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Migration-stimulating factor as a novel biomarker in salivary gland tumours

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BACKGROUND: The identification of novel stratification biomarkers would benefit the clinical management of patients with salivary gland tumours. Migration-stimulating factor (MSF) is a potent stimulator of cell invasion, matrix remodelling and angiogenesis. The aim of this study was to determine whether MSF was expressed in salivary gland tumours and its potential value as a diagnostic biomarker.

METHODS: Paraffin-embedded archival specimens of small salivary gland tumours were stained with an MSF-specific antibody. The specimens included 27 malignant and seven benign tumours; histologically normal salivary gland adjacent to the tumour was present in 16 specimens. MSF expression was assessed by consensus of 2–4 independent observers according to various indices, including 'overall MSF grade', 'percentage of area stained' and 'intensity of the staining'. The motogenic effect of MSF on a salivary gland tumour cell line, HSG, was examined in the transmembrane assay.

RESULTS: Overall MSF expression increased significantly in a step-wise fashion from normal salivary gland to benign and malignant tumours ($P = 0.04–0.0001$); with moderate/strong positive specimens representing 6%, 33% and 74% of the normal, benign and malignant specimens, respectively. MSF was heterogeneously expressed in both carcinoma and stromal cell compartments, its expression being higher in malignant than benign tumours regarding various MSF indices. In tissue culture studies, exogenous MSF stimulated the migration of HSG cells.

CONCLUSIONS: These immunohistochemical and functional studies suggest that MSF expression is a potentially useful biomarker of salivary gland tumour progression.

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Introduction

Salivary gland tumours (SGT) are relatively rare lesions, accounting for only 3–6% of all head and neck neoplasms. They are a morphologically and clinically diverse group which present a challenge to head and neck surgeons and pathologists, both in terms of diagnosis and clinical management. The majority of tumours arising from the minor salivary glands are malignant (1–4). The identification and validation of novel stratification and predictive biomarkers for SGT would consequently be of significant clinical utility.

Migration-stimulating factor (MSF), a soluble genetically truncated isoform of fibronectin, is a potent oncofoetal regulatory molecule. Its 2.1-kb message is transcribed from the fibronectin gene by read-through of intron 12, followed by premature intra-intronic cleavage. As a consequence of this post-transcriptional foreshortening, MSF message is identical to the 5′ end of fibronectin (up to and including exon III-1a), followed by a 30-bp intron-derived in-frame coding sequence and a 165-bp 3′-UTR. The 70 kDa MSF protein is consequently identical to the N-terminus of fibronectin and terminates in an MSF-unique (intron-coded) 10 amino acid sequence not present in any full-length fibronectin (5, 6).

Immunohistochemical studies (using antibodies raised against the MSF-specific C-terminal decamer) indicate that MSF is expressed by keratinocytes, fibroblasts and vascular endothelial cells during foetal development, but is generally not expressed in healthy adult skin. MSF is transiently re-expressed during acute wound healing (7) and persistently re-expressed by both tumour and stromal cells in a number of common human cancers,

including breast and oesophageal carcinomas (5, 7, 8). The expression of MSF in salivary gland tumours has not been examined previously.

Recombinant MSF exhibits a number of potent bioactivities *in vitro* and *in vivo* relevant to cancer progression and wound healing, including stimulation of cell migration/invasion, matrix remodelling, and angiogenesis (5, 7–10). The aims of this study have been to ascertain the possible presence, diagnostic significance and role of MSF in salivary gland tumours. Immunohistochemical and *in vitro* functional studies were used to: (i) compare the expression pattern of MSF in histologically normal salivary gland tissue, benign and malignant SGT, and (ii) ascertain the effects of MSF on the migration of a malignant salivary carcinoma cell line *in vitro*.

Materials and methods

Tissue specimens and reagents

Formalin-fixed, paraffin-embedded, archival tissue specimens of benign ($n = 7$) and malignant ($n = 27$) salivary gland tumours (SGT) were obtained from the Domagk-Institute of Pathology, University Clinic of Munster, Germany. The study was performed according to the Declaration of Helsinki and approved by the local Ethics Committee of the University Hospital of Essen, University Duisburg-Essen, Germany. The majority of malignant tumours were adenoid-cystic carcinomas ($n = 16$) and mucoepidermoid carcinomas ($n = 6$). The majority of benign tumours ($n = 6$) were pleomorphic adenomas. Unless otherwise stated, only these (epithelial-derived) tumours were included in the statistical analyses. An angiomyoma (or angioleiomyoma) was also examined. This is a benign tumour derived from the vascular smooth muscle cells and rarely found in the salivary gland. The anatomical location of the tumours and relevant patient information are presented in Table 1. Sixteen of the tumour specimens (15 malignant and 1 benign) included regions of adjacent histologically normal salivary gland (NSG), as indicated in Table 2.

Recombinant MSF (rhMSF) and a mouse monoclonal MSF-specific antibody (mab7.1), raised against a peptide consisting of the unique C-terminal decamer of MSF, were prepared as previously described (5).

Detection of MSF by immunohistochemistry

Formalin-fixed paraffin-embedded salivary gland tissues were sectioned (4 μm) and mounted on polylysine-coated slides (cat. no. 631-0107, BDH). De-paraffinized sections were incubated with 3% (v/v) hydrogen peroxide in phosphate-buffered saline (PBS) for 20 min

Table 1 Clinical characteristics of the malignant and benign salivary gland tumours examined

		Anatomical location								
Tumour	n	*lip*	*Oral mucosa*	*Upper Jaw*	*Palate*	*Cheek*	*Lower jaw*	*Sub Mand*	*Age Average (range)*	*Gender (F:M)*
ACC	16	2	1	8	3	1	1		57.1 (35–83)	13:3
MC	6			2	2	1		1	47.5 (32–66)	2:4
AC	2		1	1					43.5 (36–51)	1:1
BcAC	2				1	1			58–59	0:2
SA	1			1					79	1:0
All malignant	27	2	2	12	6	3	1	1	55.7 (32–83)	17:10
PA	6			2	1	1		2	41.6 (20–75)	4:2
Ang	1				1				29	0:1
All benign	7			2	2	1		2	39.8 (20–75)	4:3
All tumours	34	2	2	14	8	4	1	3	52.5 (20–83)	21:13

Malignant tumours: ACC, adenoid cystic carcinoma; MC, mucoepidermoid carcinoma; AC, adenocarcinoma not otherwise specified; BcAC, basal cell adenocarcinoma; SA, sebaceous adenocarcinoma.
Benign tumours: PA, pleomorphic adenoma; Ang, angiomyoma.

Table 2 Tumour specimens containing histologically normal salivary gland adjacent to the tumour. Normal salivary gland (NSG) was found adjacent to adenoid cystic carcinoma (ACC), mucoepidermoid carcinoma (MC), adenocarcinoma not otherwise specified (AC), basal cell adenocarcinoma (BcAC) and pleomorphic adenoma (PA)

		Anatomical location								
Adjacent tumour	n	*Lip*	*Oral mucosa*	*Upper Jaw*	*Palate*	*Cheek*	*Lower jaw*	*Sub Mand*	*Age Average (range)*	*Gender (F:M)*
ACC	9	1		6	1	1			58 (35–80)	8:1
MC	3			1	1			1	41.3 (32–59)	1:2
AC	2		1	1					43.5 (36–51)	1:1
BcAC	1					1			58	0:1
All malignant with adjacent NSG	15	1	1	8	2	2		1	50.2 (32–80)	10:5
Benign (PA) with adjacent NSG	1			1					36	1:0
All tumours	16	1	1	9	2	2		1	43.1 (32–80)	11:5

to inhibit endogenous peroxidase activity. To further reduce non-specific background staining, sections were treated with Avidin/Biotin blocking step (Avidin/Biotin Blocking Kit; Vector Labs, Peterborough, UK) and incubated with 20% (v/v) normal goat serum (NGS) in PBS for 30 min. Sections were then incubated overnight at 4°C with the MSF-specific primary antibody (mAb 7.1 at 40 μg/ml) in 20% NGS in PBS. Initial optimization of the staining (11) indicated that pre-treatment of the sections was not required for antigen retrieval. Detection was achieved by treatment with 6 μg/ml biotinylated goat anti-mouse IgG (Vector Labs) in PBS containing 20% NGS, followed by avidin–biotin complex for 30 min at room temperature. All the above stages were separated by PBS washes. Immunostaining was visualized by developing the slides in diaminobenzidin (DAB) for 10 min and counter-staining with Mayer's haematoxylin. As negative controls, sections were incubated with normal mouse IgG (nMIgG; Dako, Ely, Cambridgeshire, UK) instead of primary antibody.

Assessment of MSF expression in salivary gland tissue sections

Duplicate sections of each specimen were stained and assessed by 2–4 independent observers. Final results were obtained by consensus. MSF staining was first evaluated at ×100 magnification, scanning the whole section. The overall distribution of staining was recorded (e.g. homogeneous/heterogeneous, tumour cell- and/or stromal cell-associated, etc.) and the following semi-quantitative parameters were then evaluated by comparison to pre-selected calibration slides:

(i) MSF overall grade (0–3): Specimens were initially graded by four observers as negative (grade 0), weak (grade 1), moderate (grade 2) or strong (grade 3) positive. At least 10% of the whole area stained was chosen as the cut-off point between grade 0 and grade 1. Although such overall grade evaluation includes epithelial and stromal compartments, it reflects mainly the former, as higher magnification is required to assess the stroma. The epithelial and stromal compartments were then evaluated individually.

(ii) Epithelial MSF expression: This was defined by four indices (12). The *percentage of total area stained* (1–100%) was estimated and the *intensity of the staining* was graded from 0 (negative) to 3 (strong) by comparison to calibration slides. The *highest (or hot spot) intensity* (present in at least 10% of the epithelium) was also recorded. *Final score* (0–300) was derived by multiplying the percentage area stained by the intensity of the staining. Heterogeneous staining was common and this is reflected in the final score; for example, 50% area stained with intensity 2 and 20% area with intensity between 2 and 3 (2.5) gives a final score of 150.

(iii) Stromal MSF expression in SGT was classified, using higher magnification, as either positive or negative for three constituent stromal cell types: Fibroblasts, microvascular and inflammatory. The stromal cell types were identified on the basis of cytological characteristics, as generally accepted and used in routine pathology.

(iv) MSF expression in NSG: The staining of specific cell types in the histologically NSG was similarly classified as either positive or negative.

Tissue culture

The human salivary tumour cell line (HSG) was a gift from the laboratory of origin (Professor Mitsunobu Sato, University of Tokushima School of Dentistry, Japan) (13). HSG cells were maintained in cell culture using Eagle's minimum essential medium (MEM) growth medium supplemented with 15% (v/v) donor calf serum and 1% (w/v) glutamine, as described (14). For immunostaining, HSG cells were either stained directly on tissue culture dishes or embedded within a floating 3D collagen gel as previously described (15). In this latter case, the cells were plated at high density (2×10^6cells/2 ml gel), the gels were maintained under standard tissue culture conditions for 1–2 days and then pelleted by gentle centrifugation, formalin-fixed and paraffin-embedded. Paraffin-embedded blocks were sectioned and stained with the MSF-specific antibody (mAb 7.1) as for tissue sections. Cells on tissue culture dishes were fixed with 2% (v/v) formalin in PBS for 30 min at room temperature and permeabilized with 100% (v/v) methanol for 10 minutes at −20°C before staining (16).

Cell migration

Migration assays were performed using the transmembrane or Boyden chamber assay as previously described (17, 18). The bottom wells of the 48-well chamber (Neuro Probe Inc., Gaithersburg, MD, USA) were loaded with 30 μl of different concentrations of rhMSF (from 1 pg/ml to 1 μg/ml) dissolved in serum-free MEM containing 2 μg/ml bovine serum albumin (SF-MEM). Solvent only, containing 2 μg/ml albumin, was used as negative control. Polycarbonate nucleopore membranes (8 μm pore; Whatman, Maidstone, Kent, UK) were coated with native type I collagen (100 μg/ml) and placed in the chamber. The upper wells of the assembled chamber were then loaded with 50 μl of HSG cell suspension in SF-MEM at 1.2×10^6cells/ml. After a 5-h incubation period at 37°C in a humidified CO_2 incubator, the membranes were removed, fixed in methanol and stained with Mayer's haemotoxylin. The cells remaining on upper surface of the membrane were scraped off and the cells that had migrated through the pores to the under surface were assessed microscopically (×200) under bright field illumination. Six replicate wells were used per variable; the number of cells migrated was counted in three random fields per well (i.e. total of 18 fields) and used to calculate the mean cell number per field ± standard deviation.

Statistical analysis

Statistical analyses were carried out with the Prism 5 (Graphpad Inc. La Jolla, CA, USA) software package. Differences among groups of tissues were determined either by chi-squared and Fisher's exact tests or Kruskal–Wallis and two-tailed Mann–Whitney tests,

as appropriate. Differences in cell migration were analysed by ANOVA and Bonferroni tests. Significance differences were defined at 95% level of confidence ($P < 0.05$).

Results

Immunolocalization of MSF

Duplicate sections of benign and malignant small salivary gland tumours (SGT) (Table 1) were stained with MSF-specific antibody mab7.1 (5). Histologically NSG was presented adjacent to some of the tumours (Table 2). MSF was differentially expressed in the salivary gland tissues examined (see below). Positive staining was observed in the majority of malignant tumours, both in the tumour and in the associated stromal cells. Representative examples of MSF expression by malignant tumour cells and tumour-associated stromal cells are presented in Fig. 1A,B,G. Examples of negative and positive benign tumours were also encountered (Fig. 1C,D). Positively stained acinar cells were rarely present in NSG (Fig. 1E,F). Positively stained inflammatory cells were occasionally detected in association with malignant tumours (Fig. 1G). Sections incubated with normal mouse IgG, instead of MSF antibody, showed no staining (negative controls, not shown).

Differential expression of MSF in salivary gland tissues

MSF expression was evaluated in NSG (n = 16), benign SGT (B; n = 7) and malignant SGT (M; n = 27). For statistical analyses, only pleomorphic adenomas (n = 6) were included in the benign group. MSF expression was first graded as negative (0), weak positive (1), moderate (2) or strong positive (3) by four independent observers. To compare the different tissues, results are presented in Fig. 2A as the percentage of specimens showing the various MSF grades. This initial classification (overall MSF grade) indicated that a

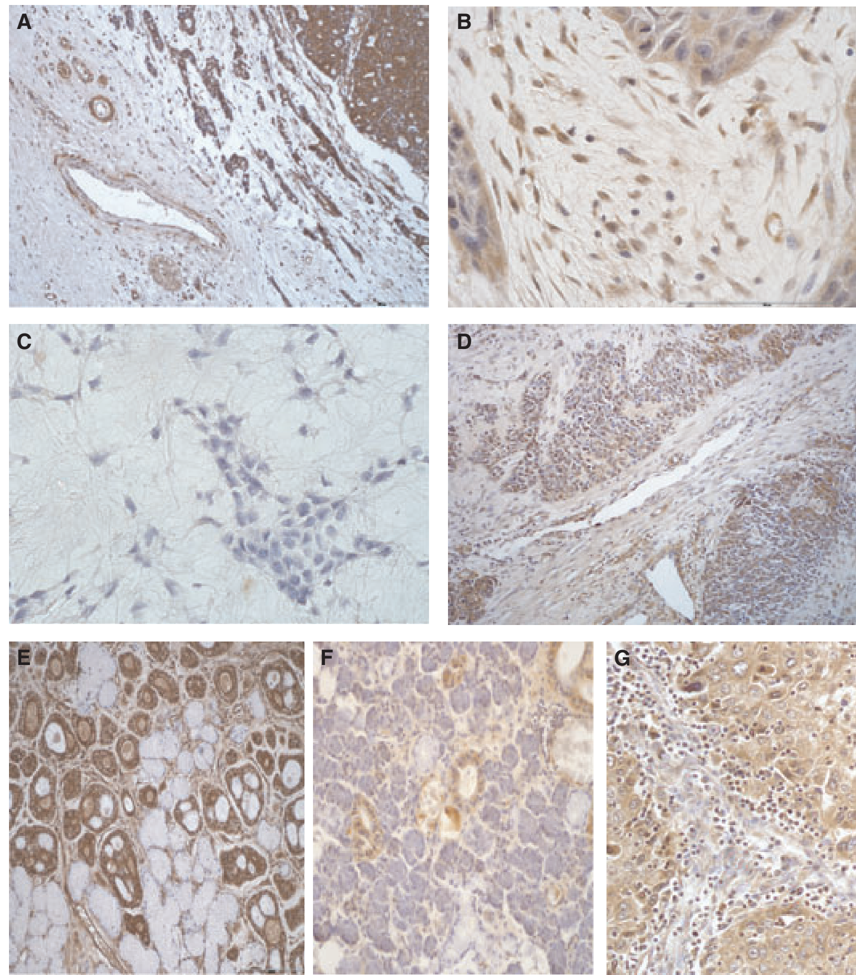

Figure 1 Immunolocalization of MSF in salivary gland tissues. (A, B) MSF expression in malignant tumours. Adenoid cystic carcinoma (A) and mucoepidermoid carcinoma (B) showing MSF staining in the tumour cells and associated blood vessels and fibroblasts. (C) Negative MSF staining in benign tumour (pleomorphic adenoma). (D) MSF-positive pleomorphic adenoma. (E) Adenoid cystic carcinoma showing negative MSF staining in histologically normal salivary gland (NSG) (mucous cells) adjacent to positive tumour. (F) Positive excretory and striated ducts next to negative serous cells in NSG. (G) Positive inflammatory cells and carcinoma cells in a mucoepidermoid carcinoma. Original photographs were taken at magnification ×100 (A, D, E, F, G) or ×400 (B, C).

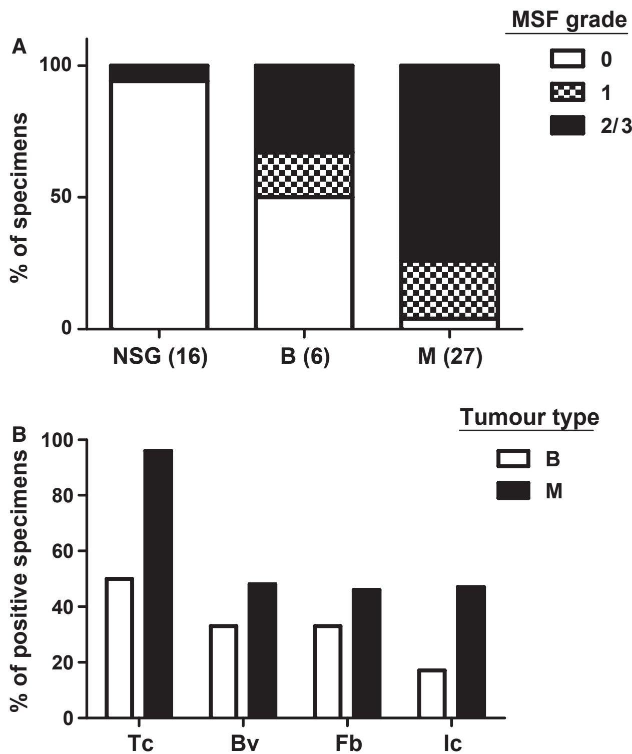

Figure 2 MSF overall grade in salivary gland tissues. Following staining with MSF-specific antibody, the tissues were graded from negative (MSF grade 0), to weak (1), moderate (2) or strong (3) positive by consensus of four independent observers. (A) Percentage of specimens showing the indicated MSF grades. The tissues examined included histologically normal salivary gland (NSG, $n = 16$), benign (B, $n = 6$) and malignant salivary gland tumours (M, $n = 27$). Chi-squared tests (MSF grades 0 vs. 1 vs. 2/3) and Fisher's exact test (MSF grades 0 vs. 1/2/3) demonstrated significant differences between M and B ($P = 0.01$–0.006), between M and NSG ($P < 0.0001$) and between B and NSG ($P = 0.04$). All P-values were $P < 0.0001$ when the percentage of specimens (as shown in the graph) were compared. (B) Epithelial and stromal MSF expression in benign (B) and malignant (M) salivary gland tumours. Results show the percentage of specimens graded positive (MSF grades 1/2/3) for different cellular compartments, including tumour epithelial cells (Tc; $n = 27$ for M and 6 for B), blood vessels (Bv; $n = 27$ M and 6 B), fibroblasts (Fb; $n = 26$ M and 6 B) and inflammatory cells (Ic; $n = 19$ M and 6 B). The difference between malignant and benign tumours was analysed by Fisher's exact test for the actual number of specimens assessed (n) and for the percentage of specimens (%, as shown in the graph). The respective P-values for (n) and (%) were: 0.01 and 0.001 for Tc; 0.6 and 0.04 for Bv; 0.6 and 0.08 for Fb; 0.3 and 0.0001 for Ic.

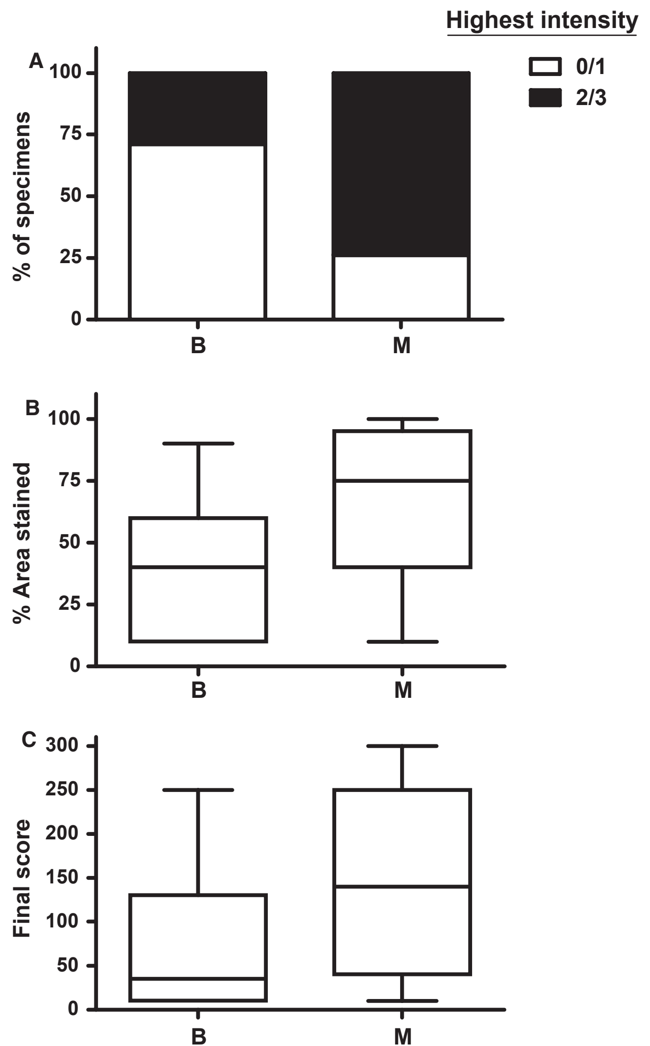

Figure 3 Epithelial MSF expression in salivary gland tumours. MSF expression in the epithelium of benign (B; $n = 6$) and malignant (M; $n = 27$) tumours was evaluated and compared. Significant differences between B and M occurred in terms of the highest intensity ($P = 0.006$, Fisher's exact test), the % of area stained ($P = 0.044$, Mann–Whitney test) and final score (area × intensity; $P = 0.044$, Mann–Whitney test).

significantly greater proportion of the malignant tumours were positively stained for MSF compared with benign lesions or NSG. A significant difference was also observed between benign tumours and NSG. Therefore, overall MSF expression increased significantly in a step-wise fashion from normal salivary gland to benign and malignant tumours ($P = 0.04$–0.0001, Fig. 2A); with moderate/strong positive specimens representing 6%, 33% and 74% of the normal, benign and malignant specimens, respectively. The group of benign SGT consisted of six epithelial-derived tumours (pleomorphic adenomas). The inclusion of an additional stromal-derived tumour (angiomyoma) brought the percentage of moderate/strong positive specimens to 29%, but did not alter the significance of the results ($P = 0.02$–0.0001). Significant differences between pleomorphic adenomas and malignant tumours were also observed even when comparing specific sub-groups of the latter (Table 1), such as adenoid cystic carcinomas ($P = 0.045$) and mucoepidermoid carcinomas ($P = 0.049$), in spite of the small number of specimens involved. Within the group of SGT specimens examined, MSF expression was not related to the anatomical site of origin or to the age or gender of the patient.

The overall MSF grade (Fig. 2A) gives a general assessment of the sections, including epithelial and stromal compartments. More detailed observations of the tumours revealed that MSF was heterogeneously distributed within both the tumour and stromal compartments (such as fibroblasts, blood vessels and inflammatory cells), with a greater proportion of the

specimens being positively stained in malignant than in benign tumours in all cellular compartments (Fig. 2B). A benign tumour derived from the vascular smooth muscle cells (angiomyoma) was positive for MSF, whereas the blood vessels of the normal salivary gland were MSF-negative. Within the epithelium, (Fig. 3) significant differences between benign and malignant tumours occurred in terms of highest (hot spot) intensity, percentage of area stained and final score, (i.e. area × intensity).

Overall MSF grade of the NSG reflects mainly the staining present in the acinar cells. More detailed observation of the different NSG cell types indicated that mucous and serous acinar cells were indeed predominantly negative for MSF, whereas ductal cells commonly exhibited a diffuse positivity, this being stronger in the luminal cells compared with basal and myoepithelial cells (Fig. 1E,F). Of 16 NSG specimens examined, 10, 11 and 15 (63%, 69% and 94%) showed positive intercalated, striated and excretory salivary ducts, respectively. Only one specimen (6%) showed MSF staining in acinar (serous) cells and nine (56%) in myoepithelial cells.

Motogenic activity of MSF on a human salivary gland tumour cell line (HSG)

HSG cells stained positively for MSF. The same results were obtained irrespective of whether the cells were embedded into collagen gels, paraffin-embedded and sectioned (Fig. 4A) or stained directly on tissue culture dishes (not shown). As with tissue sections, negative controls, incubated with normal mouse IgG, showed no staining (Fig. 4B). Exogenous rhMSF stimulated the migration of HSG cells through type I collagen-coated membranes in a dose-dependent fashion. All concentrations of MSF tested (1 pg/ml–1 μg/ml) exhibited significant motogenic activity by comparison to the negative control or baseline, with a plateau reached at 10 ng/ml (Fig. 4C).

Discussion

The present study revealed that (i) significantly higher levels of MSF were detected in malignant than in benign salivary gland tumours, (ii) MSF staining was also apparent in some histologically normal salivary gland tissue, although this was significantly less than in either benign or malignant tumours, and (iii) exogenous rhMSF stimulated the migration of a salivary gland tumour cell line in a dose-dependent fashion. Further studies will be required to determine whether MSF is also an autocrine factor on these tumour cells. The difference in MSF expression between histologically normal tissue (adjacent to tumours) and benign tumours suggests that up-regulation of MSF expression may be a significant feature of early salivary gland tumour incep-

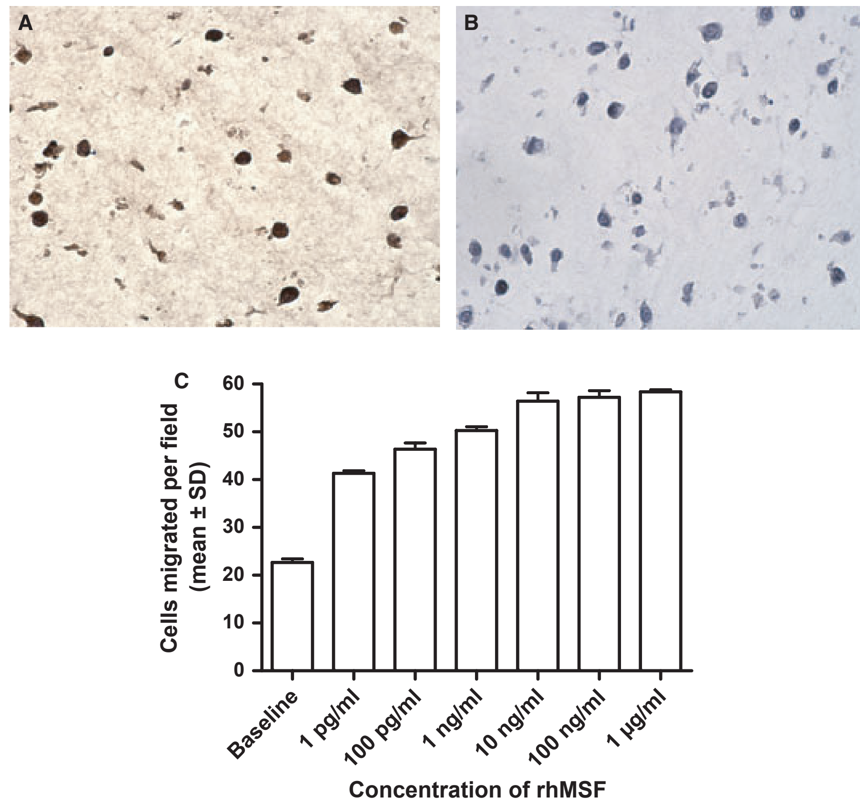

Figure 4 Expression of MSF and response to exogenous MSF by a human salivary gland tumour cell line (HSG). (A) Positive MSF staining in HSG cells embedded into collagen gels. (B) Negative controls stained with normal mouse IgG. (C) The migration of HSG in response to rhMSF in the transmembrane assay. Significant differences occurred between baseline control levels (containing 2 μg/ml bovine serum albumin) and every concentration of MSF tested ($P < 0.001$; ANOVA and Bonferroni tests). Further stepwise significant differences ($P < 0.05$–0.001) occurred from 1 pg/ml to 10 ng/ml, reaching a plateau of maximal stimulation with 10 ng/ml.

tion and/or progression. Furthermore, the observed higher expression of MSF by malignant tumours may prove to be a useful marker of later stage disease progression.

We have previously reported that both MSF protein and mRNA are expressed by the same cell types in foetal skin and breast tissues (5). As is the case with breast cancers (5, 9), MSF was over-expressed in malignant salivary gland tumours by both tumour cells and tumour-associated stromal cells. It is of particular interest that several sub-sets of stromal cells, including fibroblasts, microvascular endothelial cells and inflammatory cells, were positively stained, thus indicating the coordinated up-regulation of MSF expression during tumour progression. In this regard, it should be noted that MSF expression by stromal fibroblasts has recently been reported to be induced by epigenetic mechanisms regulated by the concerted signalling of TGF-β and matrix (6, 7, 9). Considering the strong association between tobacco consumption and head and neck cancer incidence, it is of interest that the tobacco carcinogen benzo(a)pyrene has been shown to induce MSF expression on a bronchioloalveolar carcinoma cell line (19).

Some MSF expression was associated with the ductal epithelium of histologically normal salivary gland tissue adjacent to malignant tumours. It is important to note that normal salivary gland tissue from healthy adults was not available for examination in this study. This may be of significance in light of previous observations that MSF is expressed by fibroblasts obtained from histologically normal breast adjacent to mammary carcinomas, but not by normal breast tissue from healthy adults (i.e. reduction mammoplasty biopsies) (20). It is therefore possible that MSF expression by the normal appearing salivary gland tissue represents a 'functional aberration' reflecting the proximal location of a malignant tumour and/or the result of a 'field cancerization' effect resulting from previous exposure to a carcinogenic agent (19, 21). In the case of the stromal vasculature, it is of interest that MSF was not detected in the blood vessels of the normal salivary gland, whereas a benign tumour derived from the vascular smooth muscle cells (angiomyoma) showed MSF staining. The complex histo-cytological structure of the salivary glands and their tumours leads to diagnostic difficulties (see Introduction section). A more detailed cytological study will be required to ascertain the possible relationship between MSF expression and the histological characteristics of the tumours.

In conclusion, data presented here provide an initial indication that MSF expression is up-regulated in both the epithelial and stromal cell compartments of malignant salivary gland tumours. This study provides a rational platform for subsequent more extensive investigation of the possible diagnostic and prognostic significance of MSF expression in this currently difficult to manage patient group. It also suggests that developing means to inhibit MSF expression and/or functionality may provide novel therapeutic strategies to improve the management of patients with salivary gland tumours.

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