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Immunolocalisation studies of matrix metalloproteinases-1, -2 and -3 in human melanoma

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Abstract The matrix metalloproteinases (MMPs) are considered to have an important role in connective tissue degradation and have been implicated in the mechanisms of tumour invasion and metastatic spread. We have used immunohistochemistry to examine and compare the tissue distributions of collagenase-1 (MMP-1), gelatinase A (MMP-2) and stromelysin-1 (MMP-3) in 18 specimens of malignant melanoma, viz. 10 superficial spreading and 8 nodular melanomas. MMPs-1, -2 and -3 were demonstrated within melanoma and host tissue cells, especially at the periphery of some tumours, but were usually restricted to less than 10% of total melanoma cells. The MMPs were absent from 'normal' skin tissue distant from the tumour. MMP-2 was localised to discrete groups of cells and was especially evident at the epidermal:tumour interface, whereas MMP-3 was mainly confined to the deeper margins of melanoma. No regular pattern of MMP expression was observed for either the superficial spreading or the nodular melanomas. The variable distributions of the MMPs suggested that enzyme expression was subject to local microenvironmental regulation, possibly in response to matrix components and the cellular heterogeneity observed at the tumour margins. These in situ observations add weight to the concept that specific MMPs contribute to the mechanisms of tumour invasion.

Key words Melanoma · Matrix metalloproteinases · Collagenase · Gelatinase · Stromelysin · Immunohistochemistry

Introduction

The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases, now exceeding 17 different gene products, which collectively are capable of degrading all components of the extracellular matrix [2, 3]. Degradation of connective tissues by MMPs has been implicated in the mechanisms of cell detachment and migration, essential features of tumour invasion and metastatic spread [13, 19]. Previous studies have shown good correlation between MMP expression and the invasive phenotype of tumour cells [19]. Several studies have reported MMP expression by carcinoma cells of gastric cancer, especially the production of collagenase (MMP-1), matrilysin (MMP-7), gelatinase A (MMP-2) and gelatinase B (MMP-9); but not stromelysins-1 or -2 (MMP-3 and MMP-10) [15]. Numerous studies have led to reports of MMP expression by breast carcinomas, colorectal and lung carcinomas, and a variety of skin cancers [4, 11], with the recognition that cells of the host response are a major source of degradative enzymes at tumour invasion sites [1, 4, 9, 13]. Although several studies have examined MMP production by melanoma cells in vitro, finding for example that gelatinases A and B are characteristically produced by the C8161 melanoma cell line [18], and collagenase-1 and both gelatinases are markedly up-regulated in cultures of the A2058 melanoma cells [10, 17], relatively few studies have examined MMP production by malignant melanomas in situ. However, Vaisanen et al. [23, 24] have demonstrated MMP-2 localisation in primary skin and uveal melanomas and conclude that its overexpression is a prognostic marker for metastasis and unfavourable survival rates.

The concept of tumour cell heterogeneity and different metastatic potentials within one cancer specimen is well established [8, 14], and so too is the realisation that the varying patterns of specific MMP expression relate to transient changes in local factors such as hormones, cytokines and growth factors [10, 16, 21]. The present study has employed immunohistochemical techniques to assess the distributions of three different MMPs in the two major classes of malignant melanoma. Both superficial spreading (SSM) and nodular melanomas (NM) are recognised as highly invasive, metastatic cancers, with poor prognosis once invasion in the vertical plane has

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become established [5]. We report here our observations of MMP-1, -2, -3 production by these two types of malignant melanoma, where comparative studies have emphasised the variable nature of MMP expression, specific distributions usually being restricted to local, microenvironmental sites.

Materials and methods

Formalin-fixed, paraffin-embedded specimens of primary melanoma (n=18) were provided by the Department of Pathology, University Hospital of South Manchester, UK. These comprised 10 specimens of SSM and 8 specimens of NM, as confirmed independently by two histopathologists. The SSM specimens were identified as Clark grades III and IV (n=4 and 6, respectively) whereas the NM specimens were all Clark grades IV/V [5], i.e. advanced melanomas with relatively poor prognosis.

Tissue sections 5 µm thick were cut, dewaxed, rehydrated and examined for collagenase-1, gelatinase A and stromelysin-1 (MMP-1, -2 and 3, respectively), using monospecific sheep polyclonal antibodies to MMP-1 and MMP-2 (Dr. David Taylor, The Binding Site, Birmingham) and rabbit polyclonal antibody to MMP-3 (Prof. H. Nagase, Kansas City). All antibodies were tested for lack of cross reactivity with other MMPs (-1, -2, -3 and -9) as described previously [12, 22]. Tissue sections were first incubated for 30 min in 10% (v/v) serum of the species used for the secondary conjugated antibody. Primary antibodies were applied to sections for 2 h at room temperature. After three washes in Tris-buffered saline (TBS), biotinylated swine anti-rabbit or biotinylated rabbit anti-sheep IgG (both from DAKO, Glostrup, Denmark) was added for 45 min, followed by washing with TBS. Sections were then incubated with avidin-biotin complex conjugated to alkaline phosphatase (AP) for 45 min and washed with TBS, after which the AP was developed with New Fuchsin.

For immunofluorescent studies the procedure was similar to that described, except that the secondary antibodies were FITC-conjugated rabbit anti-sheep IgG or FITC-labelled swine anti-rabbit IgG (both from DAKO). For FITC studies tissue sections were counterstained with ethidium bromide (Sigma, Poole, UK). Antigen retrieval techniques using trypsin or microwave pretreatments did not significantly improve MMP visualisation.

Control tissue sections were included in each run, receiving either TBS or non-immune immunoglobulins of the same species and concentration as the primary antibodies. The immunodetection of each enzyme was shown to be abolished by preincubation of the primary antibody with purified antigen (obtained from Biogenesis, Poole, UK).

Tissue sections were examined with ×6.3 and ×40 objectives, and at least two sections from each specimen were examined by two independent assessors. Photomicrographs were taken on a Zeiss Photomicroscope III. Black and white prints were prepared using the rapidoprint system, and colour prints were developed from slide film using the Ilfachrome system.

Results

Normal skin tissue surrounding each of the primary melanomas showed no evidence for the local production of MMP-2 or MMP-3. By contrast, each of the melanomas showed immunostaining for these enzymes, but to variable extents. Each enzyme was usually restricted to a minor proportion of all the melanoma cells of any one specimen; on no occasion was there a general or widespread production of either MMP. Figure 1 demonstrates the distributions of MMP-2 and MMP-3 in relation to the tumour cells of a superficial spreading melanoma. Whereas MMP-2 was localised to discrete groups of cells especially evident in the epidermal:tumour regions, MMP-3 was mainly confined to the deeper margins of the melanoma and to cells of the host response (Fig. 1).

The microenvironmental nature of MMP expression is further demonstrated in the photomicrographs of Fig. 2; MMP-2 was restricted to small nests of melanoma cells, either in relation to adjacent epidermal cells, some of which were also weakly stained (Fig. 2a), or to peripheral melanoma cells surrounded by stromal, collagenous matrix (Fig. 2b). MMP-3 was more commonly demonstrated at the deeper invasive margins, where host response cells at the tumour interface were occasionally seen to contribute to MMP-3 expression (Fig. 2c). However, MMP-3 production was often restricted to peripheral melanoma cells, with little evidence of immunostaining in the adjacent dermal stroma or melanoma cells (Fig. 2d). Similar observations were found for MMP-1 production (Fig. 2e), this again being more frequently observed at the deeper, invasive margins, and often associated with numerous host response cells.

Since the distributions of MMP-2 and MMP-3 showed extensive variations between specimens, an attempt was made to identify their tissue locations more accurately (Fig. 3, Table 1). For SSM, MMP-2 was more commonly observed in the upper regions where melanoma:epidermal associations were prevalent, and to a lesser extent within small groups of host response cells. By contrast, MMP-3 was usually demonstrable in the deeper, peripheral regions of SSM, especially at sites containing mixed cell types of the host tissue response. MMP-3 was not observed within the epidermis. For NM MMP-2 staining was a prominent feature of the overlying and adjacent epidermis, as well as melanoma:epidermal junctions, but was also observed in various other sites with no obvious pattern. MMP-3 production was often demonstrated within melanoma cells at the dermal invasion zone of NM specimens, as well as other microenvironmental sites throughout the tumour.

In general, the observations suggest some degree of polarisation for the expression of these two enzymes; MMP-2 being more commonly produced by the epidermis and upper melanoma locations, as opposed to the MMP-3 production usually within the deeper, potentially invasive sites of most melanoma specimens (Table 1). However, the co-localisation of both enzymes within the same location was observed for a minority of the MMP-positive cells: in 7 of the 18 specimens examined. Such locations represented no more than a few percent of all the cells stained for MMP and never exceeded approx. 10% in any 1 specimen (cf. Fig. 1). Thus no regular pattern was observed for the distribution of MMP-2, MMP-3 and the co-expression of both enzymes between different melanoma specimens. Similarly, tissue sections from different regions of the same specimen also showed extensive variations in MMP distribution. These observations indicate that no major, or potentially prognostic, differences exist for MMP expression between the SSM

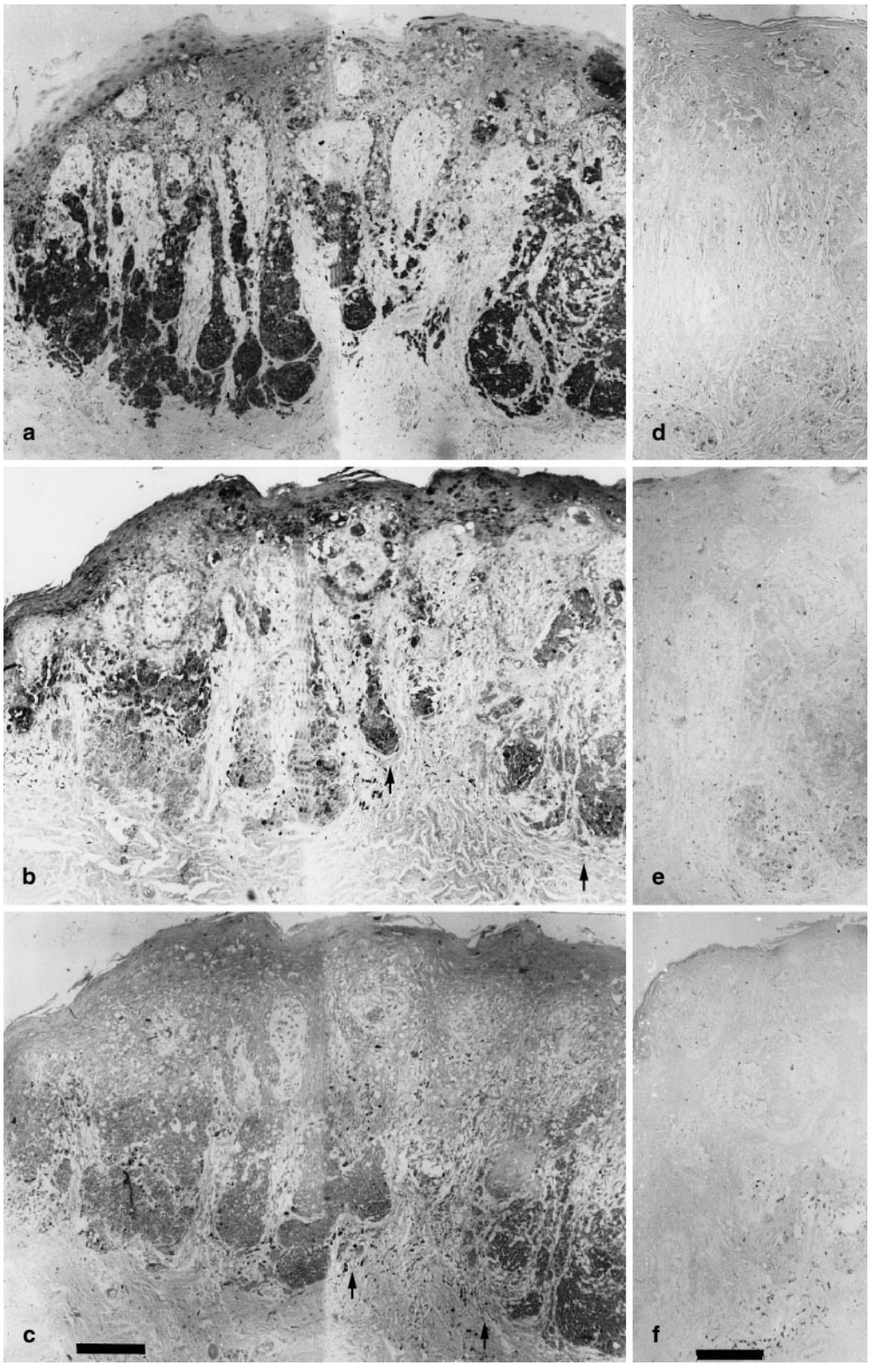

Fig. 1a–f Low-power micrographs of consecutive sections of human superficial spreading melanoma (SSM), showing the distribution of melanoma cells (S100), MMP-2 (gelatinase A), and MMP-3 (stromelysin-1). **a** Melanoma cells are demonstrated with the melanocyte marker S100 to show tumour morphology. **b** MMP-2 immunolocalised to epidermal cells and tumour cells close to the epidermis and in the deeper dermal margins (*arrows*). Note the limited distribution of MMP-2. **c** MMP-3 immunolocalised to cells of the deep tumour margins and to the host response cells around the tumour periphery (*arrows*). **d**, **e, f** Controls for **a**, **b**, and **c** respectively, using appropriate non-immune IgG in place of primary antibody. *Bars* 160 µm

and NM specimens. However, the absence of both enzymes from the epidermis and dermis of uninvolved skin tissues confirms that MMP-2 and MMP-3 contribute in some way to the pathophysiological processes associated with invasive malignant melanomas.

Discussion

The matrix metalloproteinases have long been implicated in tumour invasion and metastatic spread, and many investigations have been designed to improve our under-

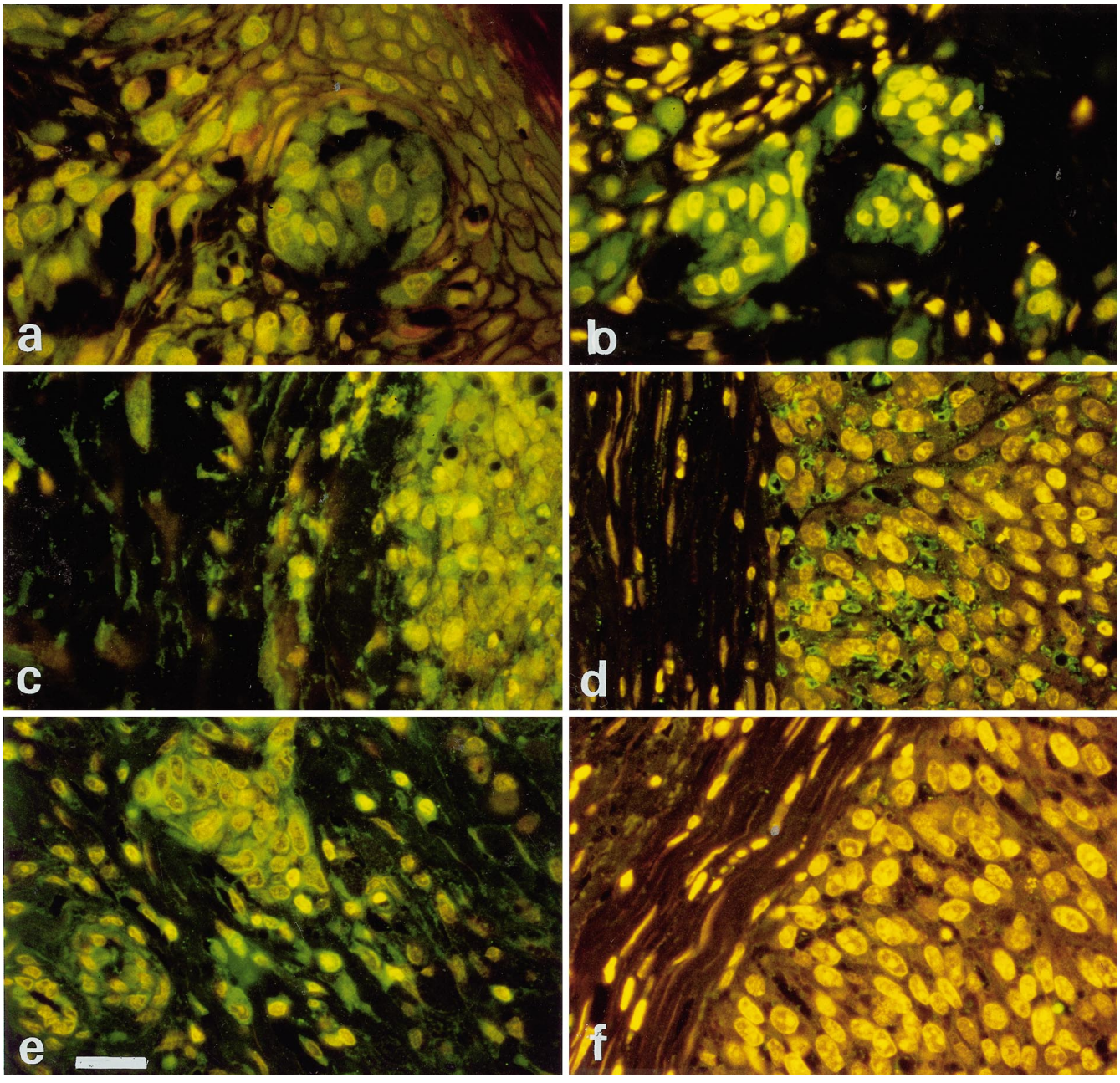

Fig. 2a–f Immunolocalisation of matrix metalloproteinases, MMP-1, MMP-2, MMP-3 in malignant melanoma. **a** Photomicrograph of MMP-2 distribution at an epidermal/melanoma junction. Note that the nest of melanoma cells is immunopositive (green FITC), with a proportion of epidermal cells showing weaker staining. **b** Photomicrograph of MMP-2 in nests of melanoma cells at the tumour margin. Note the absence of MMP-2 localisation in adjacent cells and the extracellular matrix. **c** Photomicrograph of MMP-3 immunolocalisation at the tumour periphery, showing both intra- and extracellular enzyme. **d** Photomicrograph showing immunoreactive MMP-3 at a tumour:host junction at a deep dermal invasion zone. Note enzyme distribution restricted to melanoma cells. **e** Photomicrograph of MMP-1 expression by a group of melanoma cells and a few stromal cells at an invasive margin. **f** Photomicrograph of a tissue section receiving non-immune sheep IgG as a negative control, counterstained with ethidium bromide. **a**, **b**, and **e** from SSM specimens; **c**, **d** and **f** from nodular melanoma (NM) specimens. Bar for all micrographs 25 µm

standing of the molecular mechanisms involved in these processes. The MMPs are purported to contribute to the breakdown of matrix barriers to tumour cell invasion, especially the collagenous components of stromal and basement membrane structures, thereby facilitating entry into adjacent normal tissues and vascular or lymphatic vessels [3, 13, 15, 19, 20]. In this study the production of MMP-2 was a prominent feature of the epithelial cell layer, in close apposition to tumour cells of both types of melanoma. Indeed in most specimens the basement membrane structures appeared lacking at the epidermal:melanoma junctions (cf. Fig. 2a), collagen type IV being a recognised substrate of MMP-2. By contrast, MMP-3 was rarely produced by epithelial cells of these locations, this enzyme being more frequently observed at

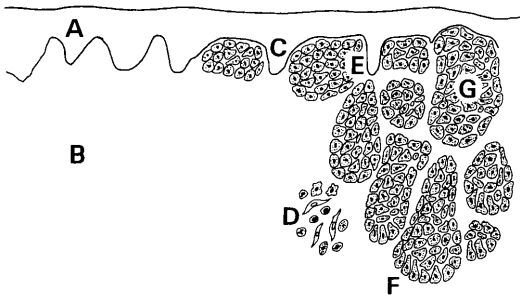

Fig. 3 Diagrammatic illustration of malignant melanoma, showing representative locations common to both SSM and NM. *A* Normal epidermis remote from melanoma; *B* normal dermis remote from melanoma; *C* epidermis closely associated with melanoma; *D* host cell response to melanoma; *E* melanoma closely associated with epidermis; *F* melanoma in dermal invasion zone; *G* melanoma cells in central tumour mass

Table 1 General summary of immunolocalisation data obtained for gelatinase A and stromelysin-1 at specific sites of superficial spreading melanoma (n=10) and nodular melanoma (n=8) (– no cellular staining, + to +++ increasing staining intensity and increasing number of cells stained at the sites described, *n/a* not applicable owing to lack of tissue in the specimens concerned

	Gelatinase A				Stromelysin-1			
	–	+	++	+++	–	+	++	+++
Superficial spreading melanoma								
A+B	10	0	0	0	10	0	0	0
C	1	2	1	6	8	2	0	0
D	4	4	2	0	3	2	5	0
E	6	1	3	1	1	3	6	0
F	5	3	2	0	2	3	5	0
G	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Nodular melanoma								
A+B	8	0	0	0	8	0	0	0
C	0	0	1	7	2	1	5	0
D	2	4	2	0	1	2	4	1
E	2	2	2	2	1	3	3	1
F	0	4	4	0	0	0	5	3
G	1	4	3	0	0	2	6	0

deeper melanoma locations associated with stromal elements of the dermis.

Using immunohistochemistry we have previously shown that collagenase-1 is occasionally produced by malignant melanomas, especially at the tumour periphery [25, 26]. The present study has confirmed those earlier observations, demonstrating a microfocal and variable production of this enzyme in both SSM and NM. Like MMP-1, the localised distributions of MMP-2 and MMP-3 suggest that their expression was subject to local microenvironmental regulation, possibly in response to the cellular heterogeneity observed at the tumour margins [7, 25]. The host response was generally more pronounced around specimens of SSM, where the cells observed included mast cells, macrophages and lymphocytes; but specific cell numbers and distributions varied both between and within the different specimens. However, there appeared to be no obvious correlations between the presence of inflammatory host cells and the sites of MMP production.

Recent studies on human carcinomas have demonstrated that extracellular proteinases, and especially MMPs implicated in the progressive invasion of local tissues, are predominantly expressed by stromal cells, and not by carcinoma cells [1, 4, 9]. Such findings have not been so apparent in the melanoma specimens examined here, where the 'balance' of MMP expression appeared to favour the tumour rather than the stromal or host cells. However, it was evident that both the transformed and the host cells provided a source of MMP-1, -2 and -3, but although MMP-2 and MMP-3 showed some suggestion of polarisation, in general it was not possible to recognise a regular pattern for the distribution of these three MMPs.

It is reported that tumour cells with different metastatic potentials, and with varying patterns of collagenolytic expression, may be generated by transient alterations in certain local factors, such as various cytokines, hormones and growth factors, resulting in the modulation of gene activity for various proteinases, receptors and inhibitors [21]. The ability to degrade basement membrane structures via gelatinases A and B is recognised as a major advantage for the invasive behaviour of malignant cells [10, 19]. The melanoma cell line A2058 was shown to respond to interferons-α and -γ with a short-term upregulation of MMP-2 and MMP-9 expression, which diminished with time but allowed the conclusion that MMP-2 was the predominant basement membrane degrading type IV collagenase in human melanoma [10]. Its distribution in both uveal melanoma and primary skin melanomas has been described, and the higher incidence of positively immunostained melanoma cells has been correlated with poor 5- and 10-year survival rates [23, 24]. Since the primary melanomas of the present study have only recently been acquired it has not been possible to relate the findings to survival rates, although there appeared to be no obvious relationship to the clinical Clark grading assessment. However, more specimens would be needed for a detailed statistical analysis. Our present study demonstrates that MMP-2 is produced in vivo, but is restricted to a relatively small proportion of melanoma cells, which is suggestive of a transient rather than a constitutive expression. While signals from the local environment can modulate the proteolytic and metastatic potentials of individual cells, for example the variable effects of interferons on the adhesive, motile and proteolytic behaviour of melanoma cells [10], relatively little is known about the proteinase-regulating factors which operate in vivo. Although this study has shown *where* MMP-1, MMP-2 and MMP-3 are produced in different melanoma specimens, we do not know as yet which factors are responsible for their expression.

The localised and variable distributions of MMP-2 and MMP-3 in human melanoma, similar to that previously reported for MMP-1 [26], suggest that MMP ex-

pression is subject to local microenvironmental regulation such as the cellular and matrix heterogeneity observed at the tumour margins [7, 25]. Further studies are required to examine whether specific host cell:melanoma cell interactions might stimulate or suppress the production of different MMPs [6]. Nevertheless, the observations reported here of MMPs located within sites where matrix breakdown and invasive behaviour would be expected adds weight to the concept that specific MMPs contribute to the mechanisms of tumour invasion and metastatic spread.

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