Collagenase-2 (matrix metalloproteinase-8) plays a protective role in tongue cancer

Squamous cell carcinoma (SCC) of the tongue is the most common cancer in the oral cavity and has a high mortality rate. A total of 90 mobile tongue SCC samples were analysed for Bryne’s malignancy scores, microvascular density, and thickness of the SCC sections. In addition, the staining pattern of cyclooxygenase-2, αvβ6 integrin, the laminin-5 γ2-chain, and matrix metalloproteinases (MMPs) -2, -7, -8, -9, -20, and -28 were analysed. The expression of MMP-8 (collagenase-2) was positively associated with improved survival of the patients and the tendency was particularly prominent in females. No sufficient evidence for a correlation with the clinical outcome was found for any other immunohistological marker. To test the protective role of MMP-8 in tongue carcinogenesis, MMP-8 knockout mice were used. MMP-8 deficient female mice developed tongue SCCs at a significantly higher incidence than wild-type mice exposed to carcinogen 4-Nitroquinoline-N-oxide. Consistently, oestrogen-induced MMP-8 expression in cultured HSC-3 tongue carcinoma cells, and MMP-8 cleaved oestrogen receptor (ER) α and β. According to these data, we propose that, contrary to the role of most proteases produced by human carcinomas, MMP-8 has a protective, probably oestrogen-related role in the growth of mobile tongue SCCs.

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However, in virtually all these previous studies, a heterogenous set of tumours have been analysed and therefore the results may not be comparable (Guttman et al., 2004; Ruokolainen et al., 2004). Identification of any of these factors as a potential prognostic marker would be useful for estimating the behaviour of the tumour and the survival of the patient with SCC in the mobile tongue. In this work, we provide evidence that collagenase-2 (MMP-8) is a protective factor in mobile tongue SCC. Furthermore, and by using mice deficient in this metalloproteinase and oral carcinoma cells producing MMP-8, we examine putative molecular and cellular mechanisms underlying the protective effect of this enzyme in tongue cancer.

MATERIALS AND METHODS

Patients

All 90 patients treated surgically (with at least 5 mm histological margins) at the Oulu University hospital with diagnosed mobile tongue SCC during the years 1981–2001 were included in this study. This tertiary care centre provides primary treatment for all cancer patients in the two northernmost provinces of Finland, covering a population of ~650,000 inhabitants. The patients (47 males, 43 females) were 26–99 years of age at the diagnosis of SCC (median 62 years). The following tumour-related factors were collected from the hospital files: tumour grade according to the recommendations of the Animal Care and Use Committee at the University of Oulu.

Animals

MMP-8 knockout mice were generated by gene targeting as previously described (Balbin et al., 2003). Wild-type mice (C57BL/6) with a similar genetic background were used as controls. All experiments were approved by and performed according to the recommendations of the Animal Care and Use Committee at the University of Oulu.

Induction of tongue SCC in MMP-8 KO mice

Forty-seven 13–16-week-old mice were exposed to tongue SCC-inducing 4-Nitroquinoline-N-oxide (4NQO, Sigma, USA) (Steidler and Reade, 1984; Gannot et al., 2004) dissolved in propylene glycol to a final concentration of 10 mg ml⁻¹. The mice were lightly anaesthetised by Isofluran (Forene® Abbott Scandinavia, Sweden) inhalation and 4NQO was smeared to the left dorsal half of the tongue 3 times per week for 12 weeks. The mice were killed at 55 weeks by cervical dislocation after inhalation of CO₂. The tongues were dissected and fixed routinely in 10% formalin, paraffin-embedded and stained with haematoxylin–eosin (HE) for histopathological diagnoses. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Oulu.

Immuno-histochemical staining

Immunohistochemical staining was done as previously described (Ylipalosaari et al., 2005). Briefly, paraffin sections were deparaffinised and pretreated. Sections were incubated with the primary antibody overnight at 4°C. Sections incubated with non-immune rabbit (polyclonal) or non-immune mouse (monoclonal) IgGs instead of primary antibodies were used as negative controls. The sections were incubated with biotinylated secondary antibodies and thereafter with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA). Finally, tissue sections were stained with diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA) or 3-amino-9-ethylcarbazole (Zymed, San Francisco, CA, USA) or 3,3’-diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA) or(JSONObject object)
In vitro ER-α and ER-β cleavage assay

Human recombinant MMP-8 (Chemicon International Inc., Temecula, CA, USA) was tested for the ability to digest human recombinant oestrogen receptor-α (ER-α) and oestrogen receptor-β (ER-β) (Invitrogen, CA, USA). In all 3.1 μg of human recombinant ER-α and 4.1 μg of human recombinant ER-β were used in the assays. The tested enzyme/substrate (E:S) molar ratios were 1:11 and 1:27 for ER-β and 1:5, 1:12, and 1:33 for ER-α. The reactions were performed in an incubation buffer (10 mM HEPES, 0.15 M NaCl, 5 mM CaCl₂ (pH 7.4)) in the presence or absence of MMP inhibitor GM6001, 10 μM (Ryss Laboratories) at 37°C for 22 h. The reactions were stopped by boiling in 4 × electrophoresis sample buffer (250 mM Tris-HCl; 8% SDS; 40% glycerol; 0.0098% Bromphenol blue (pH 6.8)) for 4 min. The samples were then subjected to SDS–PAGE and cleavage products were separated in non-reducing conditions. ER-α and ER-β were separated in non-reducing conditions. ER-α samples were then subjected to SDS – PAGE and cleavage products were then exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech, Glostrup, Denmark) for 1 h at RT, washed and incubated with incubated with anti-goat secondary antibody (1 : 1000, DAKO A/S, Inc., CA, USA) at RT overnight. The membrane was washed and incubated with 5% non-fat milk for 1 h and incubated with MMP-8 antibody (Santa Cruz Biotechnology (Millipore). The membrane was blocked with 5% non-fat milk for 1 h and incubated with MMP-8 antibody (1 : 1000, DAKO A/S, Glostrup, Denmark) for 1 h at RT, washed and 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 Pharmacia Biotech, Buckinghamshire, UK).

The proteins from the cleavage assays were separated by 12% SDS–PAGE and electrotransferred onto a nitrocellulose membrane (Millipore). To identify the digested fragments, the membranes were incubated overnight with ER-α (MC-20, Santa Cruz Biotechnology Inc., CA, USA) antibody against the COOH-terminal part of the receptor at 2 μg ml⁻¹ concentration or ER-β (Ab-24, Lab Vision, CA, USA) antibody against the COOH-terminal part of the receptor at 2.5 μg ml⁻¹ concentration. Biotinylated swine anti-rabbit immunoglobulin G (IgG) secondary antibody (1:1000 dilution) (Dako, Glostrup, Denmark) was then allowed to bind for 1 h.

Finally the membranes were incubated with avidin/biotinylated horse radish peroxidase (HRP) complex (Dako) for 1 h and the immunoreactive proteins were visualised with ECL western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

Malignancy analysis

Hematoxylin–eosin-stained sections were used for Bryne malignancy score analysis (Bryne et al., 1992) and for measuring the thickest SCC area. Malignancy score was calculated by determining five morphological features (degree of keratinisation, nuclear polymorphism, number of mitoses, pattern of invasion and lymphoplasmacytic infiltration) from each section and by giving a score (1–4) to each feature. The separate values were then summed up into the final malignancy score (5–20). Samples were

Western blotting

Serum-free HSC-3 culture medium was concentrated with 10 K centrifugal filter tubes (Millipore Bedford, MA, USA). The samples were subjected to 10% SDS–PAGE gel electrophoresis and thereafter the proteins were transferred to Immobilon P membrane (Millipore). The membrane was blocked with 5% non-fat milk for 1 h and incubated with MMP-8 antibody (Santa Cruz Biotechnology Inc., CA, USA) at RT overnight. The membrane was washed and incubated with anti-goat secondary antibody (1 : 1000, DAKO A/S, Glostrup, Denmark) for 1 h at RT, washed and 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 Pharmacia Biotech, Buckinghamshire, UK).

The proteins from the cleavage assays were separated by 12% SDS–PAGE and electrotransferred onto a nitrocellulose membrane (Millipore). To identify the digested fragments, the membranes were incubated overnight with ER-α (MC-20, Santa Cruz Biotechnology Inc., CA, USA) antibody against the COOH-terminal part of the receptor at 2 μg ml⁻¹ concentration or ER-β (Ab-24, Lab Vision, CA, USA) antibody against the COOH-terminal part of the

Table 1 The disease-specific five-year mortality from 90 tongue SCC patients

| Factors                        | n  | 5-year mortality (%) | 95% CI |
|--------------------------------|----|----------------------|--------|
| Gender                         |    |                      |        |
| Female                         | 47 | 22                   | 9–35   |
| Male                           | 43 | 25                   | 10–40  |
| Age group                      |    |                      |        |
| 26–70 years                    | 59 | 18                   | 7–29   |
| >70 years                      | 31 | 35                   | 15–55  |
| Clinical stage*                |    |                      |        |
| I and II                       | 51 | 14                   | 3–25   |
| III and IV                     | 36 | 32                   | 15–49  |
| Malignancy score (Bryne)*      |    |                      |        |
| Low score                      | 22 | 5                    | 0–14   |
| High score                     | 65 | 31                   | 18–44  |
| Thickness of SCC               |    |                      |        |
| <6.5 mm                        | 33 | 25                   | 8–42   |
| ≥6.5 mm                        | 57 | 27                   | 15–39  |

*The clinical stage was not reported and the malignancy score not analysed from three patients.

Table 2 The disease-specific five-year mortality from 80–84 tongue SCC patients for histological prognostic factors

| Factors                        | n  | 5-year mortality (%) | 95% CI |
|--------------------------------|----|----------------------|--------|
| Microvessel density            |    |                      |        |
| Inside carcinoma islands       |    |                      |        |
| Slight                         | 26 | 25                   | 5–45   |
| Moderate or abundant           | 58 | 25                   | 13–37  |
| Carcinoma marginals            |    |                      |        |
| Slight                         | 29 | 27                   | 7–47   |
| Moderate or abundant           | 54 | 24                   | 12–36  |
| ‘Normal looking’ mesenchymal tissue | |            |        |
| Slight                         | 36 | 30                   | 14–46  |
| Moderate or abundant           | 47 | 20                   | 7–33   |
| Expression of COX-2            |    |                      |        |
| Overview (× 100 magnification) |    |                      |        |
| <1% positively stained cells   | 40 | 25                   | 10–40  |
| 1–50% positively stained cells | 44 | 21                   | 7–35   |
| Detailed view from ‘hot spots’ (× 400 magnification) | | | |
| <1% positively stained cells   | 25 | 28                   | 8–48   |
| 1–10% positively stained cells | 28 | 14                   | 1–27   |
| >10% positively stained cells  | 31 | 29                   | 10–48  |
| Expression of laminin-5 γ2-chain | |                       |        |
| Overview (× 100 magnification) |    |                      |        |
| No staining in cancer cells    |    |                      |        |
| <40% of the tumour area        | 63 | 19                   | 8–30   |
| ≥40% of the tumour area        | 20 | 31                   | 10–52  |
| Cytoplasmic staining in cancer cells | | | |
| <40% of the tumour area        | 57 | 17                   | 6–28   |
| ≥40% of the tumour area        | 26 | 36                   | 13–59  |
| Staining in basement membrane  |    |                      |        |
| <40% of the tumour area        | 23 | 27                   | 6–48   |
| ≥40% of the tumour area        | 60 | 21                   | 10–32  |
| Detailed view from ‘hot spots’ (× 400 magnification) | | | |
| Negatively stained cells       |    |                      |        |
| <50% of all cancer cells       | 23 | 17                   | 0–35   |
| ≥50% of all cancer cells       | 60 | 24                   | 12–36  |
| Positively stained cells       |    |                      |        |
| <50% of all cancer cells       | 10 | 12                   | 0–36   |
| ≥50% of all cancer cells       | 73 | 23                   | 12–34  |
| Expression of αvβ6-integrin (score 0–7) | | | |
| 0                              | 7  | 31                   | 0–69   |
| 3–5                            | 28 | 12                   | 0–25   |
| 6–7                            | 45 | 29                   | 14–44  |
divided into two categories: low (5–10) and high (11–20) Byrne score. Thickness of the SCC was determined from the sections by measuring the thickest SCC area with Leica microscope using Leica IM50 Image Manager program. The sections were divided into two groups: those under 6.5 mm and those 6.5 mm or over.

Immunohistochemical evaluations

All histological evaluations were done at least two times by two to three calibrated investigators without the knowledge of the clinical information of the patients. For microvascular density assessment a method described previously (Weidner et al, 1991) was used with slight modifications. Briefly, the most highly vascularised areas (‘hot spots’) based on both factor VIII and CD31 stainings were selected and counted from three different areas: (i) inside carcinoma islands, (ii) carcinoma marginals and (iii) at the edge of the slide, ‘normal looking’ mesenchymal tissue. Cases were divided into three groups: slight, moderate and abundant microvascular density. The levels of COX-2 expression were classified first with low magnification and then with high magnification as follows; grade 0: <1%, grade 2: 1–10%, grade 3: >10–50% and grade 4: >50% of tumour cells. Immunohistochemical staining for the laminin-5 γ2-chain was evaluated by dividing each slide into negative (N; no staining within cancer cells), positive (P; cytoplasmic staining within cancer cells) and basement membrane (BM; the tumour nest periphery was partly or circumferentially stained) areas. Parts of different areas were categorised as follows: 1: <20%, 2: 20–40%, 3: 40–60%, 4: 60–80% and 5: 80–100% of the analysed tumour areas. In addition N and P areas separately were analysed with high magnification as follows: 1: <25%, 2: 25–50%, 3: 50–75%, and 4: 75–100% of all tumour cells. The levels of αvβ6-integrin expression were classified as follows: 0 = no positive staining, 1 = slight positive staining, 2 = moderate positive staining and 3 = strong positive staining in carcinoma cells. The percentage of positively stained cells of the tumour was categorised as follows: 0: <1%, 1: 1–25%, 2: 26–50%, 3: 51–75% and 4: 76–100%. Finally the score (0–7) was calculated by summaring the value of intensity and the category of positively stained cells. Immunostainings for MMPs were evaluated using MMP-2, MMP-7, MMP-8, MMP-9, MMP-20 and MMP-28 antibodies, respectively. The staining intensity and the proportion of the positively stained cells were quantified using a method described previously (Bachmeier et al, 2000). Briefly, a five-step grading score was used for the proportion of positively stained carcinoma cells as follows: score 0: <1 cell, score 1: 1 to <25 cells, score 2: 25 to <50 cells, score 3: 50 to <75 cells and score 4: ≥75 cells. A four-step grading was used for the staining intensity of carcinoma cells as follows; score 0: no positive staining, score 1: weak positive staining, score 2: strong positive staining, score 3: strong positive staining. The percentage of positively stained collagenase-2 in carcinoma cells, staining intensity and the proportion of the positively stained cells were quantified using a method described previously (Bachmeier et al, 2000). Briefly, a five-step grading score was used for the proportion of positively stained carcinoma cells as follows: score 0: <1 cell, score 1: 1 to <25 cells, score 2: 25 to <50 cells, score 3: 50 to <75 cells and score 4: ≥75 cells. A four-step grading was used for the staining intensity of carcinoma cells as follows; score 0: no positive staining, score 1: weak positive staining, score 2: moderate staining, score 3: strong staining intensity. With MMP-8 and MMP-9 antibodies positively stained cancer cells and positively stained inflammatory cells surrounding the carcinoma islands were counted separately, whereas with MMP-2 and MMP-7 antibodies only positively stained cancer cells were counted. For inflammatory cells only the amount of stained cells was counted, that is not stained on both used. MMP-20 was excluded from the analysis due to the small number of stained carcinoma cells. The score was calculated by multiplying the mean value of positively stained cells and the mean value of staining intensity. The levels of ER-β expression were classified as follows: 0 = no positive staining, 1 = slight positive staining, 2 = medium positive staining and 3 = strong positive staining in carcinoma cells and also in inflammatory cells.

Statistical analysis

Five-year mortalities from SCC itself (with 95% confidence intervals, CI) in various subgroups were estimated by the Kaplan–Meier method. The relative hazards of death from SCC (and 95% CIs) associated with each marker under study were estimated by the Cox proportional hazards regression model, adjusting for the main known prognostic factors (age, sex, and TNM stage of the tumour). Mutual bivariate associations between the various markers were evaluated by computing odds ratios (OR with 95% CIs) for pairs of the dichotomised versions of these variables. The response variable in the mice experiment had three ordered categories: no change, dysplasia, and cancer, but it was dichotomised by pooling dysplasias and cancer into one category. The differences in proportion of developing dysplasia or cancer between the MMP-8 knockout mice and the wild-type C57BL/6 mice, were estimated separately for males and females. This analysis was performed using the function twoby2 in the package Epi, version 0.7.0 (Carstensen et al, 2007) attached with the R environment for statistical computing and graphics, version 2.6.0 (R Development Core Team, 2007). All the other statistical analyses were performed using the SPSS software version 12.0.1.

RESULTS

The overall mortality from the tongue cancer up to 5 years following the diagnosis of the SCC was 23%. Case fatality was generally higher among older patients, those with a more advanced clinical stage, and/or with a higher than average Byrne malignancy score, but thickness of the tumour did not predict the outcome.

Table 3 The disease-specific five-year mortality from 80–84 tongue cancer for histological prognostic factors

| Factors                        | n  | 5-year mortality (%) | 95% CI |
|--------------------------------|----|----------------------|--------|
| MMP-2 in cancer cells, score   |    |                      |        |
| <0.32                         | 54 | 28                   | 15–41  |
| ≥0.32                         | 30 | 15                   | 1–29   |
| MMP-7 in cancer cells, score   |    |                      |        |
| <1.84                         | 52 | 24                   | 11–37  |
| ≥1.84                         | 21 | 26                   | 6–46   |
| MMP-8 in cancer cells, score   |    |                      |        |
| <0.78                         | 52 | 31                   | 17–45  |
| ≥0.78                         | 31 | 11                   | 0–23   |
| MMP-8 in inflammatory cells   |    |                      |        |
| <0.08                         | 42 | 24                   | 10–38  |
| ≥0.08                         | 41 | 24                   | 10–38  |
| MMP-9 in cancer cells, score   |    |                      |        |
| <2.62                         | 53 | 29                   | 15–43  |
| ≥2.62                         | 30 | 19                   | 4–34   |
| MMP-9 in inflammatory cells   |    |                      |        |
| <10.20                        | 41 | 32                   | 16–48  |
| ≥10.20                        | 42 | 18                   | 5–31   |
| MMP-28 in cancer cells, score  |    |                      |        |
| <0.09                         | 58 | 26                   | 13–39  |
| ≥0.09                         | 24 | 19                   | 2–36   |
| ER-β in cancer cells, staining intensity |    |                      |        |
| 0–1 = No or slight positive staining | 31 | 19                   | 4–35   |
| 2–3 = Medium or strong positive staining | 35 | 29                   | 12–45  |
| ER-β in inflammatory cells, staining intensity |    |                      |        |
| 0–1 = No or slight positive staining | 34 | 25                   | 8–41   |
| 2–3 = Medium or strong positive staining | 33 | 23                   | 8–38   |
There was no evidence for microvascular density or expression of factors such as COX-2, laminin-5 γ2-chain and zvβ6-integrin (Table 2) as being associated with the prognosis. By contrast, it appeared that subjects with positive immunostaining for MMP-2, -8, -9, or -28 in the cancer cells or MMP-9 or ER-β in inflammatory cells would have a better prognosis than other patients (Table 3). However, the statistical evidence in support of these observed contrasts was weak.

Bivariate associations between the various markers were also analysed. High Bryne malignancy score value predicted high level of zvβ6-integrin (OR 2.9, 95% CI 1.1–8.2), but MMP-9 and COX2 levels in carcinoma cells (OR 0.17, 95% CI 0.03–0.78; and OR 0.19, 95% CI 0.06–0.65, respectively) were negatively associated with high Bryne category. Likewise, high level of COX-2 predicted high MMP-9 level in carcinoma cells (OR 2.7, 95% CI 1.0–7.3), but laminin-5 γ2-chain staining within carcinoma cells was inversely associated with MMP-9 expression (OR 0.22, 95% CI 0.08–0.61).

Further analysis revealed that the only statistically significant marker for case fatality was MMP-8 (Table 3). Patients with tumours lacking MMP-8 expression in cancer cells had a relative SCC mortality rate of 3.70 (95% CI 1.04–12.5) compared to patients with some MMP-8 positive immunostaining, when adjusted for age, sex, and stage of tumour (TNM) by the proportional hazards model. MMP-8 or MMP-9 expression in inflammatory cells was not associated with survival (Table 3). In addition, for all evaluated factors only MMP-8 came up with Cox's regression model when age, gender and stage were installed as main variables. Interestingly, positive MMP-8 expression and improved survival also showed a tendency to be more prominent in female tongue cancer patients than in male tongue cancer patients, but this difference was not found to be statistically significant.

The mortality of patients with no positive MMP-8 immunostaining also increased over time during the 5-year period (Figure 1). The associations of MMP-8 with gender, oestrogen receptor levels (separately in cancer cells and in inflammatory cells), tumour thickness, TNM stage, and tumour grading were not statistically significant.

To test the hypothesis that MMP-8 plays a protective role in tongue SCC, 23 MMP-8 knockout (KO) and 24 wild-type C57BL/6 mice were subjected to chemical carcinogenesis with 4-Nitroquinoline-N-oxide (4NQO) for 12 weeks. Half (6/12) of the MMP-8 KO female mice in contrast to none of the 12 wild-type C57BL/6 female mice developed tongue cancer during the experiment (Table 4, Figure 2). Similarly, dysplasias were more frequent in the tongues of MMP-8 KO female mice (4/12) than in wild-type mice (2/12). In male mice no difference in carcinoma development was found between the mouse groups. There was a strong statistical support to the observation that the MMP-8 KO female mice developed tongue cancer more often than the wild-type mice. The estimated proportion of female MMP-8 KO mice developing either dysplasia or carcinoma was 67 percent points higher than that of the female wild-type mice (83 vs 17%; 95% CI for the difference in proportions: +21 to +85 percent points). In male mice the same contrast was observed to be 20 percent points (95% CI –21 to +55 percent points).

MMP-8 expression was further analysed in cultured oral SCC cells by confocal immunofluorescence, which localised MMP-8 immunoreactivity mainly to the tongue carcinoma cell membranes and to subcellular granules. Lack of MMP-8 was found to have different effect on male and female mice when experimental SCC was induced. We therefore investigated the effect of oestrogen on HSC-3 cells. Molecular forms of MMP-8 were identified from HSC-3 tongue carcinoma cells by using western blotting which demonstrated a 75 kDa species in HSC-3 cell membrane extracts. Oestrogen treatment induced expression of a 75 kDa MMP-8 species in HSC-3 cells as evidenced by western blotting. To verify this observation we also performed MMP-8 RT–PCR from HSC-3 cells incubated with or without 10 nM oestrogen overnight and found that MMP-8 mRNA was undetectable in resting HSC-3 cells while oestrogen treatment induced MMP-8 mRNA expression (Figure 3).

Because oestrogen acts through the oestrogen receptors, we investigated the expression of ERs in tongue SCCs. Both oestrogen receptor-α (ER-α) and oestrogen receptor-β (ER-β) were expressed in human and mouse tongue SCC cells as detected by immunohistochemical methods (Figure 4). ER-β expression was found to weakly correlate with a better prognosis (Table 3).

To test, whether MMP-8 cleaves ER-α or ER-β, and thus could have effect on their function, we performed an in vitro cleavage assay using purified recombinant MMP-8 and ERs. MMP-8 was found to cleave ER-α in vitro dose dependently (Figure 5). Two cleavage products of full length ER-α (66 kDa) were detected, with the approximate molecular weights of 44 and 26 kDa by western immunoblotting with an ER-α antibody. The intermediate cleavage product of 44 kDa was detected only with the enzyme/substrate molar ratio 1:5 (Figure 5A). Only minor cleavage of ER-β by MMP-8 could be detected (Figure 5B, only the result from E:S ratios 1:11 shown). Approximately 20 kDa and 45 kDa cleavage products of monomeric 53 kDa ER-β increased, and around 100 kDa dimeric and 200 kDa higher molecular weight forms of ER-β slightly diminished after incubating with MMP-8 (Figure 5B). The broad-spectrum MMP inhibitor GM6001 abolished the ERs degradation by MMP-8 (not shown).

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**Table 4** Histological incidence of lesions in 4NQO-treated tongue sections from MMP-8 KO and wild type mice

| Response    | Sex    | Strain     | No change | Dysplasia | Carcinoma | Total |
|-------------|--------|------------|-----------|-----------|-----------|-------|
| Male        | C57BL/6| 9          | 2         | 1         | 12        |
| Male        | MMP-8 KO| 6          | 4         | 1         | 11        |
| Female      | C57BL/6| 10         | 2         | 0         | 12        |
| Female      | MMP-8 KO| 2          | 4         | 6         | 12        |
Our investigation was based upon a unique population-based collection of 90 surgically treated SCC resection samples of the mobile tongue. Treatment of the patients in Northern Finland is very effective; the overall mortality from the disease up to 5 years was only 23% in this study, while it generally varies within about 40–55% in Finland (Dickman et al., 1999). Worldwide, head and neck cancer is the sixth most common cancer (Hunter et al., 2005). Low mortality in this case may hide the significance of some individual prognostic markers, since patients outcome is most likely affected more by the effective diagnostic and treatment strategies than by any of the biological variables. The prognosis was significantly better in younger patients (26–70 years), in

Figure 2  Histopathological and clinical analyses of 4NQO-treated tongues from MMP-8 KO and C57BL/6 mice. (A) Normal C57BL/6 male mouse mucosa stained with hematoxylin and eosin. Clinical tongue on the right. (B) MMP-8 KO male with dysplasia. (C) MMP-8 KO females with invasive SCC. (D) MMP-8 KO females with invasive SCC. Scale bar = 200 μm.

Figure 3  Localisation and molecular forms of MMP-8 in cultured oral carcinoma cells. Localisation of MMP-8 immunoreactivity in oral SCC cell membranes and in intracellular granules is demonstrated in green and red staining demonstrates actin (A). MMP-8 and β-actin RT–PCR from HSC-3 tongue carcinoma cells incubated with or without 10 nm oestrogen (E2) overnight (B). Molecular sizes of MMP-8 in HSC-3 tongue carcinoma cells analysed by western blotting (C). Lanes 1 (no E2) and 4 (E2 added) represent concentrated medium where no MMP-8 is detected. Lanes 2 (no E2) and 5 (E2 added) represent the cell membrane extracts where a 75 kDa species can be seen. Lanes 3 (no E2) and 6 (E2 added) are total proteins where a 75 kDa species is visible only after E2 treatment. Lane 7 is odontoblast medium and lane 8 human saliva used as positive controls where a 58 kDa form of MMP-8 can be detected.

DISCUSSION

Our investigation was based upon a unique population-based collection of 90 surgically treated SCC resection samples of the mobile tongue. Treatment of the patients in Northern Finland is very effective; the overall mortality from the disease up to 5 years was only 23% in this study, while it generally varies within about 40–55% in Finland (Dickman et al., 1999). Worldwide, head and neck cancer is the sixth most common cancer (Hunter et al., 2005). Low mortality in this case may hide the significance of some individual prognostic markers, since patients outcome is most likely affected more by the effective diagnostic and treatment strategies than by any of the biological variables. The prognosis was significantly better in younger patients (26–70 years), in
patients with lower clinical stages of the tumours (TNM I and II) and in patients with lower (5–10) Bryne’s malignancy score as has been shown earlier by us (Kantola et al, 2000) and other investigators (Pilifko et al, 1997). Our results were in line with Teixeira et al (1996), who also did not find a correlation between the thickness of the cancer in the deepest tumour areas and outcome of the patients, but in contrast with a study by Charoenrat et al (2003) where tumour thickness and survival of the tongue SCC patients correlated significantly. Also in line with previous tongue SCC studies, we did not observe any association between micro vessel density either within the borders or outside the invasive SCC tissue and prognosis of the patients (Leedy et al, 1994; Hogmo et al, 1999). Unlike Pannone et al (2007), we could not find a correlation between COX-2 expression and survival, but COX-2 expression was negatively associated with high Bryne’s category, which is seen as a predictor for poor prognosis (Bryne et al, 1992). Unlike published earlier (Ono et al, 1999; Katoh et al, 2002; Gasparoni et al, 2007), cytoplasmic or basement membrane zone staining for the laminin-5 β2-chain in cancer cells did not correlate with the outcome of the patient in our study. There are several reports showing increased αvβ6 integrin expression in invasive SCCs (Breuss et al, 1995; Jones et al, 1997; Ramos et al, 1997), but these findings could not be replicated here, although our results, suffering from wide error margins, were not in any conflict with the previous ones. Over 90% of our tongue cancers samples did express αvβ6 integrin but the expression level did not statistically correlate with the outcome of the patients. However, high level of αvβ6 integrin was associated with high Bryne malignancy score category and may thus be linked to more aggressive behaviour and poorer prognosis of the cancer.

MMPs are known to be overexpressed in pathological stages requiring matrix turnover (Overall and Lopez-Otin, 2002). Of the molecules analysed in this study, the statistical evidence was insufficient to make judgments on the association between the expression of MMP-2, -7, -9, and -28 with survival from SCC of the tongue. In previous studies, MMP-9 expression is associated with poor prognosis in tongue and also head and neck SCCs (Juarez et al, 1993; Kawamata et al, 1998; Nyberg et al, 2002) but there is also publications where such a correlation has not been found (Kim et al, 2006). However, in our bivariate analysis we observed a positive association between MMP-9 and COX-2 levels in carcinoma cells, but negative association with MMP-9 presence and high Bryne category suggesting that MMP-9 in these samples reflects, but not statistically significantly, better prognosis of the disease. In addition, our MMP-28 findings were in the line with Lin et al (2006). We found a slight trend between positive MMP-28 immunostaining and better prognosis of tongue squamous cell carcinoma. By contrast, parallel analysis with MMP-8 revealed that production of this protease was significantly associated with good clinical outcome in tongue cancer patients. Decock et al (2007) showed that genetic variation in the MMP-8 gene could influence breast cancer prognosis. They also noticed that MMP-8 inhibited breast cancer metastasis. These observations are also in the line with previous studies where MMP-8 expression was associated with a better prognosis of skin cancer in mice (Balbin et al, 2003) and a benign behaviour of cultured breast cancer cells (Agarwal et al, 2003). However, Stadlmann et al (2003) found that MMP-8 expression in ovarian cancer was associated with a poorer prognosis. This is probably due to the fact that different cancer types, or species for that matter, cannot always be directly

Figure 4 Oestrogen receptor-α and -β immunohistochemical staining in tongue squamous cell carcinoma. Nuclear and cytoplasmic ER-α and ER-β positivity (red staining) were detected both in mouse and human tongue SCC cells. (A) Mouse SCC stained with ER-α antibody (MC-20). (B) Human SCC stained with ER-α antibody (ab-24). (C) Mouse SCC stained with ER-β antibody (ab-24). (D) Human SCC stained with ER-β antibody (ab-24). Scale bars = 50 μm.
Figure 5 Cleavage of ERs by MMP-8 in vitro. Recombinant MMP-8 was incubated with recombinant ER-α and ER-β at different enzyme-substrate ratios. The cleavage fragments were separated by SDS-PAGE and identified by Western immunoblotting using ER-specific antibodies. (A) ER-α was incubated with an increasing amount of MMP-8. The cleavage products of approximately 44 and 26 kDa were detected with ER-α (MC-20) antibody. (B) The ER-β (ab-24) antibody detected the cleavage products of about 45 and 20 kDa. In addition, the higher molecular weight forms of ER-β with approximate sizes of 100 and 200 kDa were diminished after incubation with MMP-8.

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C/EBP has been shown to associate with ER-α and the complex then acts as a transcription factor and can regulate gene promoter activity (Chen et al., 2006). This could explain why oestrogen induces MMP-8 production in HSC-3 cells. Li et al., 2004 found the MMP-26 gene promoter activity to be stimulated by oestrogen through the ERs. In addition, MMP-26 can cleave ER-β in vitro and plays an important antitumorigenic role in hormone-regulated malignancies by regulating the amount of ER-β and thus regulating the oestrogen signalling pathway (Savinov et al., 2006). In this study we found, for the first time, that although ER-β, but not ER-α, are expressed in normal oral mucosa (Välimaa et al., 2004) they were both faintly produced in the tongue SCC islands. ER-β is expressed by lymphocytes (Törnwall et al., 1999; Ulziibat et al., 2006) and interestingly we found a weak correlation for the expression of ER-β in inflammatory cells with prolonged survival. We also found that MMP-8 can effectively cleave ER-α and some cleavage of ER-β in vitro was also detected. Additionally, a dimeric (about 100 kDa) and a higher molecular weight (around 200 kDa) form of ER-β monomer (53 kDa) diminished after incubating with MMP-8. According to these data, it is possible that MMP-8 cleaves the ER-β dimer and complex forms also in vivo. Dimers and stability of ER dimers is crucial for oestrogen receptor activation and function as a transcription factor (Tamrazi et al., 2002). These data suggest that MMP-8 protective role could be related to its ability to regulate the amount of ERs and thus regulating oestrogen-signalling pathway during tumour development, especially in hormone-regulated malignancies. This finding also at least partly explains the differences between MMP-8 KO male and female mice cancer susceptibility. Our study now supports these data also in human patients with mobile tongue SCC, the most common type of oral cancer.

In conclusion, based on both human SCC tissue sample analysis and mice in vivo carcinogenesis experiments, our study is the first to provide evidence that carcinoma cell membrane bound MMP-8 should be considered as a protective anti-tumour factor in mobile tongue SCC and its mechanism of action in tumours may be oestrogen related.
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