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An immunohistochemical study of the extracellular matrix in oral squamous cell carcinoma and its association with invasive and metastatic potential

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Abstract The expression of extracellular matrices (ECMs) laminin (LN), type IV collagen (IV C), heparan-sulphate proteoglycan (HS-PG), fibronectin (FN), tenascin (TN), decorin and vitronectin (VN) was examined immunohistochemically in 112 primary tumours and 29 metastatic cervical lymph nodes in oral squamous cell carcinoma (OSCC). In highly invasive primary tumours, the expression of LN, IV C and HS-PG in the basement membrane along the tumour-stroma borderline and the expression of decorin and VN in the tumour stroma at the invasive site were all significantly decreased. The expression of FN and TN in the tumour stroma at the same site was markedly increased. In peritumour stroma in metastatic lymph nodes, LN, IV C, HS-PG, decorin and VN were weakly expressed, while FN and TN were strongly expressed. Thus, the staining pattern of the ECMs in the metastatic lymph nodes was similar to that in highly invasive primary tumours. Furthermore, in primary tumours of metastatic cases, the expression of LN, IV C, HS-PG, decorin and VN obviously decreased, while the expression of FN and TN increased when compared with those of the non-metastatic cases. The investigation of ECMs in OSCC was valuable in predicting tumour behaviour.

Key words Extracellular matrix
Immunohistochemistry · Squamous cell carcinoma
Invasiveness · Metastasis

Introduction

Extracellular matrices (ECMs), which are composed of collagenous proteins, non-collagenous proteins and proteoglycans (Bruijn et al. 1988), constitute an extracellular environment of active structural proteins and influence tumour behaviour as well as organogenesis, embryogenesis and wound healing (Burkhardt 1985). The multifactorial properties of ECMs have been studied in numerous normal organs and malignant tumours (Pearlstein et al. 1980; Martinez-Hernandez and Amenta 1983; Iozzo 1985; Dahlbäck et al. 1986; Mackie et al. 1987; Erickson and Lightner 1988).

In oral squamous cell carcinoma (OSCC) and in other malignant tumours, the invasive and metastatic potential both play a critical role in prognosis (Shimada et al. 1990). To predict the outcome of the disease at an early stage, many studies have addressed the grading of histological malignancies in primary tumours (Anneroth et al. 1987) but no adequate histological classifications have been reported. In the present study, we examined immunohistochemically the expression of the following ECMs: laminin (LN), type IV collagen (IV C), heparan-sulphate proteoglycan (HS-PG), fibronectin (FN), tenascin (TN), decorin and vitronectin (VN), at the invasive site of OSCC, to determine whether ECMs are associated with clinicopathological features, in particular the invasive and metastatic potential.

Materials and methods

All patients in this study were referred to the Second Department of Oral and Maxillofacial Surgery, Kyushu University Dental Hospital, Fukuoka, Japan, during 1985–1990. Histological and immunohistochemical examinations were carried out on 112 initial biopsies of the primary OSCC, which included 69 non-metastatic and 43 metastatic cases. In the biopsies, particular attention was given to involve the deeper portion of the tumour at the invasive front. The primary sites were the tongue in 46 cases, the mandibular gingiva in 26, the floor of the mouth in 16, the maxillary gingiva and buccal mucosa in 10, the lower lip in 3 and the soft palate in 1. Twenty-nine metastatic cervical lymph nodes which were obtained from neck dissection were also examined. In nearly all cases, preoperative radiotherapy and chemotherapy were applied and thereafter surgical treatment was performed. The cases without metastasis were followed for at least 2 years after treatment to confirm that no distant metastasis had occurred.

The specimens were fixed in 10% formalin and embedded in paraffin. The serial sections were cut at 4 μm, deparaffinized, and

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Table 1 Primary antibodies and proteolytic treatments (*LN* laminin, *IV C* type IV collagen, *HS-PG* heparan-sulphate proteoglycan, *FN* fibronectin, *TN* tenascin, *VN* vitronectin)

Antibodies	Source	Dilution	Proteolytic treatment[a]
Rabbit anti-mouse LN (polyclonal)	Advance, Tokyo, Japan	1:800	0.4% pepsin, 37°C, 50 min
Mouse anti-human IV C (monoclonal)	Chemicon, Temecula, USA	1:50	0.1% trypsin, 37°C, 120 min
Mouse anti-bovine HS-PG (monoclonal)	Chemicon, Temecula, USA	1:25	0.05% protease type XXIV, 37°C, 30 min with 300 IU/ml hyaluronidase, 37°C, 30 min
Rabbit anti-human FN (polyclonal)	Dakopatts, Glostrup, Denmark	1:200	0.05% protease type XXIV, room temperature, 10 min
Rabbit anti-human TN (polyclonal)	Chemicon, Temecula, USA	1:150	0.05% protease type XXIV, room temperature, 10 min
Rabbit anti-human decorin (polyclonal)	Chemicon, El Segundo, USA	1:150	0.4% pepsin, 37°C, 40 min
Rabbit anti-human VN (polyclonal)	Chemicon, Temecula, USA	1:150	0.4% pepsin, 37°C, 40 min

[a] The reagents for proteolytic treatment were purchased from Sigma, St. Louis, Mo., USA

stained with haematoxylin and eosin for histological diagnosis. The avidin-biotin peroxidase complex (ABC) method was used for immunohistochemical staining, using a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA). Several types of proteolytic digestion were needed prior to the immunohistochemical procedures, as shown in Table 1. In order to abolish endogenous peroxidase activity, treatment with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min was performed. The sections were then treated with normal serum of the same species as secondary antibodies for 20 min to eliminate any non-specific binding. Thereafter, the sections were incubated with either monoclonal or polyclonal primary antibodies as shown in Table 1, with biotinylated secondary antibodies for 30 min, and then subsequently with ABC immunoperoxidase for 60 min. To visualize the immunoreactivity, 3,3-diaminobenzidine H_2O_2 (Wako Pure Chemical Industries, Osaka, Japan) substratum was used. As negative controls, normal serum of the same species as primary antibodies was used in place of primary antibodies.

The mode of tumour invasion (MI) was graded by the method reported by Yamamoto et al. (1983) as follows: 1, a well-defined borderline; 2, cords – a less marked border; 3, groups of cells – no distinct borderline; 4, diffuse invasion (4c, cordlike type; 4d, widespread type).

A statistical analysis of the data was determined by the chi-square test and a significance level of 0.05 ($P<0.05$) was used for this test.

Results

At first we examined the ECM staining pattern in 20 normal epithelia (tongue, 6; mandibular gingiva, 4; floor of mouth, 3; maxillary gingiva, 3; buccal mucosa, 4). As shown in Fig. 1, LN, IV C and HS-PG showed continuous positivity in basement membrane (BM; Fig. 1a–c), FN and TN were almost negative or restricted in the adjacent stroma of BM (Fig. 1d, e), while decorin (Fig. 1f) and VN were well preserved in the connective tissues. This pattern was consistently observed in all normal epithelia. Although the three components of BM showed a similar continuous positivity, the staining intensity of HS-PG was weaker than that of LN and IV C. In OSCC, the LN, IV C and HS-PG all showed discontinuous positivity, FN and TN were increased, while decorin and VN were decreased (Figs. 2–4). However, the ECM staining pattern in OSCC was highly heterogeneous. Then we graded the ECM staining pattern on the basis of immunoreactivity in the normal epithelia, and then examined any relationship with the invasive and metastatic potential of OSCC.

We examined the staining pattern of ECM components LN, IV C and HS-PG in BM at the tumour-stroma border and graded them as follows: + + +, continuous linear staining (compatible with normal epithelia); + +, more than or equal to 50% positive staining; +, less than 50% positive staining; ±, almost negative (see Table 2). These ECMs showed almost an equal staining pattern within the individual cases, although the staining intensity of HS-PG was slightly weaker than that of LN and IV C. A decreased expression of these ECMs was frequently observed in cases of high MI grades (4c and 4d; Fig. 5a, b) and expression was preserved in low MI grades (1 and 2; Fig. 2a–c). This association of the staining pattern with MI grade was statistically significant in LN, IV C and HS-PG. However, the staining pattern of these ECMs was heterogeneous when examining the cases individually, especially in cases with MI grade 3 (Figs. 3a–c; 4b, c).

The FN and TN expression in the tumour stroma at the invasive front was graded as follows; + + +, extensive staining; + +, continuous staining in the peritumoral stroma; +, segmented positive staining in the peritumour stroma; ±, almost negative (as normal epithelia). Increased expression of FN and TN was frequently observed in cases of high MI grades (4c and 4d;

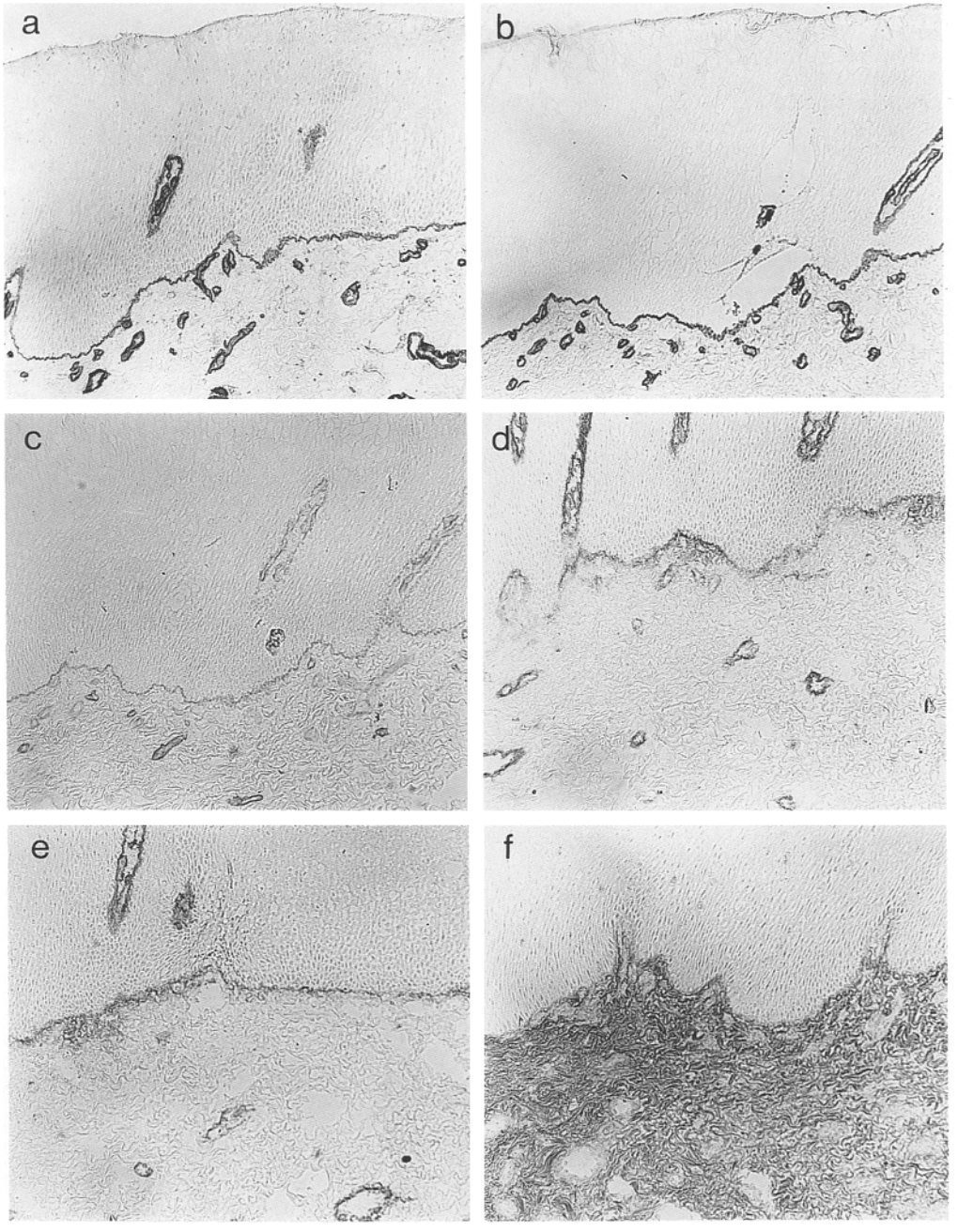

Fig. 1a–f The extracellular matrix (ECM) staining pattern of normal epithelia. **a–c** Continuous and linear staining of basement membrane (BM) components is shown [**a:** laminin (LN), **b:** type IV collagen (IV C), **c:** heparan-sulphate proteoglycan (HS-PG)]. The positive staining of stromal fibronectin (FN); **(d)** and tenascin (TN); **(e)** is restricted, beneath the BM region. Staining of decorin **(f)** is noted in the connective tissue. **a–f** ×126

Fig. 5c, d) with weak and limited expression in low MI grades (1 and 2; Fig. 2d, e). The association of staining pattern with MI grade was statistically significant in both FN and TN, although TN was more intensely stained and better defined than FN. Positive staining was detected in the peritumour stroma, while in the highly invasive cases, strong expression was observed extensively. It is of considerable interest that the FN and TN expression in cases with marked lymphocytic infiltration was almost negative, regardless of the MI grade.

We examined the staining pattern of decorin and VN at the invasive front and graded it as follows: +++, strong staining; ++, moderate staining (comparable to the normal epithelia); +, weak staining; ±, almost negative. The expression of decorin and VN decreased remarkably in cases of high MI grades (4c and 4d; Fig. 3e), remaining well preserved in low MI grades (1 and 2; Fig. 2f). The association of the staining pattern with the MI grade was significant in both decorin and VN, although it was more apparent in VN. In most peritumour stroma, the expression of decorin and VN decreased when compared with that in the connective tissue of normal epithelia. In MI grades 4c or 4d, decreased expression of decorin and VN was observed widely, even in areas distant from the invasive front. In contrast, in cases of MI grades 2 or 3 (Fig. 3f), decreased expression was observed in limited areas of the invasive front. The staining pattern of decorin and VN was simi-

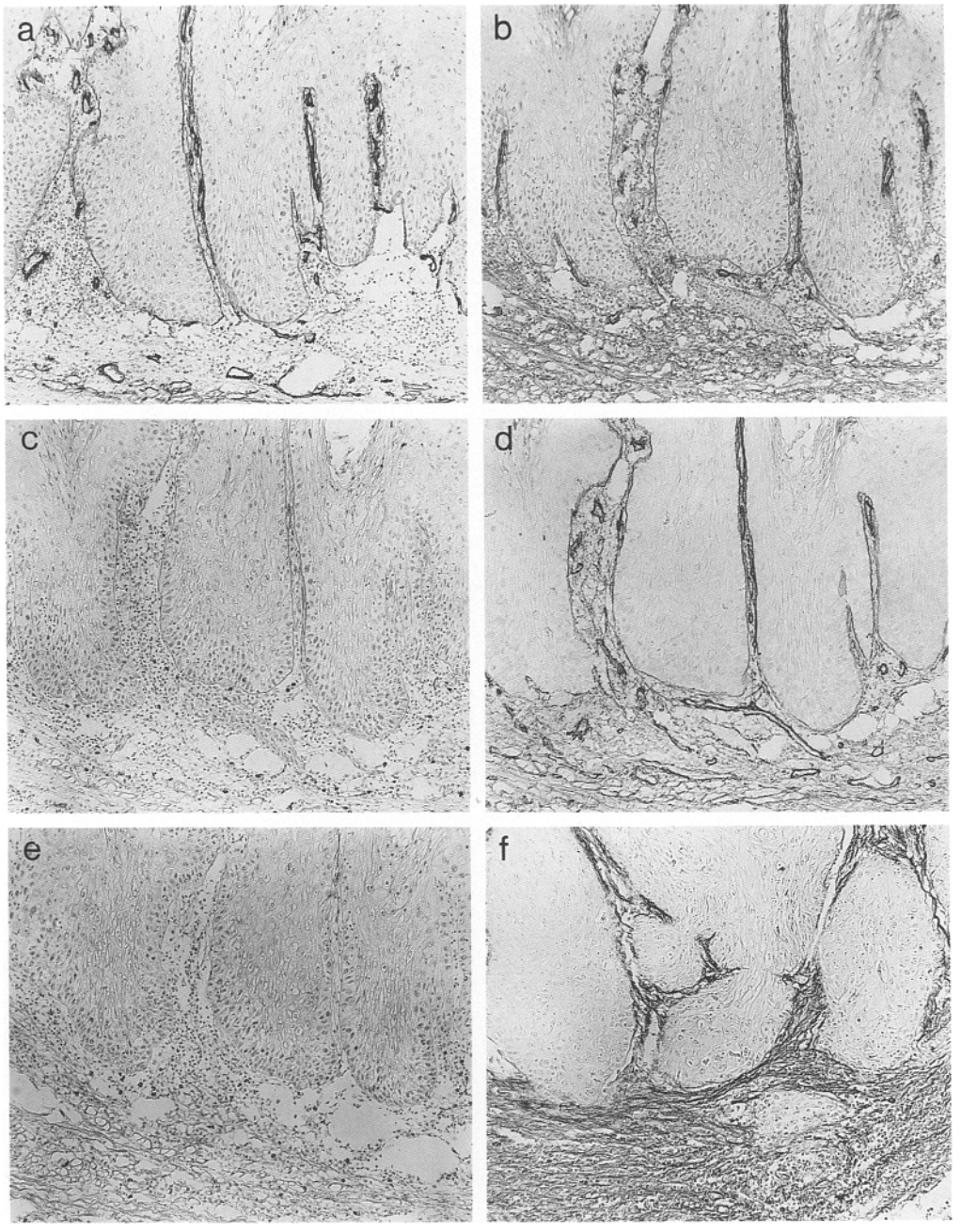

Fig. 2a–f The ECM staining pattern of primary tumours [tumour invasion (MI) grade 2]. **a–c** Continuous staining (+ + +) of BM components is shown (**a** LN, **b** IV C, **c** HS-PG). Staining of stromal FN **(d)** and TN **(e)** is almost negative (±). VN **(f)** staining is strongly positive (+ + +) where FN and TN staining are negative. **a–f** ×118

lar within the individual cases. Furthermore, it is notable that the reciprocal correlations in which FN and TN were strongly expressed while the expressions of decorin and VN were lost (Figs. 4d–f, 5c–e).

As shown in Table 3 ± and + stainings were predominant in LN, decorin and VN, in metastatic lymph nodes, while + + and + + + stainings were predominant in TN and FN (Fig. 6a–f). Thus, the staining pattern in these nodes was similar to that of primary tumours with high MI grade. In addition, lymph nodes with extranodal spread of tumour cells showed distinct expression patterns of ECMs to that of intranodal proliferation as follows; for extracapsular spread – LN (− ~ +), FN and TN (+ + ~ + + +), decorin (− ~ +), VN (− ~ +); for intranodal proliferation – LN (+ ~ + +), FN and TN (+ ~ + +), decorin (− ~ + +), VN (− ~ +).

As shown in Table 4, ECM staining pattern in primary tumours was compared between metastatic and non-metastatic cases, in order to determine whether or not ECMs are associated with metastatic potential. In primary tumours of cases with metastases, a decreased expression of LN, IV C and HS-PG, decorin and VN and an increased expression of FN and TN was observed frequently (Figs. 4a–f, 5a–e). In contrast, in primary tumours of non-metastatic cases, an increased expression of LN, IV C, HS-PG, decorin and VN and a decreased expression of FN and TN was observed (Fig. 3a–f).

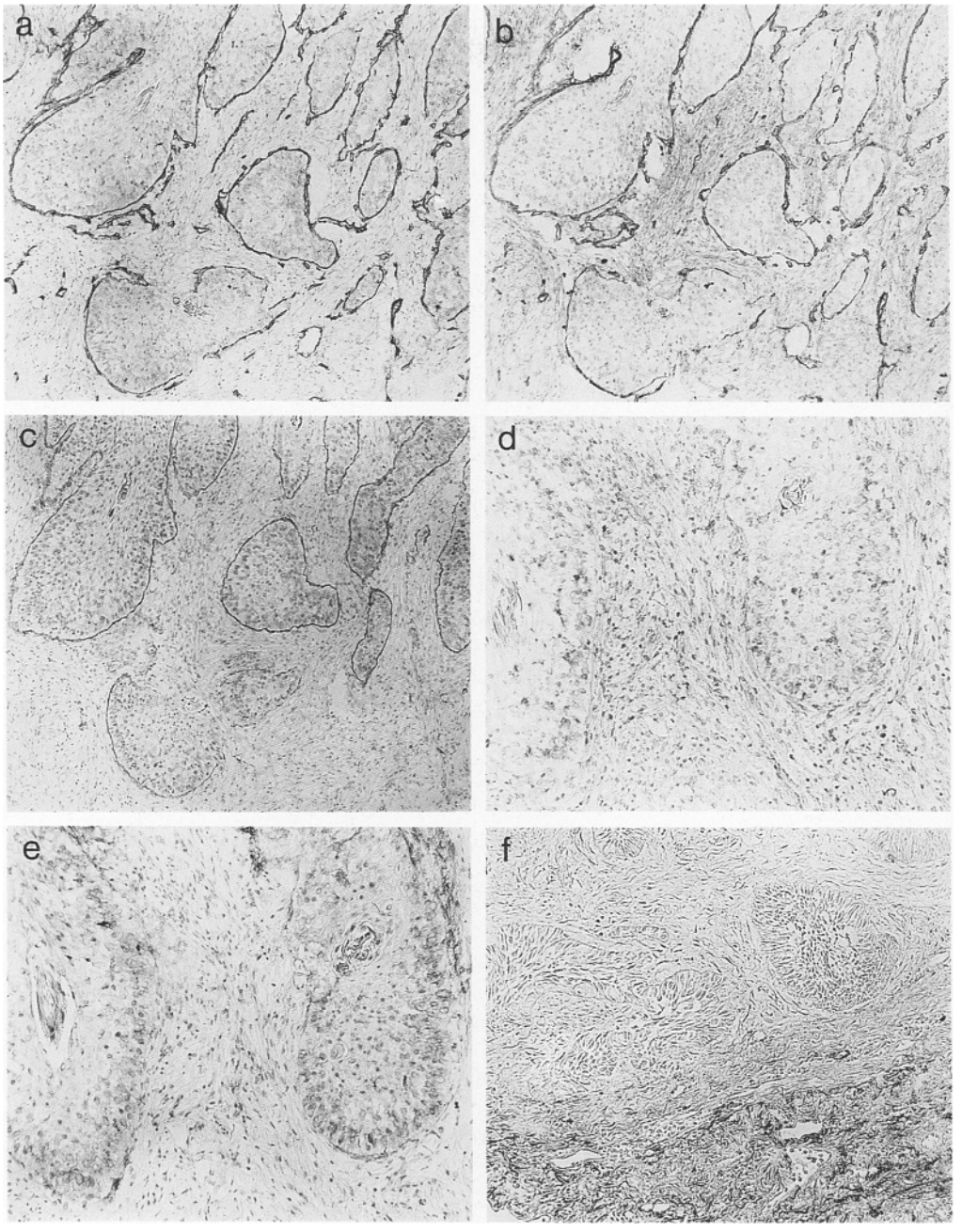

Fig. 3a–e The ECM staining pattern of primary tumours (MI grade 3) which did not show any nodal involvement. More than 50% of positive staining (+ +) of BM components (**a** LN, **b** IV C, **c** HS-PG), moderate staining (+ +) of decorin **(f)**, and the almost negative staining (±) of FN **(d)** and TN **(e)** are shown. **a–e** × 130

Thus, a significant relationship of the staining pattern of ECMs to the incidence of metastasis was detected. Although the frequency of metastasis increased significantly in cases of advanced stage (T category), the staining pattern of ECMs was not associated with the T category (data not shown). This suggests that ECMs are associated with metastatic potential independently of the T category. To confirm this, we investigated ten cases from the T1 category which developed metastasis at an early stage of the disease (Table 5). Primary tumours of these cases showed a high invasiveness (MI grades 3, 4c or 4d), a decreased expression of LN, IV C, HS-PG, decorin and VN and an increased expression of FN and TN, in most cases.

Discussion

Degradation of BM occurs when carcinomas invade the stroma (Bruijn et al. 1988; Campbell and Terranova 1988) and it has been suggested that it is essential for metastasis (d'Ardenne et al. 1986; Forster et al. 1986; Zuk et al. 1989; Hirota et al. 1990; Skalova and Leivo 1992). However, some reports have showed that BM was well preserved in invasive carcinomas (Gusterson et al. 1984; Sakr et al. 1987). In this study, we examined the expression of three kinds of ECMs (LN, IV C and HS-PG) within the BM region of OSCC. Although a variable expression of these BM components was observed from case to case, our results indicate that a loss

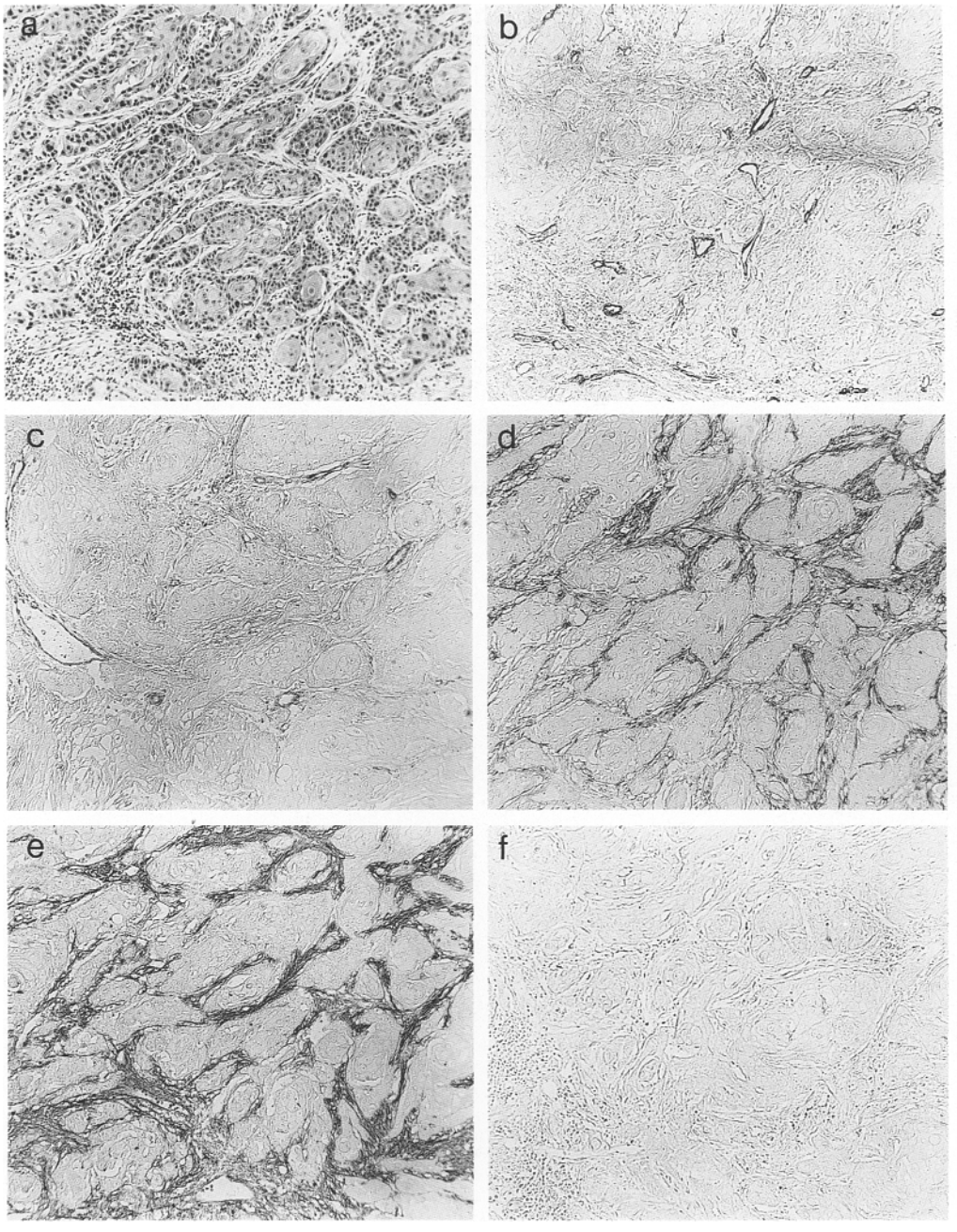

Fig. 4a–f The ECM staining pattern of primary tumours (MI grade 3) which showed nodal involvement. **a** Haematoxylin and eosin (H&E), ×86. The almost negative staining (±) of LN **(b)**, IV C **(c)** and decorin **(f)**, and extensive staining (+ + +) of FN **(d)** and TN **(e)** are shown. **a–f** ×86

of the BM components correlates with an increase in the invasive and metastatic potential and examination of BM components is suggested as a marker for predicting tumour behaviour. Further studies are needed to clarify how the degradation of BM components is induced and involved in tumour invasion and metastasis.

The expression of FN in the tumour stroma was increased in highly invasive OSCC, which is compatible with previous reports by Stenman and Vaheri (1981), d'Ardenne et al. (1983) and Forster et al. (1984). However, no relationship of stromal FN expression with metastasis has yet been reported. In this study, it was shown that the expression of FN was significantly increased in primary tumours of highly invasive and metastatic cases and in metastatic lymph nodes, especially in extranodally invaded lymph nodes. Furthermore, all the metastatic cases in the T1 category showed the extensive staining of FN. These results indicate that an increase in the stromal FN is a good marker for high metastatic potential as well as invasiveness.

The expression of TN was closely restricted beneath the BM in normal and mildly dysplastic epithelia (Chiquet-Ehrismann et al. 1986; Erickson and Lightner 1988; Mackie et al. 1988), while increased dysplastic changes in epithelia resulted in abundant and broad detection of TN in the mesenchymal connective tissue (Anbazhagan et al. 1990). Thus, the previous reports showed the close association of TN expression with dys-

Table 2 Relationship of extracellular matrix (ECM) staining pattern to tumour invasion (MI) in the primary tumour[a]. (For description of staining pattern symbols see text)

Staining pattern	Grade of MI				P value (χ^2)
	1	2	3	4c+d	
LN					
±	0[b]	0	2	12	
+	0	1	25	18	<0.01
++	1	8	23	1	(70.650)
+++	4	9	8	0	
IV C					
±	0	1	2	11	
+	1	4	27	19	<0.01
++	2	8	25	1	(48.061)
+++	2	5	4	0	
HS-PG					
±	0	0	5	17	
+	1	5	35	13	<0.01
++	1	8	14	1	(61.847)
+++	3	5	4	0	
FN					
±	2	3	5	1	
+	2	9	18	7	<0.05
++	0	4	15	9	(9.054)
+++	1	2	20	14	
TN					
±	0	1	2	0	
+	4	10	8	3	<0.01
++	1	5	23	9	(32.515)
+++	0	2	25	19	
Decorin					
±	0	0	9	9	
+	0	6	22	13	<0.05
++	3	6	19	8	(19.637)
+++	2	6	8	1	
VN					
±	0	1	14	18	
+	3	12	28	7	<0.01
++	1	3	15	6	(28.924)
+++	1	2	1	0	

[a] A total of 112 primary tumours were studied
[b] The number of cases is indicated

Table 4 Relationship of the ECM staining pattern in the primary tumour to the presence of nodal involvement[a]

Pattern of staining	Number of cases without lymph node involvement	Number of cases with lymph node involvement	P value (χ^2)
LN			
±	4	10	
+	24	20	<0.01
++	20	13	(20.488)
+++	21	0	
IV C			
±	8	6	
+	24	27	<0.01
++	26	10	(13.252)
+++	11	0	
HS-PG			
±	6	16	
+	30	24	<0.01
++	21	3	(26.082)
+++	12	0	
FN			
±	10	1	
+	27	9	<0.01
++	17	11	(13.675)
+++	15	22	
TN			
±	3	0	
+	22	3	<0.01
++	24	14	(15.663)
+++	20	26	
Decorin			
±	4	14	
+	27	14	<0.01
++	24	12	(15.600)
+++	14	3	
VN			
±	11	22	
+	34	16	<0.01
++	20	5	(18.086)
+++	4	0	

[a] A total of 112 primary tumours, which included 69 non-metastatic and 43 metastatic cases, were studied

Table 3 ECM staining pattern in the metastatic lymph nodes[a]

ECM	Staining pattern (number of cases)			
	±	+	++	+++
LN	7	18	4	0
FN	3	5	8	13
TN	1	2	10	16
Decorin	13	12	2	2
VN	20	9	0	0

[a] A total of 29 metastatic lymph nodes were studied

Table 5 ECM staining pattern in the primary tumour of the metastatic T1 cases[a]

ECM	Staining pattern	
	± ~ +	++ ~ +++
LN	9	1
IV C	8	2
HS-PG	10	0
FN	2	8
TN	1	9
Decorin	8	2
VN	10	0

[a] Ten metastatic T1 cases were studied

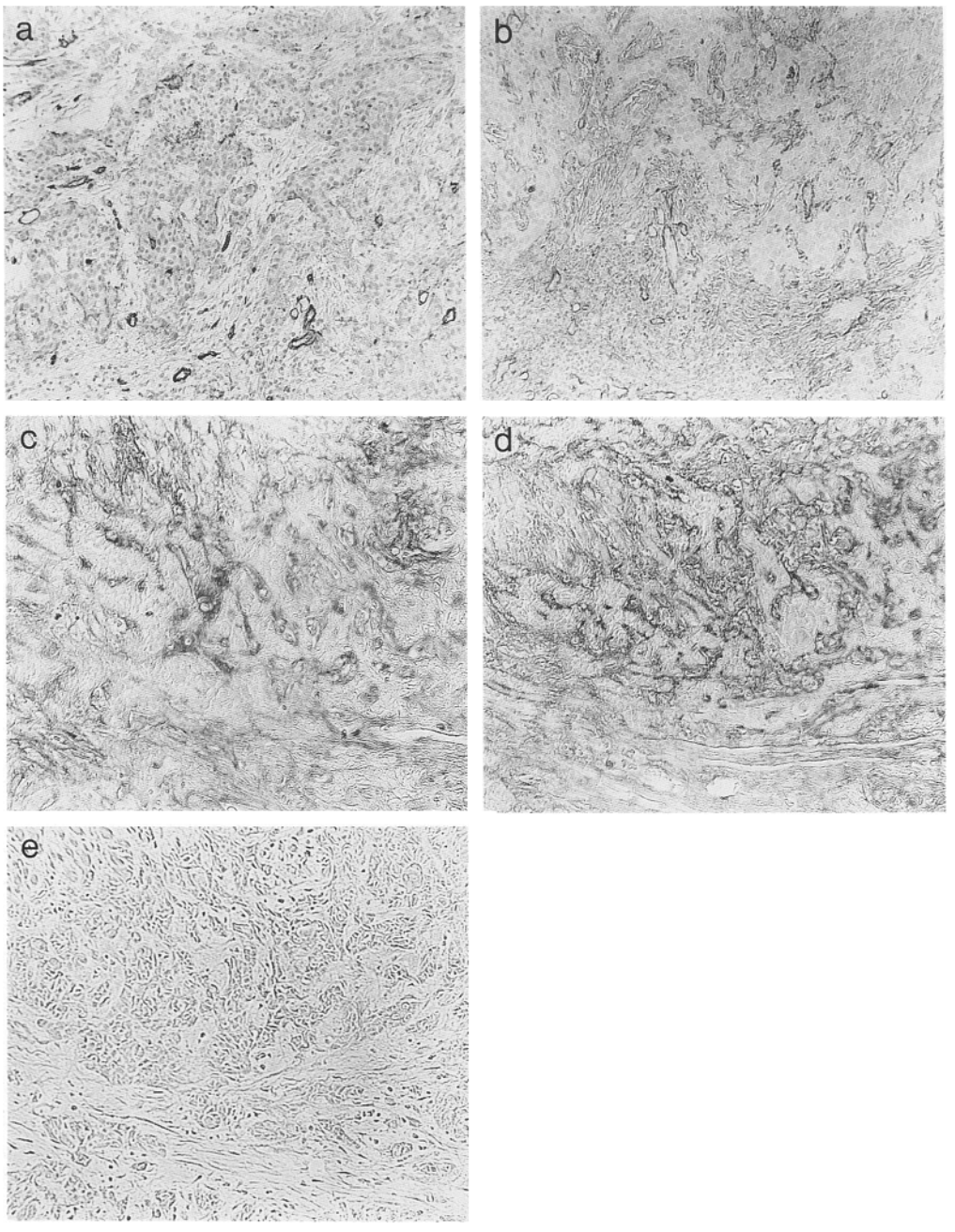

Fig. 5a–e The ECM staining pattern in primary tumours (MI grade 4c). Almost negative staining (± ~ +) of LN **(a)**, IV C **(b)** and decorin **(e)**, strong staining (+ + ~ + + +) of FN **(c)** and TN **(d)** are shown. **a, b** × 104; **c–e** × 130

plastic changes of the epithelia. In this study, the increased expression of TN at the invasive front of primary tumours was observed in highly invasive and metastatic OSCC. In the metastatic lymph nodes, especially those with extranodal spread, the extensive staining of TN was observed. These results indicate that the expression of TN is associated with invasive and metastatic potential of OSCC as well as with dysplastic changes.

With regard to FN and TN, it is of additional interest to note that both expressions were weak, regardless of the MI grade, in cases with marked infiltrating lymphocytes. Thus, the expression of FN and TN may be regulated by infiltrating lymphocytes, probably lymphocyte-derived cytokines, as well as invasive potential of tumour cells. In either case, the expression of FN and TN seems to be closely associated with the metastatic process of tumour cells, because the cases with marked lymphocytic infiltration showed a low frequency of nodal involvement (Anneroth et al. 1987; Shimada et al. 1990).

Decorin and VN are considered to be related to matrix assembly or adhesion (Iozzo 1985; Dahlbäck et al. 1986), and are present in normal tissues and showed weak expressions in malignant tumours. However, immunohistochemical localization of decorin and VN in carcinomas has not been reported to our knowledge. In this study, the expression of decorin and VN, which

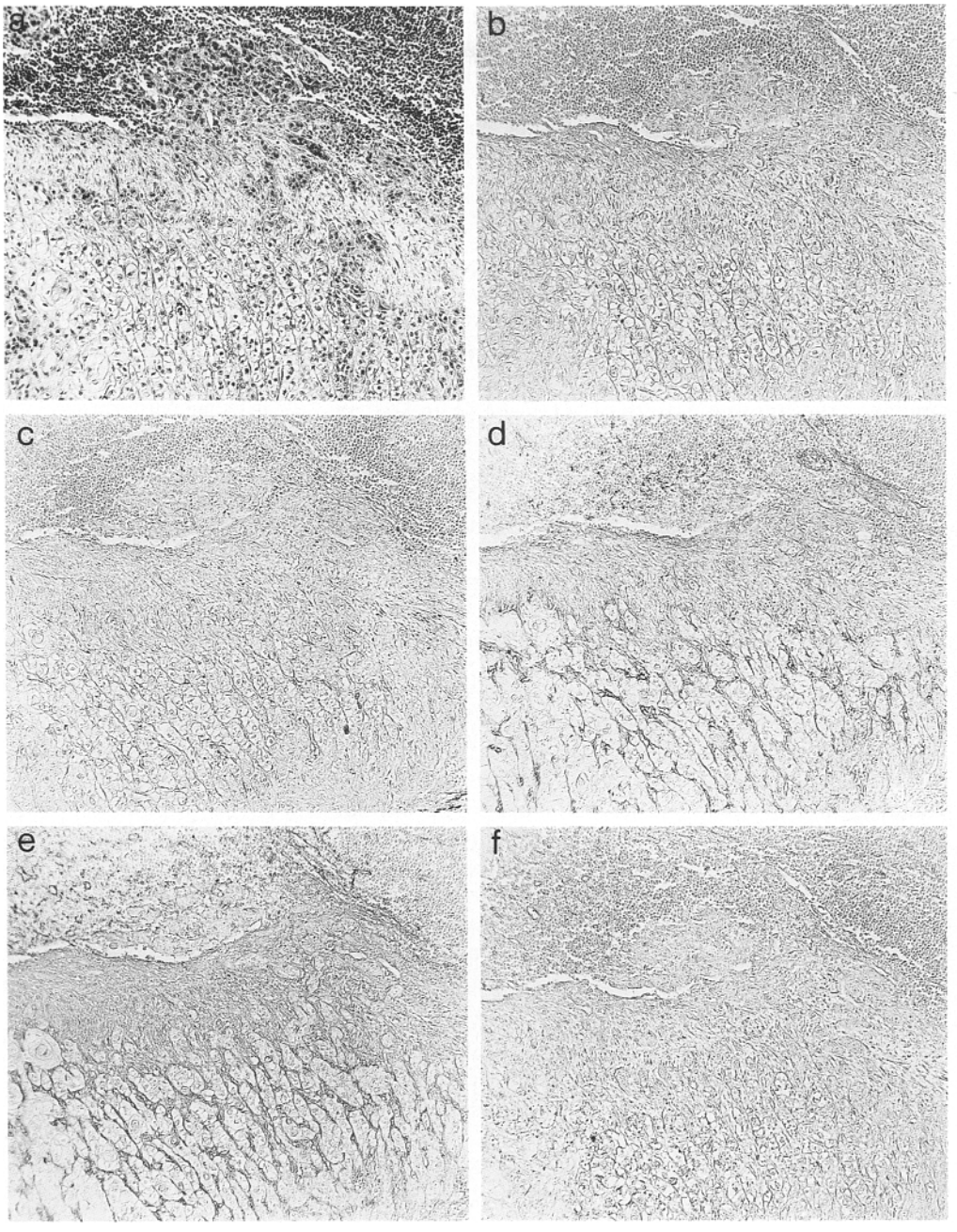

Fig. 6a–f The ECM staining pattern of the metastatic cervical lymph nodes. **a** H&E, ×90. The almost negative staining (±) of LN **(b)**, IV C **(c)** and decorin **(f)**, extensive staining (+ + +) of FN **(d)** and TN **(e)** are shown. **a–f** ×90

were detected in a similar staining pattern, was correspondingly reduced in highly invasive and metastatic primary tumours. Additionally, the expression of decorin and VN was decreased notably in the peritumour stroma of metastatic lymph nodes. It is also noteworthy that the expression of FN and TN was prominent where the expression of decorin and VN was lost. The way in which decorin and VN are involved in tumour invasion and metastatis has to be clarified and an investigation of decorin and VN might be valuable in predicting the invasive and metastatic potential of OSCC.

We examined the expression of ECMs at the invasive front of primary tumours. This seems to be important in estimating the invasive and metastatic potential of tumour cells since carcinomas represent a collection of heterogeneous cells. When the expression of ECMs was compared between primary tumours and the metastatic cervical lymph nodes, a similar staining pattern was observed. This observation is consistent with our previous report (Shinohara et al. 1993) and another report (Yamamoto et al. 1984) which demonstrated that histological features of tumour invasion were similar between the primary lesions and metastatic lymph nodes in OSCC. Thus, an apparent expression correlation between the invasive front of primary tumours and metastatic lymph nodes was observed, suggesting that the invasive front of the primary tumour reflects the invasive and metastatic potential of the primary tumour.

We used the mode of tumour invasion reported by Yamamoto et al. (1983) to evaluate the invasiveness of tumour cells. This classification is a modification of the criteria established by Jakobsson et al. (1973). However, we considered that MI grade 3 should be subdivided, because more than 50% of cases (58/112) were classified as grade 3 and showed highly heterogeneous staining patterns of ECMs. For this reason, we tried to subdivide MI grade 3 into two types as follows: type I – LN and IV C, $++\sim+++$; HS-PG, $+\sim++$; FN and TN, $+\sim++$; decorin and VN, $++\sim+++$, (which were similar to the staining pattern of MI grade 2); type II – LN, IV C and HS-PG, $-\sim+$; FN and TN, $++\sim+++$; decorin and VN, $-\sim+$, (which were similar to the staining pattern of MI grade 4c and d). As a result, the cases of type II had a significantly higher incidence of nodal involvement than did the cases of type I (submitted for publication). These results strongly suggest that the expression of ECMs is closely associated with the metastatic potential of OSCC.

Our study suggests that ECMs are significantly involved in invasion and metastasis and that the detection of a series of ECMs is valuable. It provides objective data to evaluate the invasive and metastatic potential of OSCC, in addition to conventional histological grading. Collective rather than separate evaluation of individual ECMs will help to predict the properties of OSCC. The present study could only clarify the relationship of individual ECMs to invasion and metastasis. The mutual interrelationship of ECMs with regard to invasion and metastasis has to be clarified. Further studies, for example, on integrin families, which are receptors for ECMs, and on metalloproteinases, which degrade ECMs, may help us to determine how ECMs are involved in tumour invasion and metastasis.

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