). The mixture was heated at 95°C for 20 min. Then, centrifugation was carried out at 10 500 ***g*** for 20 min. The collected pellet was electrophoresed on 12.5% polyacrylamide gels according to the method of Laemmli (14). Gels were then electroblotted onto a polyvinylidene difluoride (PVDF) membrane for immunoblot analysis. After blocking for 1 h with 5% non-fat dry milk in Phosphate Buffered Saline Tween 20 (PBST) (Bio-Rad Inc.), blots were probed with a primary antibody diluted in 0.5% milk in PBST (for 1.5 h at 25°C) and then HRP-conjugated goat antimouse or antirabbit IgG, diluted 1:50 000. The primary antibody for involucrin (1:1000) or BMP-4 or TGF-β1 (1:1000) was used for detection of each signal. Signals were detected by chemiluminescence using the ECL western blotting detection reagents from Amersham Pharmacia (Indianapolis, ID, USA).

The cells treated with 100 ng/ml BMP-4 protein were used for DNA fragmentation assay. The control was untreated cells. Cellular DNA was extracted using Wizard genomic DNA purification kit (Promega Corporation, Madison, WI, USA), according to the protocol provided by the manufacturer. Briefly, cells were harvested, washed in PBS, and lysed in 600 μl of nuclei lysis buffer. The nuclear lysate was treated with 3 μl of RNase A solution at 37°C for 30 min and then incubated with 200 μl of protein precipitation solution on ice for 30 min. The lysate was centrifuged at 15 000 *g* at 4°C to remove proteins from the lysate. The clear supernatant containing cellular DNA was then precipitated with 600 μl of isopropanol, washed with 70% ethanol, air dried, and dissolved in DNA rehydration solution. To analyse fragmented DNA, 20 μg of cellular DNA extracted as described above was electrophoresed through a 1.5% agarose gel, and DNA in the gels was visualized under UV light after staining with 5 μg/ml of ethidium bromide solution.

Quantification and statistical analysis

To compare the expression differences of BMP-4, MMP-1, and MMP-3 between NOM and OLP, the entire tissue section was examined. The expression for each antibody was defined as being negative if the positive cells comprised 0–25%, and positive when the proportion of positive cells comprised >25%. The positive cells should express protein in whole cytoplasm, not focally. Two investigators, who were unaware of the antibody information, examined all the stains independently. These findings were analysed using a chi-square test.

For quantification of organ culture, the number of TUNEL, MMP-1, and MMP-3 positive cells in the epithelium of control and BMP-4 bead group were counted. The values were calculated as a percentage of keratinocytes positive for each protein or TUNEL. At least 500 epithelial cells were counted. The mean values were compared by means of two-tailed *t*-test. The significant differences were defined as the *P*-values were < 0.05.

Results

RT-PCR, immunohistochemistry and in situ hybridization

First, we screened the expression of BMP-4 mRNA in NOM and OLP by RT-PCR. All samples expressed *BMP-4* in both NOM and OLP (Table 2). Its localization was shown by immunohistochemistry and *in-situ* hybridization. In NOM, BMP-4 was strongly expressed in epithelium (Fig. 1a) and its level was especially high in the inflammatory epithelium (Fig. 1b). The immunopositive cells were observed from the middle layer of the oral epithelium. BMP-4 was not expressed in basal layer of oral epithelium. In OLP, the expression of BMP-4 was higher in OLP than NOM (Fig. 1c). The expression of BMP-4 was also observed in the subepithelial

Table 2 RT-PCR results for each primer set

Case	*Age*	*Sex*	*Site*	*BMP-4*	*GAPDH*
MSJ	50	F	Control	+	+
			OLP	+	+
JOK	62	F	Control	+	+
			OLP	+	+
KMY	59	M	Control	+	+
			OLP	+	+
JSJ	50	F	Control	+	+
			OLP	+	+
LCO	29	M	Control	+	+
			OLP	+	+

OLP, oral lichen planus; BMP, bone morphogenic protein; FGF, fibroblast growth factor.

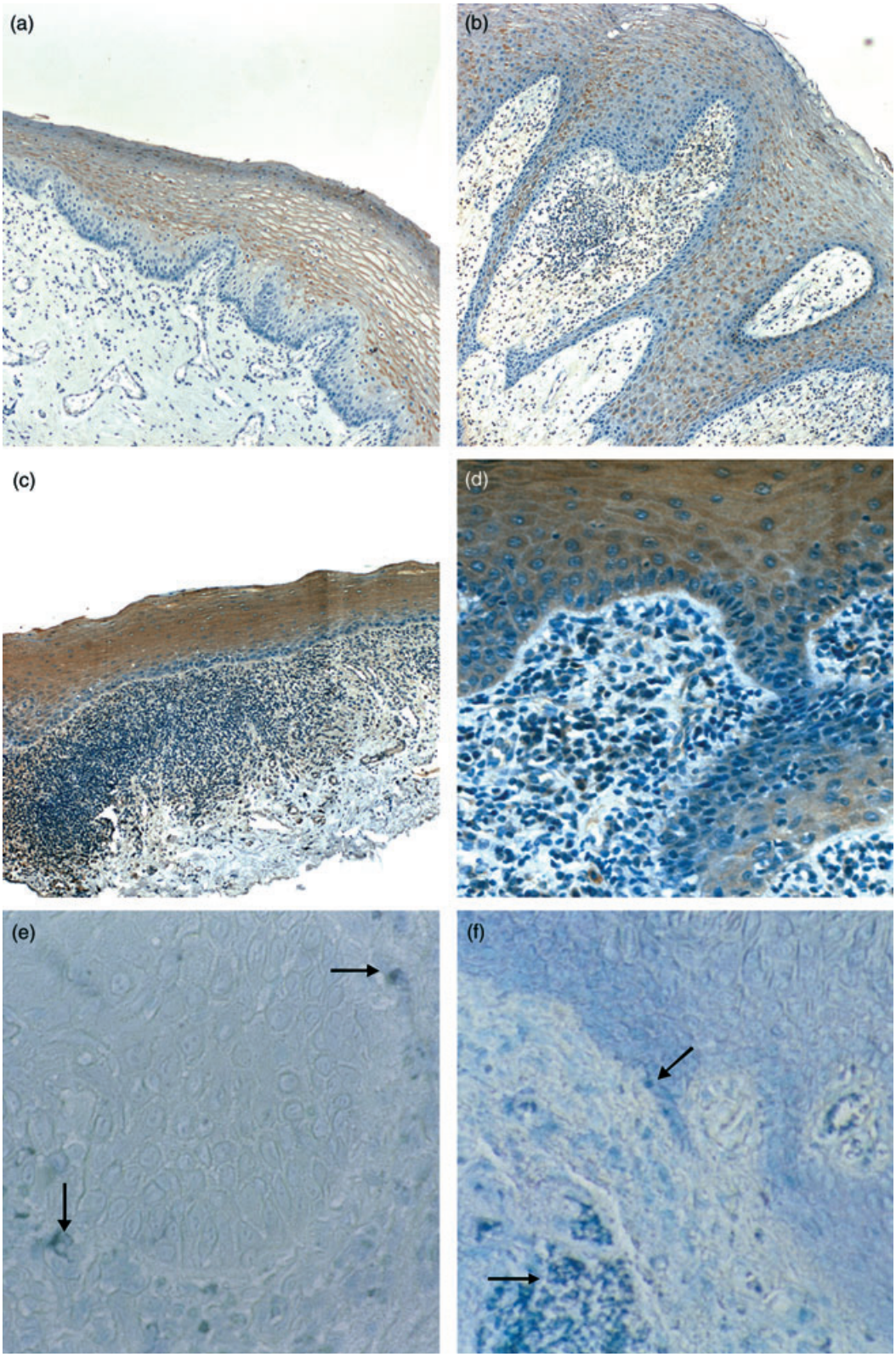

Figure 1 Expression of BMP-4 in normal oral mucosa (NOM) and oral lichen planus (OLP) by immunohistochemistry and *in situ* hybridization. (a) BMP-4 expression in NOM (×40). This pattern of expression was assumed as a negative in the comparative analysis. (b) BMP-4 expression in inflammatory oral mucosa (×40). Superficial layer kept its pattern of expression like NOM. However, its expression was increased in the rete peg of inflammatory region. (c) BMP-4 expression in OLP (×40). (d) BMP-4 expression was also observed in thick inflammatory cells in OLP (×200). (e) BMP-4 mRNA was expressed in the basal layer of epithelium and lymphocytes (×200). (f) BMP-4 was highly expressed in the collected T-lymphocytes (×200). (g) p53 expression was sparsely observed in early OLP (×200). (h) p53 expression was evident in established lesion (×200). (i) MMP-1 expression was observed in the epithelium of OLP (×100). (j) MMP-3 expression was observed in the epithelium of OLP (×100).

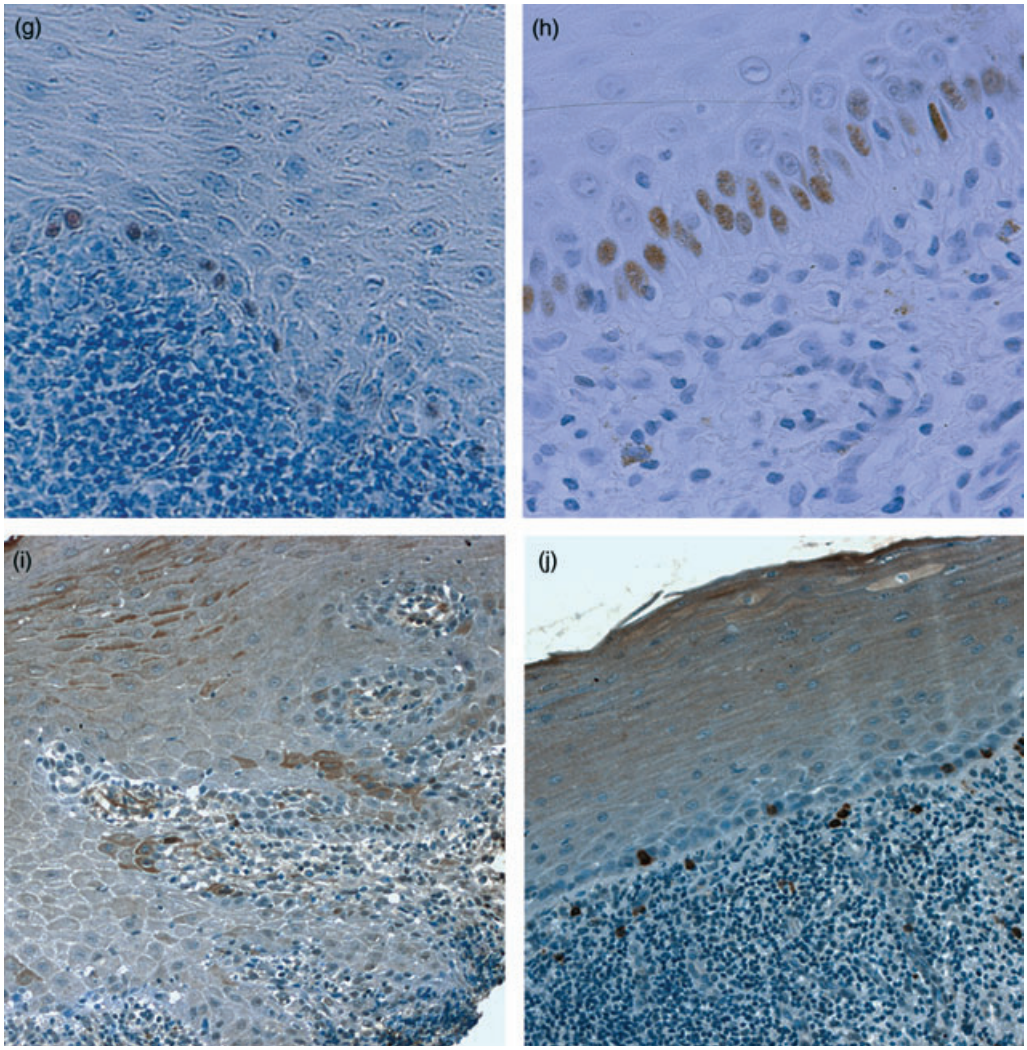

Figure 1 Continued.

inflammatory tissues (Fig. 1d). BMP-4 mRNA in OLP was detected in both epithelial cells and subepithelial inflammatory cells (Fig. 1e,f).

In apoptosis, the expression of p53 and MMPs has been known as important parameters in tissue. As shown in Fig. 1g, the p53 expression was sparsely observed in early stage. However, almost all cells in the basal cell layer and in the entire OLP section were immuno-positive in established lesion of OLP (Fig. 1h). Eleven samples from OLP showed MMP-1 positive (Fig. 1i: 55.0%). Seven samples from OLP showed MMP-3 positive (Fig. 1j: 35.0%). The expression of MMP-1 or MMP-3 was not detected in two samples (10.0%). The difference in the expression of MMP-1 and MMP-3 between OLP and NOM was significant (Table 3; $P < 0.001$). Taken together, these results indicate that BMP-4 expression is higher in OLP than NOM and abnormal upregulated BMP-4 expression may induces apoptotic genes p53, MMP-1 and MMP-3 in OLP.

In vitro organ culture of oral mucosa

Based on BMP-4 expression pattern in patient's samples, we further studied the effect of BMP-4 on

Table 3 The immunoreactivity of BMP-4, MMP-1, and MMP-3 in the normal oral mucosa and the oral lichen planus

	BMP-4			*MMP-1*			*MMP-3*		
	Negative	*Positive*	*P-value*	*Negative*	*Positive*	*P-value*	*Negative*	*Positive*	*P-value*
NOM	28	3	0.000	30	1	0.000	29	2	0.022
OLP	7	14		10	11		14	7	

NOM, normal oral mucosa; OLP, oral lichen planus.

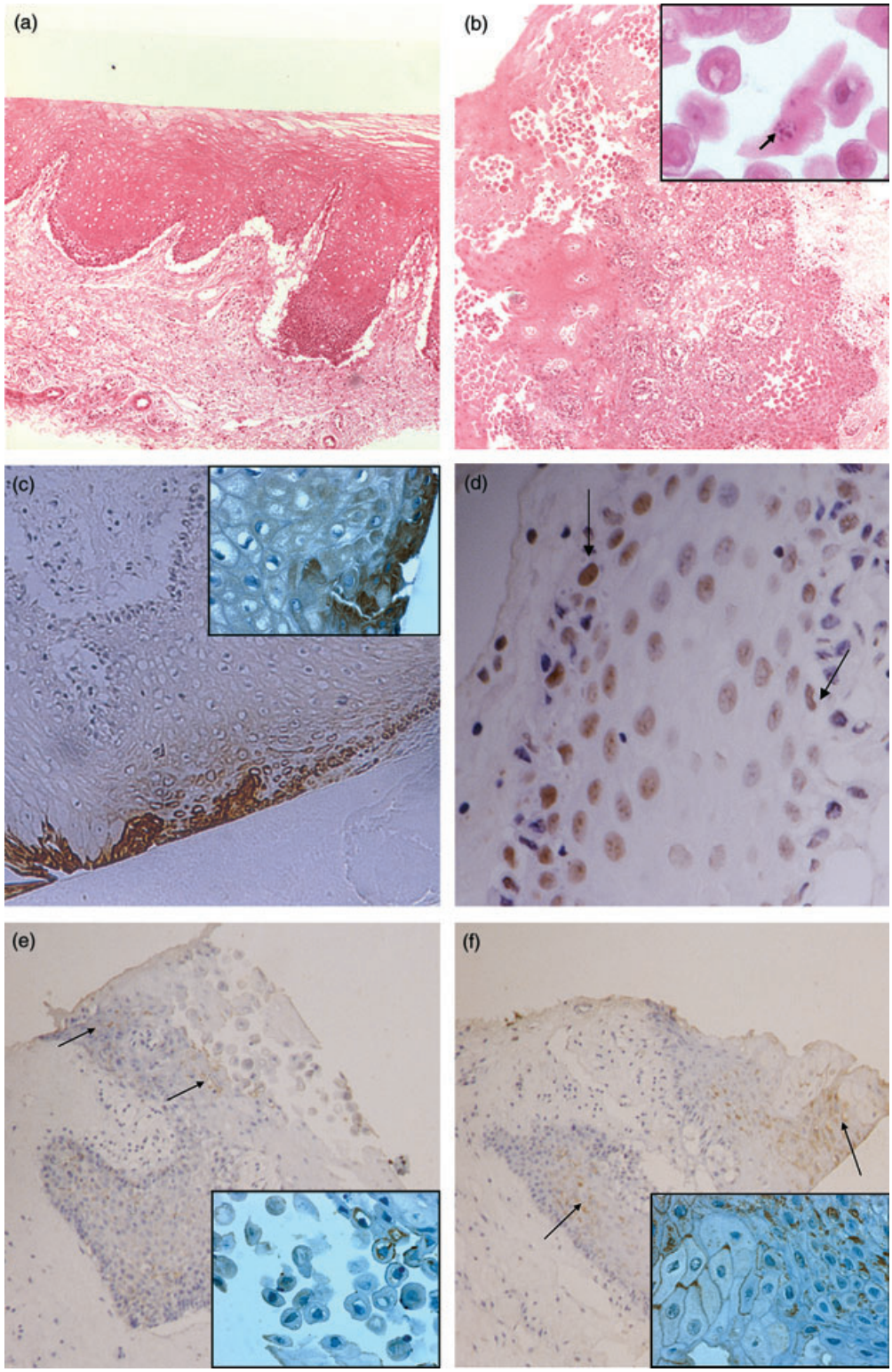

Figure 2 BMP-4 induced the expression of p53 and MMPs in oral mucosa organ culture. (a) The specimens of BMP-4 bead application (control, ×40). (b) The acantolysis was observed in 1 μg/ml of BMP-4 bead (×40). Interestingly, some cells showed cellular division (arrow) and the chromatin were distributed as dots in the nucleus (small box, ×200). (c) p53 was highly expressed in 10 μg/ml of BMP-4 bead (×100). Interestingly, only cytoplasm was shown p53 positive (small box). (d) TUNEL assay positive cells (arrows) were observed in 10 μg/ml of BMP-4 bead (×400). (e) MMP-1 was expressed in epithelial cells (arrows, 1 μg/ml of BMP-4 bead, ×40). Its expression was observed in cellular membrane (small box, ×400). (f) MMP-1 was expressed in epithelial cells (arrows, 10 μg/ml of BMP-4 bead, ×40). Its localized distribution was observed (small box, ×400). (g) MMP-3 was expressed in epithelial cells (arrows) (0.1 μg/ml of BMP-4 bead, ×40). Interestingly, the expression was localized in the basal layer (small box, ×400). (h) MMP-3 was expressed in epithelial cells (arrows, 1 μg/ml of BMP-4 bead, ×40). Its expression was observed in the cellular membrane of acantolytic cells (small box, ×400).

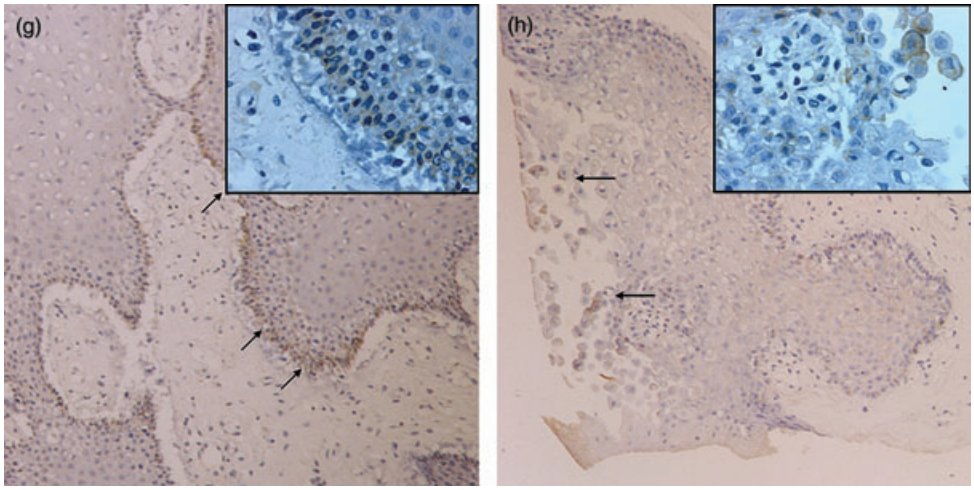

Figure 2 Continued.

oral epithelium with respect to the expression of apoptotic parameters using *in vitro* organ culture of oral mucosa.

There was no difference in gross morphology among control, 0.1 and 10 μg/ml group when haematoxylin and eosin staining was carried out (Fig. 2a). The 1 μg/ml group showed acantolysis in gross examination (Fig. 2b). Among acantolytic cells, some showed fragmented chromatin in the nucleus (Fig. 2b). The p53 was detected only in the 10 μg/ml group and it was localized mainly in the cytoplasm (Fig. 2c). We could not detect p53 in the other samples. The cells positive to TUNEL assay were observed in 1 and 10 μg/ml group (Fig. 2d). These cells showed eccentrically condensed chromatin or lobulated chromatin. MMP-1 was induced in 1 and 10 μg/ml of BMP-4 beads (Fig. 2e,f). MMP-3 was induced in 0.1 and 1 μg/ml of BMP-4 beads (Fig. 2g,h). Interestingly, the acantolytic cells of 1 μg/ml BMP-4 beads showed both MMP-1 and MMP-3 expression (Fig. 2e,h). These results suggest that BMP-4 can induce apoptosis through upregulation of MMPs and p53 (Table 4).

Effects of BMP-4 and TGF-β1 on keratinocyte differentiation and apoptosis

To determine whether BMP-4 has an effect on differentiation of oral epithelial cells, we measured involucrin expression in human oral keratinocytes by western blot analysis. As shown in Fig. 3a, both TGF-β1 and BMP-4 increased the expression of involucrin in oral keratinocytes, and TGF-β1 had more potent effect on involucrin expression. This result means that BMP-4 has a stimulatory effect on differentiation in oral epithelial cells. Furthermore, DNA fragmentation assay was a positive in BMP-4 treated group (Fig. 3b). Thus, BMP-4 could induce apoptosis in cultured oral keratinocytes.

Table 4 The quantitative analysis of TUNEL assay, MMP-1, and MMP-3 expression in organ cultured samples

	TUNEL			*MMP-1*			*MMP-3*		
	Mean	*SD*	*P-value*	*Mean*	*SD*	*P-value*	*Mean*	*SD*	*P-value*
Control	0.04	0.01		0.03	0.00		0.13	0.04	
0.1 μg/ml	0.11	0.04	NS	0.08	0.06	NS	0.51	0.02	0.000
1.0 μg/ml	0.56	0.06	0.004	0.54	0.10	0.001	0.74	0.07	0.000
10 μg/ml	0.74	0.10	0.000	0.85	0.04	0.001	0.07	0.02	NS

NS, not significant.

Discussion

In this study, we found that BMP-4 is expressed in oral epithelial cells of middle layer epithelium and has a stimulatory effect on keratinocyte differentiation. High concentration of BMP-4 induced apoptosis of oral epithelial cells in oral mucosa organ culture. BMP-4 also induced apoptosis related genes including p53, MMP-1 and MMP-3. Therefore, higher expression of BMP-4 in OLP than NOM is one factor for apoptosis of oral epithelium through the upregulation of p53 and MMP-1 and MMP-3.

Expression of BMP-4 was higher in OLP than NOM

Both BMP-4 and BMP-4 mRNA were detected in NOM and OLP (Table 2). The pattern of BMP-4 expression in NOM was overlapped with K1 and K10 expression in previous report (15). Considering that BMP-4 increases the expression of involucrin in cultured oral keratinocytes (Fig. 3), BMP-4 might be related to the maturation of oral keratinocytes. BMP-4 expression was significantly higher in OLP than NOM ($P < 0.001$; Table 3). The sporadic expression in K1 was also reported in OLP (15). Altered expression of BMP-4 in OLP might result in sporadic expression of K1. In *in situ* hybridization, the basal epithelial cells and subepithelial inflammatory cells were positive to BMP-4 mRNA (Fig. 1e,f). In OLP, these inflammatory cells were reported as mainly T-lymphocytes (16). T-lymphocyte could express BMP-4 (17). Thus, the inflammatory cells that expressed BMP-4 in OLP could be T-lymphocytes

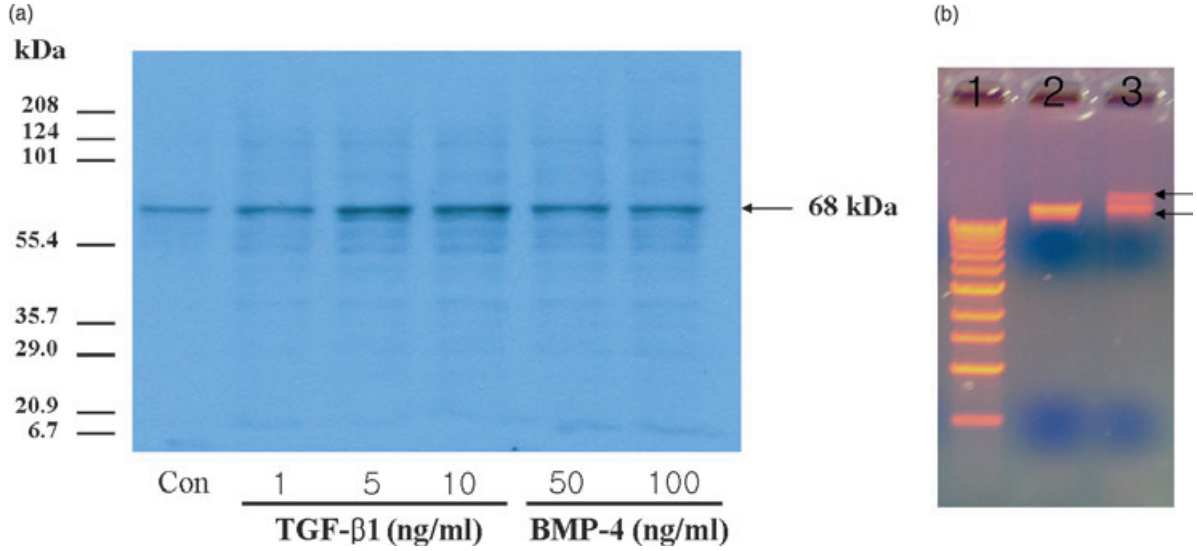

Figure 3 (a) Involucrin was upregulated by TGF-β1 or BMP-4. Confluent human keratinocyte cells were treated with various concentrations of TGF-β1 or BMP-4 for 24 h. Total protein extract (30 μg per lane) were electrophoresis and involucrin expression was determined by western blot analysis as described in meterials and methods. Control (Con). (b) Cellular DNA was fragmented in 100 ng/ml of BMP-4 (arrows in lane 3). Lane 1 was 1.5 kbps DNA ladder and lane 2 was control.

in subepithelial layer and the concentration of BMP-4 in OLP could be higher than in NOM because of their active production.

High concentration of BMP-4 induced p53 and apoptosis in oral epithelium

The protein of the TGF- β family shows different pattern of action as its concentration. BMP-4 can act as an apoptosis signal in some stage of development (8, 9). Thus, the apoptosis of the basal cell in the OLP may be related to the over-expression of BMP-4 from combined tissue of epithelium and infiltreated T-lymphocytes in subepithelial layers. The organ culture was designed to prove this hypothesis. BMP-4 soaked beads were implanted in the subepithelial tissue and they simulated to BMP-4 expressing T-lymphocytes. In results, p53 was detected in the 10 μg/ml group (Fig. 2c). The pattern of immunopositive p53 signal in NOM is different from the findings in OLP. The p53 was detected in the nucleus and cytoplasm of each cell in OLP, however, it was detected only in cytoplasm in NOM (Figs 1g,h and 2c). The presence of p53 in the nucleus has been known in special situation such as the damaged DNA (18). Otherwise, p53 will be degraded rapidly in the nucleus. BMP-4 could induce p53 and p53 protein was detected in the cytoplasm (Fig. 2c). Its production might be dependent on the signal from extracellular BMP-4 concentration. The basal cells were not always expressed p53 in the early stage of OLP (Fig. 1g). T-lymphocytes infiltration was already established in this stage. Thus, p53 expression in OLP was followed by T-lymphocytes infiltration and it might be induced by cytokine from T-lymphocytes. Considering that high concentration of BMP-4 could induce p53 (Fig. 2c) and T-lymphocytes in OLP could produce BMP-4 (Fig. 1e,f), p53 expression in OLP might be induced by BMP-4 from T-lymphocytes. TUNEL positive cells were also observed in 1 and 10 μg/ml group (Fig. 2d). The cells showed a typical apoptotic cells phenotype as dot-like fragmented chromatin (Fig. 2b). In case of caspase-mediated apoptosis, a large-scale DNA fragmentation can be observed (19). The application of BMP-4 to cultured oral keratinocytes showed a positive to DNA fragmentation assay (Fig. 3b). Thus, the action of BMP-4 was concentration dependent and high concentration of BMP-4 could induce the apoptosis in normal oral epithelium probably via p53-mediated pathway. In OLP, prominent p53-positive staining was seen in all biopsies in our samples (Fig. 1g,h) and the previous report (20).

BMP-4 induced MMP-1 and MMP-3 dose-dependently

Another feature of OLP is hyperkeratosis or erosive in oral epithelium. It may be due to the exfoliation disorder of oral epithelial cells. MMP families can do the breakdown of intercellular bridge. The cultured oral cancer cell produces at least two matrix-degrading enzymes, which are identified as MMP-2 and MMP-3 (21). TGF-β family can regulate the expression of MMP (22) and BMP-4 is a family of TGF-β. In our experiments, BMP-4 beads did not induce MMP-2 (data not shown). MMP-1 was induced in 1 μg/ml and 10 μg/ml of BMP-4 beads (Fig. 2e,f). MMP-3 was induced in 0.1 μg/ml and 1 μg/ml of BMP-4 beads (Fig. 2g,h). The expression of MMP-1 is observed in OLP and oral squamous cell carcinoma (OSCC) (23). The expression of MMP-3 is also observed in OSCC (24). Interestingly, the epithelium of 1 μg/ml BMP-4 beads showed both MMP-1 and MMP-3 expression and they showed acantolysis (Fig. 2b). The acantolysis may induce ulcer or erosive change in mucosa. In this study, the expression of MMP-1 and MMP-3 was also observed in OLP (Fig. 1i,j). Though we did not find

the sample that expressed both, if the lesion expressed both, they might undergo erosive change because of the exfoliation of epithelial cells. Thus, the level of expression of BMP-4 may be important to determine the phenotype of OLP.

Based on our results, it may be possible that over-expression of BMP-4 in OLP can induce apoptosis through induction of p53 and BMP-4 mediated upregulation of MMPs can disrupt basement membrane (23). Recently, we found that BMP-4 was highly expressed in the down-growing epithelium and the daughter cysts of the odontogenic keratocyst (25) and we also found the expression of MMPs in these samples (data not shown). BMP-4 is detected in the basal layer in NOM and it may be related to the terminal differentiation of oral epithelium. If the stress like chronic chemical or physical irritation, or viral infection may increase the expression of some genes like BMP-4 together with immune response. The abnormal cells can induce T-lymphocyte mediated immune reaction and this can induce accumulation of BMP-4 in the subepithelial layer. The high concentration of BMP-4 can induce the apoptosis in basal cell and epithelial detachment via MMP expression. The apoptosis of basal cell is a common finding in OLP and these cells are TUNEL positive (2, 3). Though p53 expression was only observed in 10 μg/ml BMP-4, we could not rule out the probability that it might be expressed in low BMP-4 concentration too. In summary, over-expression of BMP-4 is one causing factor for apoptosis of oral epithelial cells through upregulation of p53, MMP-1 and MMP-3 in OLP.

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