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Collagen XVIII modulation is altered during progression of oral dysplasia and carcinoma

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BACKGROUND: Collagen XVIII is a ubiquitous basement membrane (BM) component and a precursor of endostatin.

METHODS: Using immunohistochemistry and *in situ* hybridization, we studied the expression and localization of collagen XVIII in different stages of normal oral wound healing, epithelial dysplasia and squamous cell carcinoma (SCC).

RESULTS: In mild epithelial dysplasias collagen XVIII appeared as a continuous signal in the BM, whereas in severe epithelial dysplasias and in the invasive areas of oral SCCs collagen XVIII was absent. *In situ* hybridization showed that collagen XVIII mRNA expression did not decrease in severe dysplasia or oral carcinoma samples when compared with the mild dysplasias.

CONCLUSIONS: The results indicate that the absence of collagen XVIII protein in severe oral dysplasias is related to the processing of the protein rather than to changes in mRNA expression.

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Introduction

Oral epithelial dysplasia is a potentially malignant lesion that is graded as mild, moderate or severe. The severe form is considered to be a risk factor for oral carcinoma, which is highly invasive and has a high mortality rate. Impaired distribution of basement membranes (BMs) has been associated with the progression of dysplasias and carcinomas. Of the most common BM components, collagen IV, laminin-1 and laminin-5 are expressed discontinuously and weakly in the BM in invasive front of severe dysplasia and carcinoma (1, 2). Protein distribution of collagen VII, a BM anchoring fibril component, is impaired in oral carcinomas, but mRNA is expressed at high levels. Collagen VII has also been detected surrounding epithelial tumour cell islands (3). In contrast to these proteins, collagen XVII, a hemidesmosomal component, is upregulated in suprabasal keratinocytes in moderate and severe dysplasias and at the invasive front of squamous cell carcinomas (SCCs) (4).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteases that degrade several components of the extracellular matrix and the BM. MMPs are often upregulated in different normal and pathological conditions that require matrix remodelling, such as in carcinomas. Significantly higher MMP-9 mRNA levels have been detected in oral dysplasias that progressed to oral carcinoma than in those that did not (5). Elevated MMP-9 levels have also been detected in oral carcinoma samples and the enzyme may be linked with aggressive tumours and shortened survival prognosis (6). High MMP-9 expressions in tumour cells also correlate with metastasis in oral SCC patients (7).

Collagen XVIII is a non-fibrillar collagen that is expressed in the epithelial and vascular BMs throughout the body (8). Endostatin is a C-terminal fragment of collagen XVIII that inhibits angiogenesis and tumour progression. Endostatin has been shown to suppress cell migration and invasion of head and neck SCC cells (9) and it inhibits the activation of MMP-2 and MMP-9 in human tongue SCC cells (10). Endostatin has also been shown to interact with $\alpha 5$ and $\alpha(V)$ integrins of human endothelial cells, supporting cell migration when immobilized and inhibiting migration when soluble (11). MMPs, such as MMP-9, are known to generate endostatin fragments from human collagen XVIII (12).

To study the expression pattern of collagen XVIII and MMP-9 in different stages of oral dysplasia and carcinoma, we analysed surgical tongue SCC and oral dysplasia samples and SCC cell lines. The study demonstrates that collagen XVIII protein is degraded during

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the breakdown of epithelial BM in the oral mucosa in severe oral dysplasias and oral SCC, but no significant change is detected in the mRNA expression in these conditions when compared with normal oral epithelium. No significant correlation was seen between MMP-9 protein levels and the severity of dysplasia.

Materials and methods

Tissue samples

The use of human tissues was approved by the Ethical Committee for the Faculty of Medicine, University of Oulu. Clinically and histologically diagnosed oral dysplasia (mild, $n = 3$; moderate, $n = 6$; and severe, $n = 7$) and tongue SCC samples ($n = 10$) were surgically removed as a part of clinical treatment and were obtained from the Department of Pathology, University of Oulu. The wounds were made to the oral mucosa of healthy volunteers and collected 1, 5 and 7 days after the wound was inflicted at the Institute of Dentistry, University of Oulu. The tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin wax or alternatively frozen and embedded in TissueTek® (Sakura Finetek, Zoeterwoude, the Netherlands). The sections were cut to 6-μm thickness and placed on glass slides. The paraffin slides were kept overnight at 37°C and the frozen sections were kept at −70°C.

Cell culture

HaCaT cells (13) (a kind gift from Dr N. Fusenig, German Cancer Research Center, Heidelberg, Germany) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated foetal calf serum, 250 ng/ml fungizone, 100 units/ml penicillin and 100 μg/ml streptomycin. Human SCC cell lines SCC-25 (tongue, CRL-1628, ATCC, Rockville, MD, USA) and HSC-3 (tongue, JRCB 0623, Japan Health Science Research Resources Bank, Osaka, Japan) were cultured in 1:1 DMEM and Ham's Nutrient Mixture F-12, which was supplemented with 10% heat-inactivated foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 units/ml nystatin, 250 ng/ml fungizone, 1 mM sodium pyruvate, 2 mM L-glutamine (all from Gibco, Invitrogen Corporation, Paisley, Scotland, UK) and 0.4 ng/ml hydrocortisone (Diosynth, Oss, the Netherlands). The subconfluent cells were cultured in normal medium for 24 h and then incubated in a serum-free medium with or without 100 nM PMA (tumour promoter phorbol-12-myristate-13-acetate, Sigma, Poole, UK) for 1 or 3 days. All cell culture experiments were performed three times. Total RNA was isolated from the cells for polymerase chain reaction (PCR) and conditioned cell culture media was used for Western blotting and zymography. For Western blotting, the media was concentrated by using Amicon Ultra-4 10 000 MWCO centrifugal filter tubes (Millipore, Bedford, MA, USA).

RNA isolation and PCR

Total RNA was extracted from the cells that were cultured for 1 day using Trizol® kit (Gibco BRL, Life Technologies Inc., Roskilde, Denmark). Five micrograms of total RNA was reverse-transcribed by using Superscript II RNaseH-Reverse Transcriptase (Gibco BRL) and random primers. PCR was performed with Dynazyme DNA polymerase (Finnzymes Oy, Espoo, Finland) using primers designed against the common region of all collagen XVIII variants 5′-CGTGAC-TTCTCACTGCTGTTC-3′ and 5′-CACGTGGAGAC-AGAATCTGA-3′ (14) and β-actin control primers (15).

Antibodies

Polyclonal MMP-9 antibody was purchased from Neomarkers (Fremont, CA, USA). Generation of monoclonal antibody DB144-N2 that detects all three variants of collagen XVIII has been reported earlier (16), as has the polyclonal anti-all huXVIII collagen XVIII antibody QH48.18 that detects all three variants of collagen XVIII (14). HES.6 polyclonal anti-human endostatin antibody has also been reported earlier (12). A novel polyclonal anti-human endostatin antibody Q101.1 was generated as follows. A fragment corresponding to amino acid residues 1205–1516 of human collagen XVIII (C-terminal non-collagenous domain NC11) (17) was amplified by PCR, cloned into pQE-41 vector containing dihydrofolate reductase (DHFR) fusion part (Qiagen, Santa Clarita, CA, USA), expressed in *Escherichia coli* as suggested by the manufacturer, and purified using nickel-chelating resin (ProBond, Invitrogen, Leek, the Netherlands) and HiTrapQ anion-exhange column (Amersham Pharmacia Biotech, Uppsala, Sweden). A rabbit was injected subcutaneously with the purified fusion protein in complete Freund's adjuvant (Sigma Chemical Co., St Louis, MO, USA), followed by booster injections in incomplete Freund's adjuvant at an interval of 2 weeks. For affinity purification of the rabbit antiserum, an additional PCR-amplified polypeptide corresponding to residues 1333–1516 of human collagen XVIII (endostatin fragment) was expressed in *E. coli* from the pQE-31 vector without the DHFR leader sequences (Qiagen), and purified as described above. The polyclonal antiserum was purified on a CNBr-activated Sepharose 4B column (Amersham Pharmacia Biotech) coupled with the purified endostatin fragment as described elsewhere (16). The specificity of the affinity-purified polyclonal anti-human endostatin antibody named Q101.1 was verified by Western blotting against hepatoblastoma-derived human collagen XVIII (12) and recombinant human collagen XV produced in insect cells (Huhtala Pirkko et al., unpublished observations).

Immunohistochemical staining

All washes were performed twice in PBS. Paraffin sections were treated with xylene three times for 5 min and rehydrated with ethanol. The tissue sections were incubated in 0.3% H_2O_2 in methanol for 3 h (30 min for frozen sections) to block the endogenous peroxidase activity. The sections were microwaved in 0.01 M citrate (for anti-all huXVIII QH48.18) and washed. Non-specific binding was blocked with 2% normal goat serum for 30 min at room temperature (RT). Thereafter,

the sections were incubated with the primary antibody (diluted in PBS with 1% BSA) o/n at 4°C in a humid chamber. Sections incubated with non-immune rabbit IgGs instead of primary antibodies were used as negative controls. The sections were incubated with biotinylated secondary antibody solution (Anti-Rabbit IgGs, Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. After washes the sections were treated with Vectastain Elite ABC reagent (Vector Laboratories) for 30 min at RT. The tissue sections were stained with AEC (Zymed, San Francisco, CA, USA) for 10 min according to the kit instructions. The sections were counterstained with Mayer's haematoxylin (Histolab Products AB, Göteborg, Sweden). Different cells, such as plasma cells and granulocytes were identified by the cellular structure with a light microscope using the largest magnification.

In situ hybridization

A 272-bp fragment detecting all variants of collagen XVIII was subcloned to plasmid Bluescript SK and linearized (14). The digoxigenin-11-UTP-labelled (Boehringer, Mannheim, Germany) antisense and sense RNA probes were prepared with T3- and T7-RNA polymerases respectively. The paraffin sections were deparaffinized with xylene, rehydrated with ethanol and fixed with 4% paraformaldehyde. After PBS wash the sections were treated with proteinase K (10 μg/ml, Roche Diagnostics, Mannheim, Germany) for 10 min at 37°C and the reaction was stopped with 0.2% glycine. The sections were acetylated with acetic anhydride (0.5% in 0.1 M triethanolamine) and incubated in 4x SSC. The hybridization took place at 56°C overnight and after 0.1x SSC washes the hybridized DIG-labelled probe was detected with the antibody (100 mM Tris–HCl, 150 mM NaCl, pH 7.5, with 0.1% Triton X-100, 1% normal goat serum, 1:200 anti-digoxigenin-alkalic phosphatase Fab fragments, Roche Diagnostics, Mannheim, Germany). The AP-conjugated antibody was detected by Fast Red colour substrate (Boehringer Mannheim) and the sections were counterstained with Mayer's haematoxylin (Histolab Products AB).

Western blotting

The concentrated 3-day culture medium samples were heated at 60°C for 20 min in sample buffer containing 10% SDS and then an equal amount of total protein was loaded onto a 10% (anti-all) or 12% (anti-endostatin) SDS-PAGE gel. After electrophoresis, 0.5% Coomassie Brilliant Blue R-250 staining was performed and the proteins were transferred to Immobilon P membrane (Millipore, Bedford, MA, USA). The membrane was treated with 5% non-fat milk for 1 h, incubated with the primary antibody (anti-all huXVIII QH48.18 or DB144-N2 or anti-human endostatin Q101.1 or HES.6) at RT o/n, washed and incubated with anti-rabbit secondary antibody (for polyclonal antibodies, 1:1000, DAKO A/S, Glostrup, Denmark) or anti-mouse secondary antibody (for monoclonal antibodies, 1:1000, DAKO A/S) for 1 h at RT. After washes the membrane was incubated with ABComplex/HRP (1:1000, DAKO A/S) for 1 h. The membrane was treated with ECL Western blotting detection reagent for 1 min and then exposed to Hyperfilm-ECL (Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK).

Zymography

Gelatin zymography was performed three times for the 1- and 3-day culture medium samples using an equal volume of medium on a 10% SDS-PAGE gel that had been prepared with 1 mg/ml fluorescently labelled (with 2-methoxy-2,4-diphenyl-3-[^{2}H]furanone, Fluka, Ronkonkoma, NY, USA) gelatin. Purified MMP-9 (1:80) from human gingival keratinocyte culture media and purified MMP-2 (1:80) from human gingival fibroblast cultures were used as controls to identify the proteins (18). After electrophoresis SDS was removed from the gel with 2.5% Triton X-100 and the gel was incubated in 50 mM Tris–HCl buffer (pH 7.8, 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$) o/n at 37°C. The degradation of gelatin was visualized under UV light.

Results

Collagen XVIII and endostatin in oral dysplasia, SCCs and wound healing

Immunohistochemical analysis demonstrated that collagen XVIII protein was continuously distributed in the BM of oral mucosa in mild dysplasias and normal control samples (not shown). In moderate dysplasias the staining for collagen XVIII was mostly continuous (Fig. 1A). In severe dysplasias collagen XVIII was absent from the affected regions, suggesting degradation of this BM component (Fig. 1B). *In situ* hybridization showed no significant change in collagen XVIII mRNA expression levels in the epithelium in mild (not shown), moderate and severe dysplasias (Fig. 1C, D). In oral SCCs collagen XVIII protein was absent from the invasive parts (Fig. 1E), but present in the BMs of blood vessels and surrounding some tumour cell islands (Fig. 1F). In SCC samples collagen XVIII mRNA expression was detected in the basal cells (Fig. 1G), in some individual tumour cells and in tumour cell islands (Fig. 1H). During oral mucosal wound healing collagen XVIII protein was not present in the wound area during day 1, but was detected again during day 7 (Fig. 2A, B).

MMP-9 in oral dysplasia and carcinoma

Immunohistochemical staining of oral dysplasias showed MMP-9 expression in the epithelial BM zone of both mild (Fig. 3A) and severe (Fig. 3C) dysplasias. Expression was seen in the basal cells (Fig. 3B), but the staining was discontinuous (Fig. 3C) and absent in some studied cases (not shown). MMP-9 was also detected in some capillary blood vessels (Fig. 3D). In dysplasias and tongue carcinoma samples MMP-9 was also present in plasma cells and neutrophil granulocytes, surrounding tumour cell islands, and in individual cancer cells, with some of the cells staining more strongly than the adjacent ones (Fig. 3E, F).

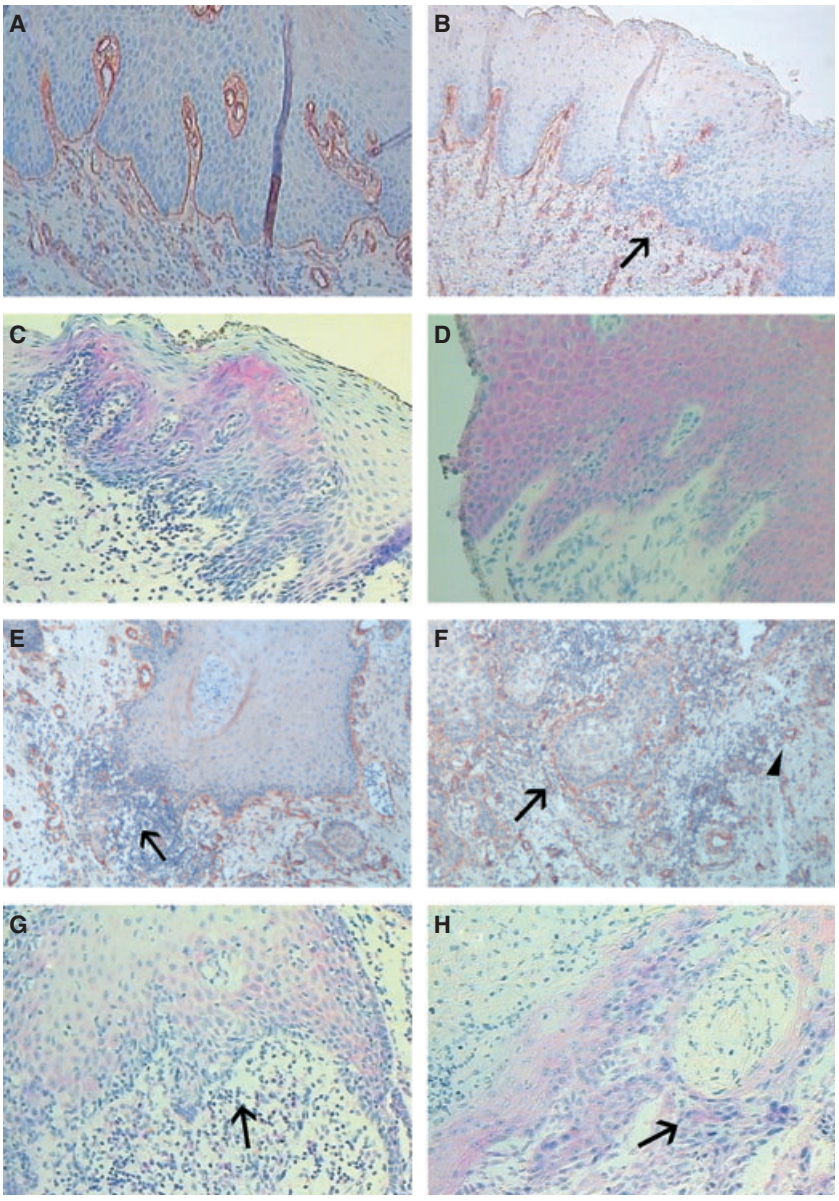

Figure 1 Collagen XVIII mRNA and protein expression in dysplasias and SCCs. Immunohistochemical staining with anti-all huXVIII antibody of moderate (A) dysplasias detected collagen XVIII protein in the mucosal BM, but in severe (B) dysplasias collagen XVIII was absent (arrow). *In situ* hybridization detected mRNA expression in suprabasal and basal cells in moderate (C) and severe (D) cases. Immunohistochemical staining of SCC samples showed absence of collagen XVIII protein in some areas of the invasive front (arrow) (E), but the staining was continuous in the BM of small blood vessels (F, arrowhead) and in the BM of separate tumour islands (F, arrow). Collagen XVIII mRNA was expressed in the SCC samples in the basal cells (G, arrow) and within SCC cells of larger islands (H, arrow). Original magnification (A–C, E) 100×, (D, F–H) 200×.

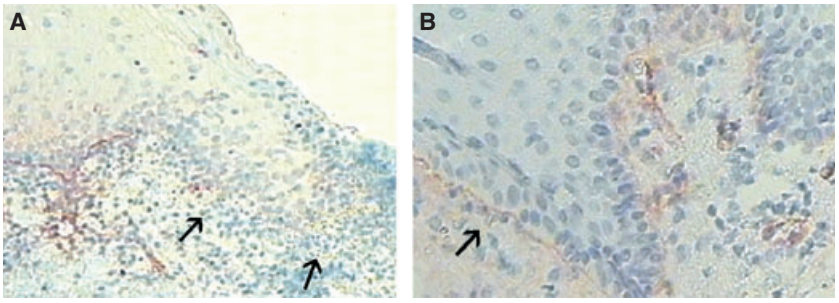

Figure 2 Collagen XVIII protein expression in oral wound healing. During wound healing collagen XVIII protein was absent from the mucosal BM of a 1 day old wound (A) but was present again in the BM of a 7-day-old wound (B, arrow). Original magnification (A) 100×, (B) 200×.

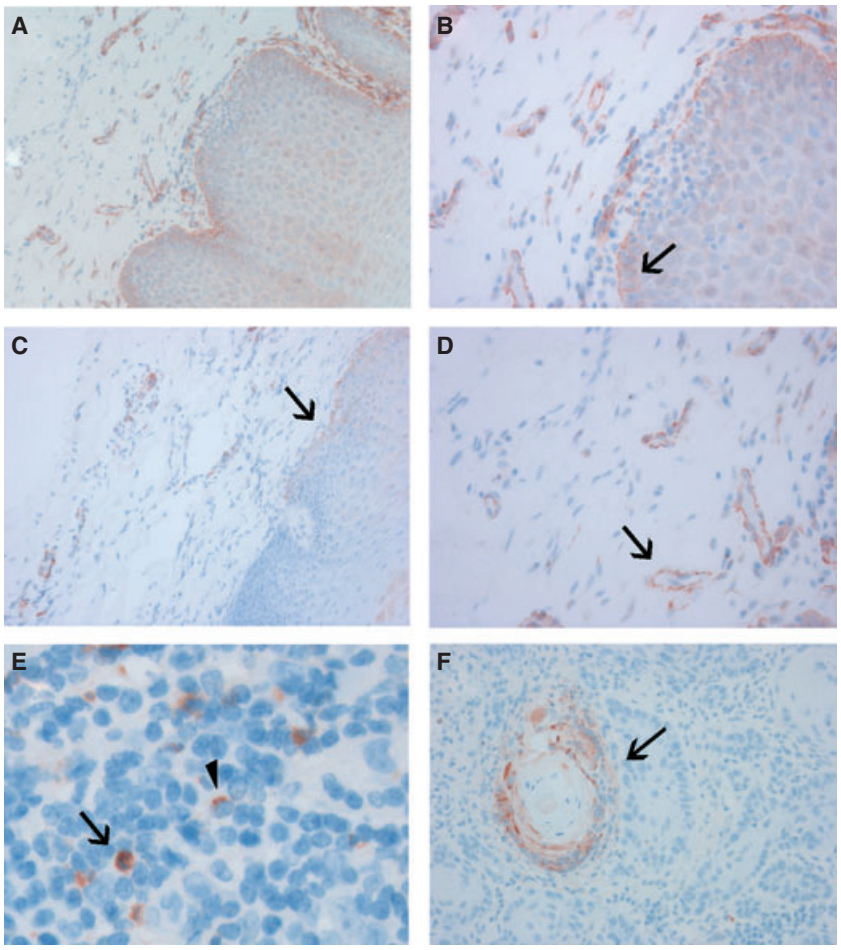

Figure 3 Matrix metalloproteinases-9 expression in oral dysplasia. In mild dysplasias MMP-9 was detected in the epithelial BM zone (A) and in the basal cells (B, arrow), and in the severe dysplasias it was expressed in the basal cells (C, arrow). MMP-9 expression was also detected in some capillary blood vessels (D, arrow). In tongue SCC samples MMP-9 was present in neutrophil granulocytes (arrow, E) and plasma cells (arrowhead, E) and surrounding SCC islands (F, arrow). Original magnification (A, C) 200×, (B, D–F) 400×.

Cell culture

Reverse transcriptase-polymerase chain reaction analysis showed that collagen XVIII mRNA was expressed in HaCaT, SCC-25 and HSC-3 cell lines (Fig. 4A). With both anti-all huXVIII collagen XVIII antibodies Western blotting detected collagen XVIII protein in all cell lines, but the full-length protein (approximately 200 kDa) that was detected in HaCaT and SCC-25 cells was less visible in HSC-3 cells and the fragmentation of the protein was different between the cell lines (Fig. 4B). With both anti-endostatin antibodies the same degradation pattern was detected, where a 90-kDa endostatin-containing fragment was clearly more visible in HSC-3 cells than in HaCaT or SCC-25 cells and several 20- to 30-kDa endostatin fragments were seen in all cell lines in differing amounts (Fig. 4B). PMA treatment had no effect on the expression of collagen XVIII mRNA (Fig. 4A) or protein, nor on the degradation of the collagen XVIII (not shown). Zymography of the same medium samples detected MMP-2 expression in HaCaT cells, whereas HSC-3 cells expressed mostly MMP-9 and in SCC-25 cells only MMP-9 was detected (Fig. 4C). PMA treatment increased the expression and especially the activation of MMP-2 in HaCaT and HSC-3 cells, and also slightly increased the production of MMP-9 in HSC-3 and SCC-25 cultures (Fig. 4C).

Discussion

Oral SCC is the most common form of malignant tumour of the oral cavity and it often develops from the potentially malignant lesions, such as dysplasias. In this study, we analysed collagen XVIII and MMP-9 expression during different stages of oral dysplasia and SCC. Continuous collagen XVIII signal was detected in the BM of mild dysplasias and in the moderate cases the staining was mostly continuous. However, the protein was completely absent from some areas of severe dysplasia, and from the invasive front of SCC where epithelium was spreading to the underlying matrix. No significant change was seen in the mRNA expression when comparing severe dysplasias to the milder ones, indicating that the absence of collagen XVIII is related to the processing of the protein rather than to changes in mRNA expression. Although collagen XVIII mRNA was present in different cells

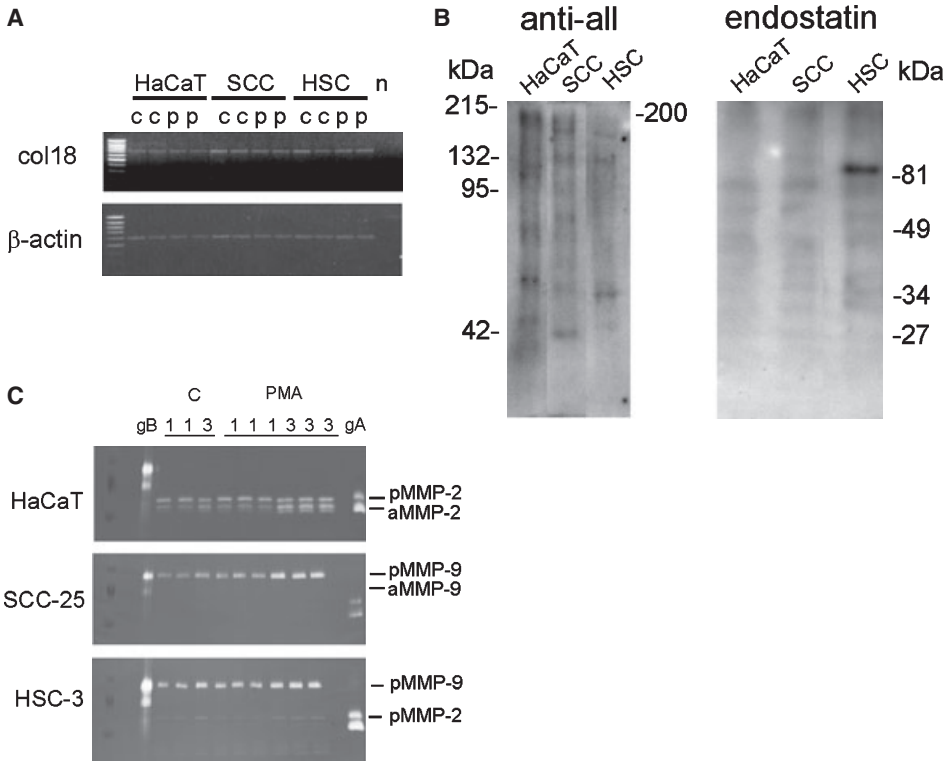

Figure 4 Collagen XVIII in cultured cells. RT-PCR analysis (A) showed collagen XVIII mRNA expression in HaCaT, SCC-25 and HSC-3 cells. No difference was seen between control (c) or PMA-treated (p) cells. Western blotting (B) with anti-all huXVII antibody QH48.18 showed that collagen XVIII was differentially processed in SCC-25 and HSC-3 cells when compared with HaCaT cells, with full-length protein detected at 200 kDa and smaller fragments between 40 and 130 kDa. Western blotting with anti-endostatin antibody Q101.1 detected 20- to 30-kDa endostatin fragments and several bigger endostatin-containing fragments in HSC-3 cells, the most prominent with a molecular weight of 90 kDa. Zymography (C) detected MMP-2 expression in HaCaT cells, with PMA increasing the expression and activation in 3-day culture but not in 1-day culture. MMP-9 was expressed in SCC-25 cells and HSC-3 cells, with weak MMP-2 expression seen in HSC-3 cells. c = control, p = PMA, n = negative control, gB = MMP-9, gA = MMP-2, 1 = 1-day culture, 3 = 3-day culture, pMMP-2 = pro-form of MMP-2, pMMP-9 = pro-form of MMP-9, aMMP-2 = active form of MMP-2, aMMP-9 = active form of MMP-9.

throughout the epithelium in all dysplasias, it is not necessarily processed into protein in all cells, as the mRNA and protein levels in tissues are not directly comparable. Protein distribution of collagen VII, an anchoring fibril component, is similarly impaired in SCC, while mRNA is synthesized at high levels (3). mRNA expression results for collagen XVIII are also similar to expression of alpha 1 gene of collagen VII, which is upregulated in mild lingual dysplasia, but for which no further increase was reported in severe dysplasia. On the other hand, in severe dysplasia a different gene for collagen VII, alpha 3, was found to be downregulated (19).

Other BM proteins, such as laminin-5, are degraded during the progression of oral dysplasia and carcinoma in a similar manner as collagen XVIII (1), indicating that the absence of collagen XVIII in the invasive areas is related to the general breakdown of the BM. Previously, it has been demonstrated that primary tumours of metastatic oral SCCs have decreased protein levels of collagen XVIII and endostatin in tumour cells when compared with non-metastatic cases. The decreased protein levels in these cells were suggested to affect angiogenesis, as endostatin is a known angiogenesis inhibitor (20). The metastatic capability is most likely also related to the absence of collagen XVIII from the BM surrounding the invasive tumour cells, as evidenced in this study.

Matrix metalloproteinase-9, which digests endostatin fragments from the full-length collagen XVIII molecule (12), was also present in oral SCC cell islands and in the basal cells and BM area in the dysplasias, but neither consistently in all studied samples nor in relation to the severity of the dysplasia. A previous study has indicated that MMP-9 mRNA levels rise in oral dysplasias that progress to carcinoma (5), but in this study no clear correlation could be detected between MMP-9 protein expression and the stage of the dysplasia, possibly because of the small number of samples. MMP-9 expression has been linked to poor prognosis and metastasis in oral carcinoma (6, 7), but there are also studies that have found no correlation between MMP-9 expression and the disease status of oral cancer (21). MMP-9 may degrade collagen XVIII in the invasive areas along with other proteinases that degrade collagen XVIII. Some of these enzymes, such as MMP-3, MMP-13, MMP-7 and cathepsin L have been previously detected in oral carcinomas (12, 22–25), and MMP-7 is also upregulated in mild lingual dysplasia (19). Besides the enzymes that cleave endostatin fragments off the

molecule, there may also be other proteinases that participate in the removal of collagen XVIII from epithelial BM.

During oral mucosal wound healing collagen XVIII protein was absent from the general wound area during days 1–5, but was present again during day 7. These results are in agreement with collagen XVIII expression during mouse corneal wound healing (26). The end of this timeframe most likely correlates with the BM being intact again, as has previously been demonstrated for the distribution of collagen IV and VII (27). Another BM component, laminin-5, is present at the leading edge of the migrating epidermis in the wounds of the oral mucosa during day 4 (28), suggesting that in human mucosal wound healing collagen XVIII is expressed in similar manner as other BM proteins.

In cell culture studies Western blotting showed the presence of collagen XVIII protein in both HaCaT cells and in oral carcinoma cell lines SCC-25 and HSC-3, but in the oral carcinoma cells the protein showed a distinct degradation pattern from HaCaT cells, and in HSC-3 cells less full-length protein was detected than in SCC-25 cells. Several endostatin fragments between 20 and 30 kDa and larger were detected in all cell lines and in HSC-3 cells there was also a strong signal for a 90-kDa endostatin-containing fragment that was not visible in the other cell lines. The increased degradation of collagen XVIII in HSC-3 cells may be related to the fact that HSC-3 cells are more invasive than SCC-25 cells (29). No significant differences were seen in the mRNA expression between these cells. These results are in agreement with *in situ* hybridization studies, which did not show significant variation in the mRNA expression between different stages of dysplasia and carcinoma. Zymography was performed to detect possible differences in the enzyme production between the cell lines. Pro-form of MMP-9 was expressed in both carcinoma cell lines, but was not present in the HaCaT cells, in which MMP-2 was detected predominantly. Gelatinolytic activity has been detected in placental tissue, although all the MMP-9 extracted from the tissue was in pro-form, and the purified pro-form of MMP-9 has been shown to possess activity against substrates (30). Thus the expression of pro-MMP-9 may be one of the factors responsible for the increased collagen XVIII degradation in the HSC-3 cells. Although direct conclusions cannot be made about the cell cultures concerning the dysplasias as the *in vivo* situation generally differs from *in vitro* studies, it is nevertheless likely that MMP-9 participates in the degradation of collagen XVIII in both cases.

In this study, we demonstrated that collagen XVIII is increasingly degraded from the BM as the stage of oral dysplasia progresses from mild to severe and to oral carcinoma, allowing the tumour cells spread to the underlying matrix. MMP-9 most likely has a role in the degradation of collagen XVIII, but further studies would be needed to identify other enzymes that participate in this process.

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