

Expression of Matrix Metalloproteinases in the Microenvironment of Spontaneous and Experimental Melanoma Metastases Reflects the Requirements for Tumor Formation

Uta B. Hofmann, Andreas A. O. Eggert, Katharina Blass, Eva-B. Bröcker, and Jürgen C. Becker

Department of Dermatology, Julius Maximilian's University, Würzburg, Germany

ABSTRACT

Expression of matrix metalloproteinases (MMPs) and their activation in tumor cells, as well as tumor surrounding stromal cells have been implicated in tumor cell invasion and metastasis. By means of a syngeneic tumor model for either experimental or spontaneous metastases, the differential expression of MMPs and tissue inhibitors of MMPs (TIMPs) in relation to the microenvironment and the way of metastasis induction was characterized.

In vitro characterization revealed that increased levels of secreted MMP-2, MMP-9, and TIMP-1 were only detectable in the most aggressive cell line, B16G3.12BM2. Remarkably, active MMP-2 was restricted to this cell line, whereas TIMP-2 and membrane type (MT) 1-MMP expression was comparable in all three of the spontaneously metastasizing melanoma cell lines investigated. *In vivo* analysis demonstrated that MMP-2, MMP-9, and MT1-MMP were predominantly expressed at the tumor-stroma border of s.c. tumors. Furthermore, functional active MMP-2 was restricted to this invasive front. In spontaneous lymph node or lung metastases, however, MMP-9 was expressed both in the center and the periphery of tumors; these areas were largely negative for MMP-2 and MT1-MMP. Notably, tumor cells of experimental lung metastases did not express MMP-9 at all.

These results indicate that expression of MMPs in melanoma metastases is not only influenced by their localization but also the nature of tumor induction, suggesting that individual MMPs serve specific roles during the different stages of metastasis formation.

INTRODUCTION

Remodeling of the ECM¹ is an essential step for cell migration (1). MMPs belong to a rapidly growing family of zinc-dependent endopeptidases that are capable of degrading a variety of ECMs (2). On the basis of their structure and substrate specificity, they have been subgrouped into soluble and MT-MMPs (3, 4). MT-MMPs are localized to the cell surface via a transmembrane domain or a glycosylphosphatidylinositol anchor (4, 5). In general, MMPs are secreted in latent forms (pro-MMPs) and require specific proteolytic activation (6). MT1-MMP was first discovered as a physiological activator of MMP-2 on the cell surface; in addition, it facilitates clustering of active MMP-2 at invadopodia of tumor cells (7). MMP activity is additionally regulated by four structurally related tissue inhibitors (TIMP-1 to TIMP-4; Ref. 8).

Recent studies have shown that expression of MT1-MMP and activation of MMP-2 in melanoma cells is correlated with tumor progression (9). Both molecules are found predominantly at the tumor invasion front (10). However, their expression is not restricted to

tumor cells, but surrounding and/or infiltrating stromal as well as immune cells also express MMPs and TIMPs. Recent *in vitro* experiments suggest that tumor cells may use the MMP-2 produced by adjacent normal stromal cells to facilitate their own invasion (11). However, until now the complex interactions between tumor cells and tumor surrounding stromal cells *in vivo*, as well as the differential expression of MMPs in relation to their microenvironment during progression are not fully understood (12). Therefore, we took advantage of a syngeneic murine melanoma model (13), which is characterized by the possibility to induce experimental and spontaneous metastases in various tissues, to scrutinize the expression of MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 *in vivo*. This analysis revealed differential patterns of MMP expression during different steps of metastasis formation.

MATERIALS AND METHODS

Cell Lines, Culture Conditions, and Animals. The spontaneous metastatic clones of the B16 melanoma cell line, designated B16G3.26, B16G3.12II, and B16G3.12BM2, were obtained originally from Christopher W. Stackpole (New York Medical College, Valhalla, NY). Of these cell lines B16G3.12BM2 showed the most aggressive metastatic behavior *in vivo* (13). Human melanoma cell line MV3 has been described previously and was used as a positive control for TIMP-1 (14). Human fibrosarcoma cell line HT1080 was obtained from the American Type Culture Collection (Rockville, MD) and served as a positive control. HT23 cells were derived from HT1080 cells by stable transfection with MT1-MMP cDNA (kindly provided from Dr. Alex Y. Strongin, The Burnham Institute, La Jolla, CA) were used as positive controls. Cells were cultured in RPMI 1640 (PAN Biotech GmbH, Aidenbach, Germany), supplemented with 10% FCS, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) in an atmosphere of 95% humidified air and 5% CO₂ at 37°C. For passaging, cells were detached with 0.05% trypsin and 0.02% EDTA in PBS for 5 min at 37°C.

Female C57BL/6 mice were obtained from The Jackson Laboratory (Charles River, Sulzfeld, Germany) at the age of 4–6 weeks and housed under specific pathogen-free conditions. Five × 10⁴ cells in 100 μl PBS were injected s.c. into the flanks of the mice. s.c. tumors (*n* = 8 per cell line) were surgically removed when they reached a diameter of ~1 cm and snap-frozen in liquid nitrogen. After an additional 3 months the animals were sacrificed and examined for spontaneous metastases. Spontaneous lymph node (*n* = 4 per cell line) and lung metastases (*n* = 4 per cell line) were isolated, snap-frozen, and stored at –80°C. For experimental lung colonization (*n* = 4 per cell line) 5 × 10⁴ tumor cells in 100 μl PBS were injected into the lateral tail vein. The animals were sacrificed after 11 and 15 days, and the lungs were snap-frozen and stored at –80°C.

Antibodies. For MMP-2 a rabbit polyclonal antibody (#AB808; Chemicon International Inc., Temecula, CA) was used in Western blot analysis and a rabbit polyclonal antibody (#AB809; Chemicon International Inc.) in immunohistochemistry. For Western blot analysis a mouse monoclonal antibody (IM37L; Oncogene Research Products, Cambridge, MA) and in immunohistochemistry a rabbit polyclonal antibody (#AB19047; Chemicon International Inc.) were used against MMP-9. For MT1-MMP a rabbit polyclonal antibody (#AB815; Chemicon International Inc.) was used in immunohistochemistry, Western blot analysis, and flow cytometry. For Western blot analysis monoclonal antibodies against TIMP-1 (#IM32L; Oncogene Research Products) and TIMP-2 (#IM11L; Oncogene Research Products), and in immunohistochemistry rabbit polyclonal antibodies against TIMP-1 (#AB800; Chemicon Inter-

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Requests for reprints: Uta B. Hofmann, Department of Dermatology, Julius Maximilian's University, Josef-Schneider-Str. 2, D-97080 Würzburg, Germany. Phone: 49-931-201-26731; Fax: 49-931-201-26700; E-mail: hofmann_u1@klinik.uni-wuerzburg.de.

¹ The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT, membrane-type; TIMP, tissue inhibitor of metalloproteinase; TRP, tyrosinase-related protein; SFCM, serum-free conditioned medium.

national Inc.) and TIMP-2 (#AB19029; Chemicon International Inc.) were used. Polyclonal rabbit antibody α PEP8 (generously provided by Dr. Vincent J. Hearing, National Cancer Institute, Bethesda, MD) was used as a control for mouse TRP2-specific staining on tumor cells.

Flow Cytometry. To determine cell surface expression of MT1-MMP on melanoma cell lines, cells were detached from the tissue-culture flask nonenzymatically with EDTA 2 mM for 10 min. Two $\times 10^5$ cells were incubated with antibody against MT1-MMP in 0.1% BSA in PBS for 45 min on ice, followed by centrifugation for 5 min at $1500 \times g$ at 4°C . After incubation with FITC-labeled antirabbit immunoglobulin antibody (#111-096-045; Jackson ImmunoResearch Laboratory) for 45 min on ice, cells were centrifuged for 5 min at $1500 \times g$ at 4°C . Upon washing, FITC-labeled cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Western Blot Analysis. Western blot analysis was performed as described (10). Briefly, the protein amount in SFCM was measured using BCA Protein Assay Reagent (Pierce, Rockford, IL). Samples of $12 \mu\text{l}$ of $10 \times$ concentrated (Centricon 10 microconcentrator; Amicon, Beverly, MA) SFCM ($10 \mu\text{g}$ total protein per lane) were electrophoresed on 7.5% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked and were probed with antibodies to the indicated MMPs. After incubation with peroxidase-labeled swine antirabbit immunoglobulin or rabbit antimouse immunoglobulin (Dako, Hamburg, Germany) secondary antibodies, bands were visualized using by chemiluminescence (Boehringer Mannheim, Penzberg, Germany). Western blot analysis was performed three times with different cell extracts giving comparable results.

Immunohistochemistry. To study the expression of MMP-9, MMP-2, MT1-MMP, TIMP-1, and TIMP-2 in tissue, $6\text{-}\mu\text{m}$ serial cryostat sections were stained. Immunohistochemistry was performed as described elsewhere (10). Sections were scored independently by two observers (U. B. H. and J. C. B.). Discrepancies were found in only four cases that were re-evaluated jointly until agreement was reached. For each section the percentage of positive melanoma cells was estimated by counting 500 consecutive malignant cells. A lesion was considered as positive if minimally 1% of tumor cells stained moderately or markedly. Weak staining was disregarded. For each section, the pattern of tumor cell staining was judged as diffuse or focal positivity, and the involvement of tumor center and/or tumor border was noted. For stromal cells, the extent of staining and the type of positive cells (fibroblast-like cells, macrophages, and endothelial cells) were recorded. For each cell line, three consecutive sections per tumor were stained, and they showed comparable results.

Zymography. Activation status of MMP-2 and MMP-9 was studied by zymography analysis and was performed as described before (10). Briefly,

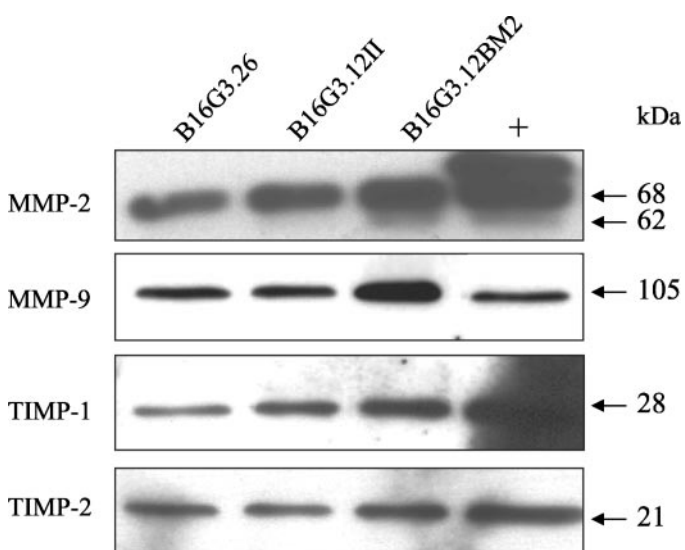


Fig. 1. Western blot analysis on SFCM. For each sample equal amounts of protein ($10 \mu\text{g}$) were loaded on gels. Increased levels of secreted MMP-2, MMP-9, and TIMP-1 were detected in the highly metastasizing cell line B16G3.12BM2 as determined by densitometry. Notably, active MMP-2 (62 kDa) was present only in this cell line. Comparable amounts of TIMP-2 were secreted by all cell lines. SFCM of HT1080 or MV3 cells were used as positive controls (+).

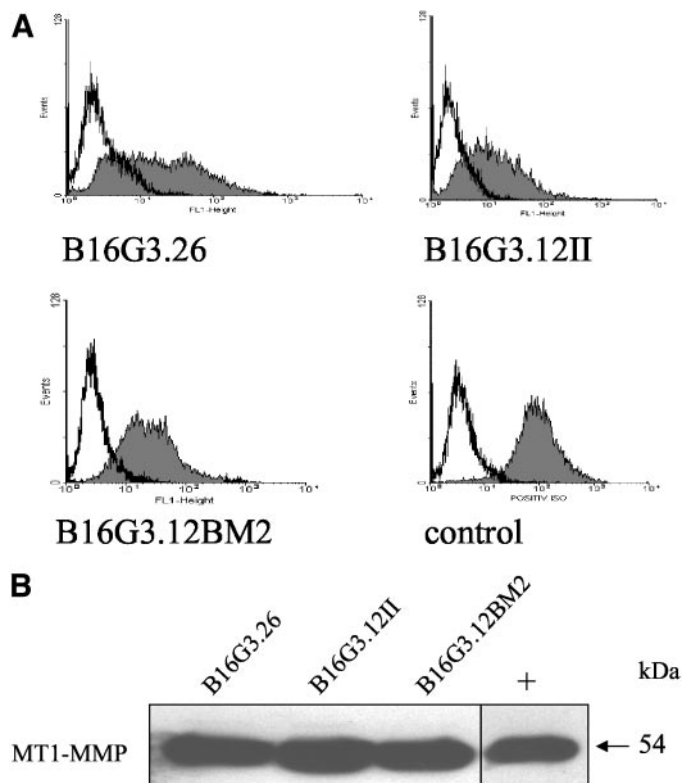


Fig. 2. Fluorescence-activated cell sorter analysis on cells and Western blot on SFCM. **A**, cell surface expression of MT1-MMP was observed on melanoma cells of all cell lines at similar levels (filled line). HT23 cells, which were stable transfected with MT1-MMP, were used as a control. **B**, comparable levels of MT1-MMP were secreted by the different B16 melanoma cell lines. HT23 cells served as positive control (+).

$5\text{-}\mu\text{l}$ aliquots of SFCM were diluted 1:1 in sample-buffer. Samples were electrophoresed at 4°C on SDS-PAGE gels containing 50 mg/ml gelatin. After electrophoresis, gels were washed three times in 2.5% Triton X-100 for 10 min to remove SDS. After rinsing twice in substrate buffer [50 mM Tris-HCl (pH 7.8), containing 5 mM CaCl_2 and 0.1% Triton X-100], gels were incubated at 37°C for 18 h in the same buffer under gentle agitation. Gels were stained for 45 min in 40% methanol/10% glacial acetic acid containing 0.1% (w/v) Coomassie Brilliant Blue R 250 and destained in the same solution without Coomassie Brilliant Blue. MMP-9 (M_r 105,000, murine, and M_r 92,000, human), latent pro-MMP-2 (M_r 66,000), and activated MMP-2 (M_r 62,000) were identified by using the pefGOLD protein marker (peqlab, Erlangen, Germany) and SFCM of HT1080 cells as a control. For zymography analysis of tumor tissue, eight serial tissue cryostat sections ($6 \mu\text{m}$) were cut from total tumor tissue, tumor interior, and periphery, weighed, and homogenized in $4 \times$ sample buffer, centrifuged for 1 min at $10,000 \times g$, and pellets were incubated at 37°C for 30 min. Samples were mixed with distilled water (1:1) and heated at 55°C for 20 min. After centrifugation (1 min; $10,000 \times g$) pellets were discarded, and supernatants were subjected to zymographic analysis. Zymography was performed at least three times for each sample.

RESULTS

In Vitro Expression of MMP-9, MMP-2, MT1-MMP, TIMP-1, and TIMP-2. Protein concentrations of different MMPs and TIMPs were determined by Western blot analysis in $10 \times$ concentrated SFCM derived from 1×10^7 cells of three subclones of murine B16 melanoma, *i.e.*, B16G3.26, B16G3.12II, and B16G3.12BM2, characterized by different metastatic behavior *in vivo* (13). MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 were released by all of the cell lines; however, an increased expression of MMP-2, MMP-9, and TIMP-1 was restricted to the most aggressive cell line, *i.e.*, B16G3.12BM2. Additionally, only in this cell line active MMP-2 (M_r

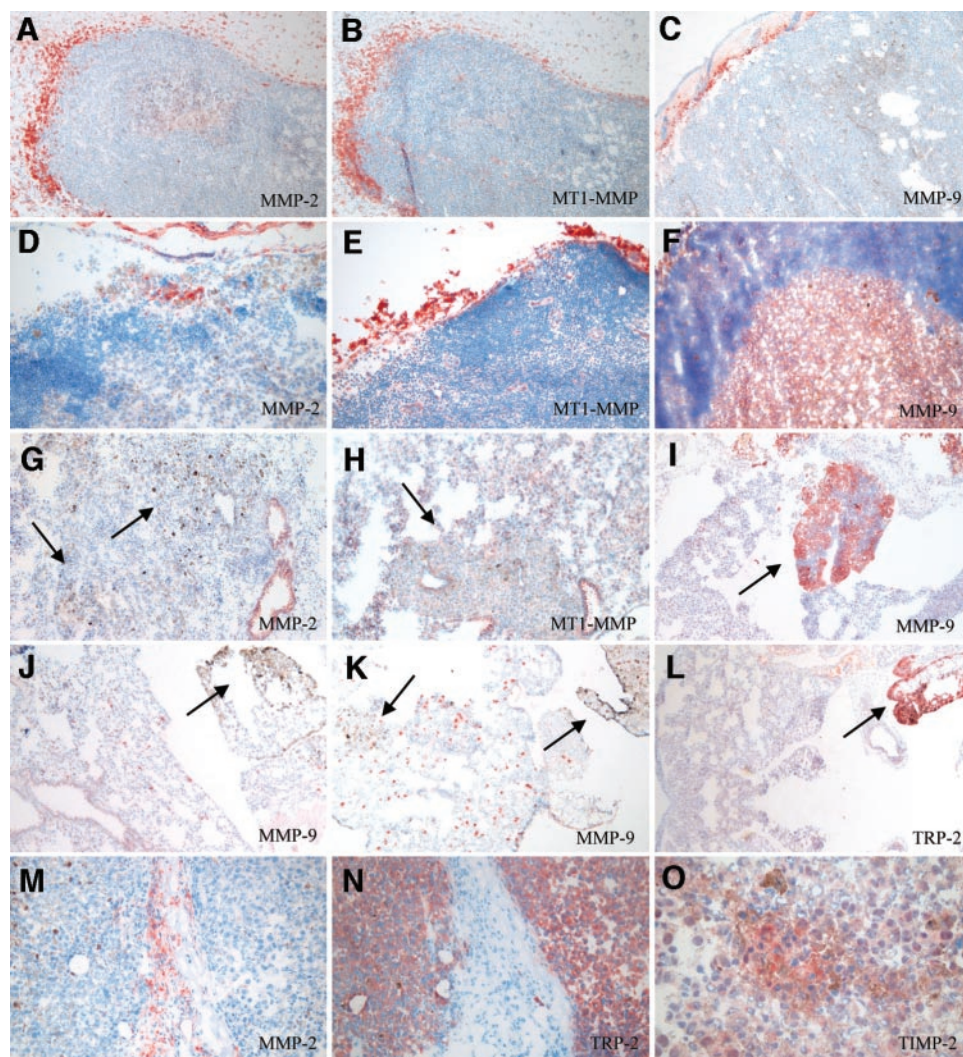


Fig. 3. Immunostainings of s.c. tumors derived from melanoma cell lines. Increased expression of MMP-2 (A), MT1-MMP (B), and MMP-9 (C) at the tumor-stroma interface at the invasive front of s.c. melanoma lesions. Similar expression of MMP-2 (D) and MT1-MMP (E) in lymph node metastases with an increased expression of both components at the tumor border. MMP-9-positive tumor cells were observed in the center and periphery of lymph node metastases (F). In spontaneous lung metastases tumor cell clusters were negative for MMP-2 (G) and MT1-MMP (H), only bronchial epithelia and lung macrophages stained positively, whereas a strong staining of MMP-9 was found in tumor cells (I). In experimental lung metastases expression of MMP-9 was restricted to immune cells localized distributed in the lung tissue, whereas the tumor cell clusters were negative. The number of MMP-9-positive cells was increased at least 2-fold on day 15 (K) compared with day 11 (J). Strong staining of tumor cell clusters with TRP-2 in experimental lung metastases (L). Staining of serial sections for MMP-2 (M) and TRP-2 (N) revealed that mainly tumor surrounding stromal cells expressed MMP-2. Expression of TIMP-2 in tumor cells (O). Arrows indicate the pulmonary metastases. Magnification: $\times 40$ (A-C and I-L); $\times 100$ (D-H); $\times 200$ (M-O).

62,000) was present as determined by an antibody recognizing active MMP-2 (Fig. 1). TIMP-2 protein was secreted in equal amounts by all of the cell lines. Flow cytometry and Western blot analysis showed comparable levels of membrane-bound and soluble MT1-MMP (Fig. 2, A and B). As measured by flow cytometry the mean fluorescence per cell of B16G3.26 was 27.00, B16G3.12II 16.44, and B16G3.12BM2 25.78; the mean fluorescence of the positive control, *i.e.*, HT23 cells, was 92.54.

In Vivo Expression of MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 in Experimental Metastases. Serial sections of s.c. tumors derived from all three of the melanoma cell lines were used to study the expression of MMP-2, MMP-9, MT1-MMP, TIMP-1, and TIMP-2 *in vivo*. Immunohistochemistry for MMP-2 and MT1-MMP showed moderate to strong staining of individual cells in the tumor center and a strong staining of mainly tumor surrounding stromal cells at the tumor border (Fig. 3, A, B, and M). Staining for the melanoma-associated antigen TRP-2 on serial sections allowed us to discriminate between tumor and stromal cells (Fig. 3N). To this end, tumor surrounding stromal cells, isolated tumor cells, and tumor-infiltrating cells in the tumor center, as well as some tumor cells at the tumor border stained strongly positive for MMP-9 (Fig. 3C). In s.c. tumors a higher number of cells were positive for MMP-2 and MT1-MMP compared with MMP-9. In all of the lesions studied, $\sim 50\%$ of the tumor cells in the tumor center or at the tumor border were positive for

TIMP-2 (Fig. 3O). A moderate immunoreactivity against TIMP-1 was found in some tumor cells at the tumor border, whereas a strong staining was observed in endothelial cells, nerves and sebaceous glands adjacent to the tumors (data not shown). Notably, s.c. tumors derived from all three of the cell lines showed comparable staining results for each component.

Zymography was performed on tissue sections of the total tumors as well as of microdissected tumor interiors and peripheries to determine the activation status of MMP-9 and MMP-2. In total tumor extracts, MMP-9 (M_r 105,000), and both latent (M_r 66,000) and active form (M_r 62,000) of MMP-2 were present in similar amounts (Fig. 4A). Higher levels of MMP-2, however, were observed at the invasive front as compared with the central part of the tumors (Fig. 4B). Moreover, the presence of activated MMP-2 was restricted to the invasive front.

To scrutinize the effect of the surrounding tissue on the expression of MMPs, we studied the expression of MMP-2, MMP-9, and MT1-MMP in experimental lung metastases. Surprisingly, the strongest expression for MMP-9 was observed for immune cells that were localized in the lung tissue. The number of these MMP-9-positive cells increased 2-fold on day 15 compared with day 11 (Fig. 3, J and K). Notably, in these experimental lung metastases tumor cells were virtually negative for MMP-9 (Fig. 3, J and K). The same applied for MMP-2 and MT1-MMP (data not shown). The presence of experi-

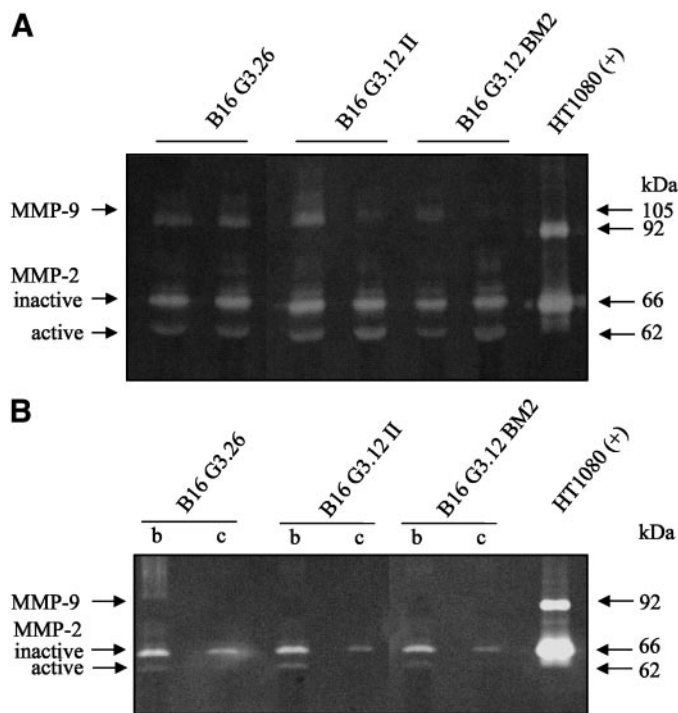


Fig. 4. Zymography on tissue extracts derived from s.c. tumors of different melanoma cell lines. A, equal levels of MMP-2 and MMP-9 were detectable in total tissue extracts. B, increased levels of inactive (66 kDa) and active (62 kDa) MMP-2 were present at the tumor border (b) compared with tumor center (c).

mental lung metastases was confirmed by staining for TRP-2 (Fig. 3L). No differences between any of the three cell lines with regard to MMP expression in the tumor cells or the induction of these enzymes in the reactive inflammatory infiltrate were observed.

Expression of MMP-9, MMP-2, and MT1-MMP in Spontaneous Metastases. After ~3 months all three of the murine melanoma cell lines spontaneously formed lymph node and lung metastases after s.c. injection into the flank of C57BL/6 mice. It should be noted, however, that the incidence and degree of metastasis varied as had been described previously with B16G3.12BM2 being the most aggressive cell line (13). Immunohistochemistry for MMP-2, MMP-9, and MT1-MMP of spontaneous lymph node and lung metastases allowed for the comparison of the differential expression of MMPs in experimental and spontaneous metastases, as well as in relation to their microenvironment. In spontaneous lymph node metastases MMP-2 and MT1-MMP display a similar distribution pattern with a positive staining of individual tumor cells within the tumor and a strong immunoreactivity at the invasive front as experimental s.c. tumors (Fig. 3, D and E). In 8 of 12 cases (66.6%) of lymph node metastases a high number (50–100%) of tumor cells stained moderately positive for MMP-9 (Fig. 3F). Similar to experimental lung metastases, tumor cell clusters of spontaneous lung metastases were negative for MMP-2 and MT1-MMP (Fig. 3, G and H). Bronchial epithelia and lung macrophages stained positively for both components. A very striking difference, however, between experimental and lung metastases was that in 10 of 12 cases of spontaneous tumors ~50% of the tumor cells expressed MMP-9 both in the tumor center and the tumor periphery (Fig. 3I). Notably, metastases derived from all three of the cell lines showed comparable MMP-9 expression in the spontaneous metastases irrespective of the different expression pattern *in vitro* or the different metastatic potential, suggesting the *in vivo* selection of metastatic subclones.

DISCUSSION

Melanoma is a highly invasive tumor with a high capacity for both lymphatic and hematogeneous metastasis formation. By means of spontaneous and experimental metastases models we attempted to at least partially dissect this process to establish the relevance of MMPs during the different phases of tumor spread.

Several lines of evidence suggest that proteolytic enzyme systems are important for melanoma progression (15). In human and murine melanoma cell lines increased expression of MMP-2, MT1-MMP, and MMP-9 was shown to correlate with an invasive phenotype (9, 14, 16). Because activation of MMP-2 is dependent on overexpression of MT1-MMP our observation that in human melanocytic lesions the expression of both MMP-2 and MT1-MMP was increased with tumor progression and that positive tumor cells were predominantly localized at the invasive front were in line with this hypothesis (10). *In situ* zymography studies confirmed that activated MMP-2 is located at the tumor-stroma border (17). The present study confirms in a syngeneic mouse model that MMP-2 and MT1-MMP expression is indeed stressed at the tumor border, and that presence of functionally active MMP-2 was restricted to the invasive front.

The balance between levels of activated MMP and free inhibitors appears to be critical for tumor progression (18). Accordingly, overexpression of TIMP-1, TIMP-2, and TIMP-3 has been shown to inhibit tumor-mediated proteolysis and melanoma invasion (19–21). However, no evident correlation of TIMP-1 or -2 expression with activation of MMP-2 and MMP-9 was observed in the present *in vivo* model. Morrison *et al.* (22) have demonstrated recently the existence of a TIMP-2-independent pathway for cellular activation of MMP-2 involving MT2-MMP. However, additional studies are needed to define the role of TIMPs and MT-MMPs for MMP-2 activation.

MMP expression is not restricted to cancer cells but is also found in the stromal cells, suggesting a contribution of host-derived proteases to tumor progression (12, 23). Studies with MMP-2-deficient mice have revealed that host MMP-2 synthesis is indeed important for experimental metastasis formation (24). We observed in s.c. tumors, spontaneous lymph node, and both experimental as well as spontaneous pulmonary metastases a strong immunoreactivity for MMP-2 and MT1-MMP on stromal and immune cells. The mechanisms by which malignant cells up-regulate MMP-2 expression in normal remain elusive, but signaling via cell surface molecules such as ECM metalloproteinases inducer seem to be involved (25, 26). In this context, it should also be noted that Saad *et al.* (11) have shown that tumor cells induce the release of MMP-2 from normal fibroblasts via cancer cell-associated fibronectin. Thus, once displaced from the fibroblast-associated collagen, MMP-2 is activated by MT1-MMP/TIMP-2 complexes expressed by tumor cells (11).

Conflicting data have been reported on the role of MMP-9 expression for melanoma progression. In humans MMP-9 was exclusively expressed in the horizontal but not in the vertical growth phase of primary tumors (27). In murine xenograft models, however, MMP-9 was expressed by, or was inducible in, cell lines derived from advanced but not early primary melanoma lesions (28). Furthermore, in cell lines constitutively expressing MMP-9, an enhancement of lung colonization was observed in experimental lung metastasis models, and in MMP-9-deficient mice, metastases formation was suppressed (29, 30). These results suggest that MMP-9 produced either by neoplastic or stromal cells is important for metastasis formation. However, the differential expression and localization of MMPs in relation to the microenvironment and the nature of the metastasis, *i.e.*, experimental or spontaneous, have not been addressed to date. It is important to note that these factors are likely to influence the requirements of protease activity, as outlined above. Our data indicate that these

requirements seem to be more dependent on the metastatic process rather than the microenvironment. In spontaneous lymph node and pulmonary metastases the majority of tumor cells expressed MMP-9, whereas experimental metastases did not express MMP-9 at all. Thus, the *in vivo* selection process for metastatic subclones favors those expressing MMP-9. Because this selection is independent of the microenvironment and is not seen for experimental metastases, MMP-9 seems to be involved in the early steps of metastasis formation but not the later steps such as extravasation.

In summary, our data support the notion that expression of MMP-2 and MT1-MMP at the tumor-stromal border is important for the progression and invasion of s.c. tumors, whereas MMP-9 is essential for spontaneous metastasis formation. Thus, expression of MMPs is not only influenced by localization but also the nature of metastasis induction, suggesting that these different MMPs serve individual and specific roles during the different stages of metastasis formation.

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