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MMP-2 expression in uveal melanoma: differential activation status dictated by the cellular environment

Mélanie Bérubé,[1] Alexandre Deschambeault,[3] Maryse Boucher,[1] Lucie Germain,[3] Eric Petitclerc,[4] Sylvain L. Guérin,[1,2]

[1]Oncology and Molecular Endocrinology Research Center and [2]Unit of Ophthalmology, CHUL, Center Hospitalier Universitaire de Québec (CHUQ) and Laval University, [3]Laboratoire d'Organogénèse Expérimentale (LOEX), Hôpital du Saint-Sacrement, CHA and Department of Surgery and Ophthalmology, Laval University, [4]Unité de Biotechnologie et de Bioingénierie, CHUQ and Laval University, Québec, PQ, Canada

Purpose: MMPs are recognized to play a major role in tumor progression and metastasis of many forms of cancers. The purpose of this study was to compare the expression and activity of MMP-2 in uveal melanoma cell lines grown either in vitro on plastic culture plates or in vivo as tumors produced in chick embryos.
Methods: The chick chorioallantoic membrane (CAM) model was used to evaluate the tumorigenic potential of uveal melanoma cell lines derived either from the primary uveal melanoma tumor isolated from three different patients (cell lines SP6.5, SP8.0, and TP31) or from a metastatic lesion derived from the liver of a patient diagnosed with uveal melanoma (cell line H79). The presence of MMP-2 in the vicinity of the tumor cells was determined by immunofluorescence analyses. Gelatin zymography was used for the detection of latent and activated forms of MMP-2 in uveal melanoma cell lines when grown in vitro on plastic, or in the solid tumors these cell lines produced in vivo on the CAM of the chick embryo. The gelatinase activity was quantified by densitometric analyses and the active/(active+pro-form) ratio was calculated as the MMP-2 activation ratio. Western blot analyses were performed to confirm the zymographic profile.
Results: Only the inactive form of MMP-2 was expressed and secreted in vitro by all uveal melanoma cell lines, higher levels being found for the liver-derived H79 cell line whereas SP8.0 only expressed MMP-2 to a very low level. On the other hand, all solid tumors produced in the CAM from these cell lines expressed and secreted, although to varying levels (SP6.5 and SP8.0, TP31 and H79), primarily the active form of MMP-2. Gelatinolytic activities of active MMP-2 were significantly higher in uveal melanoma tissues than in the non-neoplastic CAM, as revealed by the measurement of the activation ratio. The immunolocalization of MMP-2 revealed that all cell lines were MMP-2-positive although a reduced and more diffuse staining was observed for H79 and SP6.5 than in SP8.0 and TP31 cells.
Conclusions: These results suggest the activation of proMMP-2 as an important event in the process of uveal melanoma progression. An elevated active to inactive MMP-2 ratio in the tumor environment of uveal melanoma suggests that a potential MMP-2 activity could be related to the progression of this type of cancer.

Uveal melanoma, although rare, is the most common primary intraocular malignancy, with a mean-adjusted incidence in the United States of approximately 4.3 new cases per million individuals [1]. Even after the tumor is excised, the threat of death is attributable to the growth of the metastatic cancer cells that disseminated early during the progression of the primary tumor. Indeed, about 40% of patients diagnosed with posterior uveal melanoma will develop metastatic melanoma to the liver within 10 years after initial diagnosis of the primary tumor [2]. Metastatic disease of the liver remains the leading cause of death in uveal melanoma patients, as up to 95% of the patients diagnosed with uveal melanoma also will have developed liver metastases at the time of death [3]. In the case of uveal melanoma, as for other cancers, metastatic spread of tumors continues to be a major obstacle to successful treatment of malignant tumors.

Correspondence to: Dr. Sylvain L. Guérin, Oncology and Molecular Endocrinology Research Center, CHUL, 2705 Laurier Boulevard, Ste-Foy (Québec) G1V 4G2, Canada; Phone: (418) 654-2296; FAX: (418) 654-2761; email: Sylvain.guerin@crchul.ulaval.ca

The escape of tumor cells from the primary tumor and invasion into the surrounding stroma represent early events in the process of metastasis formation [4]. These events are dependent on the interaction of tumor cells with the ECM, which consists of a three dimensional network of interstitial collagens, fibronectin, and other proteins that are interspersed between cells [4,5]. Cellular invasion, whether physiological or pathological as in tumor invasion, depends on the ability of cancer cells to penetrate the ECM. Thus, a key determinant in cellular invasion is the requirement for proteolysis of the ECM [5].

Over the years, the MMP family of proteases has been recognized to play a major role in tumor progression of many forms of cancers. Collectively, the members of the MMP family, also known as the matrixins [5], can degrade a wide variety of ECM and non ECM substrates [4]. These zinc endopeptidases play key roles in both physiological (wound healing) and pathological tissue degradation. Under pathological conditions such as cancer, tumor cells use MMPs to break down and remodel tissue matrices thus facilitating neoplastic cell invasion and metastasis. This increase in matrix turnover is thought to promote tumor cell invasion [5].

Most of these enzymes are secreted by cells from a variety of connective tissues (including fibroblasts, osteoblasts, chondrocytes and endothelial cells), inflammatory cells (including macrophages, neutrophils and lymphocytes), and by cancer cells [5,6]. Most MMPs appear to be secreted from cells in their inactive proforms, making their activation a key step in regulating the amount of degradative activity outside the cell [7]. They are then activated extracellularly by either a plasminogen cascade system or by another member of the MMP family. However, MMP-2 is refractory to activation by serine proteinases and is instead activated at the cell surface through a unique multistep pathway involving MT-MMPs and TIMP-2, a member of the endogenous TIMP family of enzymes [6,8]. Once activated, the proteinases are further regulated by TIMPs in the extracellular environment, or by α_2-macroglobulin in the plasma [5].

Although all five major classes of proteases (serine, aspartic, cysteine, threonine, and metalloproteinases) are involved in metastasis, a great deal of emphasis has been dedicated to the type IV collagenases, MMP-2 and MMP-9 (also known as gelatinase-A and gelatinase-B) both of which cleave collagen type IV (a major structural protein from the basement membrane and ECM) and type V, and denatured collagens (gelatins) [5,9]. Thus far, the relative expression levels of MMPs seem to increase with tumor progression and numerous studies have linked elevated MMP-2 and MMP-9 levels with an increased metastasis. Increased MMP-2 expression has been demonstrated in many different human tumors, including carcinomas of the colon, pancreas, prostate, bladder, skin, breast, and ovary [5]. The presence of activated MMP-2 correlates strongly with tumor cell invasion and metastasis in a variety of cancers [5,10-14]. In uveal melanoma, Cottam et al. [15] observed that all the 15 studied cell lines secreted MMP-2 in vitro, whereas only nine secreted MMP-9. In addition, the expression of MMP-2 in tissue sections of uveal melanoma is associated with a poor prognosis [16]. The level of proMMP-2 compared with active MMP-2 also plays a role in determining the invasive and metastatic ability of the tumor cell. Indeed, the MMP-2 activation ratio in tumor tissues was found to be higher in patients with pancreatic carcinoma that also have positive regional lymph nodes than in those without metastasis [17]. The invasive potential of breast cancer cell lines is also reported to correlate with their ability to activate proMMP-2 [18].

Few studies have made a comprehensive comparison of the factors regulating the metastatic progression of uveal melanoma and the presence of degradative enzymes in the tumor environment. Besides, little is known about the involvement of an increased MMP-2 activity in the progression of uveal melanoma. In the present study, we examined whether the state of MMP-2 activation changes when uveal melanoma cell lines that are maintained in an in vitro environment are grown into an in vivo environment. For this purpose, MMP-2 expression and activity was followed in the primary uveal melanoma cell lines SP6.5, SP8.0, and TP31, and in the liver-derived metastatic cell line H79. All solid tumors produced on the chick CAM showed more activated than nonactivated MMP-2 whereas no activated MMP-2 was observed in vitro. The importance of this activation in vivo for the metastatic spreading of uveal melanoma is discussed.

METHODS

This study was conducted in accordance with our institution's guidelines and the Declaration of Helsinki, and the protocols approved by the institution's Committee for the Protection of Human Subjects.

Cell culture and reagents: The mixed epithelioid-spindle uveal melanoma cell lines SP6.5, SP8.0, and TP31 were initially cultured each from a single primary uveal tumor isolated from the enucleated eye of three different patients (77, 48, and 62 years old, respectively, Table 1) [19]. They were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Oakville, Ontario, Canada) supplemented with penicillin-streptomycin-glutamine (1X; Gibco, Invitrogen, Burlington, Ontario, Canada), and 5% fetal bovine serum (FBS; Gibco, Invitrogen). Cultured cells were grown at 37 °C under 5% CO_2. The epithelioid H79 cell line was isolated postmortem from a patient diagnosed with liver metastases derived from a primary uveal melanoma. Samples from the liver metastatic lesions were fragmented into small pieces, seeded in T25 flasks in supplemented DMEM and allowed to grow. As non-neoplastic cells from the liver can not be propagated for more than a few passages without a feeder layer, it is likely that the cell line establish by serial passaging was derived from the cancer cells that invaded the liver and which initially originated from the primary uveal tumor. The melanocytic origin of the spontaneous metastatic cell line was then confirmed by immunochemical analyses as a positive staining for the melanoma differentiation marker S-100, using an antibody (Ab) directed against this protein (data not shown). However, as the S-100 marker is very sensitive but sometimes lacks specificity as it also stains a variety of other tissues, we also used the HMB45 marker, a useful melanocyte marker that proved to be more specific but much less sensitive than the S-100 protein [20], in order to confirm further the melanocytic origin of this cell line. As non-neoplastic cells from the liver can not be propagated for more than a few passages without a feeder layer, it is likely that the cell line established by serial passaging was derived from the cancer cells that invaded the liver and which initially originated from the primary uveal tumor. As is often the case with uveal melanoma, the SP6.5, SP8.0, TP31, and H79 lost their ability to produce melanin. However, the melanocytic origin of the SP6.5, TP31 and SP8.0 has already been established by the detection of premelanosomes and melanosomes structures through electronic microscopy [19]. Their origin was further confirmed by immunochemical analyses as a positive staining for the melanoma differentiation marker S-100, using an antibody (Ab) directed against this protein (data not shown).

Chick embryo tumor growth assay and tissue preparation: Fertilized chicken eggs were incubated in a rotary incubator at 38 °C with 60% of humidity for 10 days. At this time, the CAM was dropped by drilling a small hole through the eggshell into the air sac and by applying a mild vacuum to the

hole. Single cell suspensions of SP6.5, SP8.0, TP31 and H79 uveal melanoma cell lines ($5x10^6$ cells per embryo) were detached by trypsinization and applied in a total volume of 40 µl of complete DMEM to the CAMs of 10-day-old chick embryos as described [21,22]. Tumors were allowed to grow for a total of 7 days. The resulting tumors were then removed and trimmed free of surrounding CAM tissue, and their wet weights measured before they were frozen and stored at -80 °C. Tumor experiments were performed three to four times with 12 to 18 embryos per condition.

Indirect immunofluorescence assays: Fresh samples from the tumor tissues, derived from the SP6.5, SP8.0, TP31, and H79 cells upon their inoculation in chick embryos, were frozen and an indirect immunofluorescence assay was performed on paraformaldehyde-fixed and acetone permeabilized cryosections as previously reported [23]. Primary Abs included a rabbit anti-MMP-2 Ab, a mouse anti-human nuclei Ab (Chemicon International, Temecula, CA) and a mouse anti-melanoma cells Ab (clone HMB45, Cedarlane Laboratories Limited, Hornby, Ontario, Canada). Secondary Abs used included a sheep FITC-conjugated anti-rabbit Ab (Chemicon International), and Alexa 594-conjugated goat anti-mouse Ab (Molecular Probes, Eugene, OR). Cell nuclei were also labeled with Hoechst reagent 33258 (Sigma-Aldrich) following immunofluorescence staining. Negligible background was observed for controls (primary Abs omitted). Immunofluorescence was measured using a Nikon Eclipse TE2000-U microscope equipped with epifluorescence, photographed with a numeric CCD camera (ORCA-ER, Hamamatsu, Bridgewater, NJ) and analyzed with SimplePCI 5.0 C-imaging software (Compix Inc., Cranberry Township, PA) and Adobe Photoshop 6.0 (Adobe System Inc., San Jose, CA).

Histology: A hematoxylin-eosin staining was performed on ethanol/formol-fixed cryosections (5 µm thick) of tumor tissues was performed on ethanol 70%/formol 3.7%-fixed cryosections.

Preparation of conditioned media and tissue lysates: Cells were cultured until they reached 80-90% confluence and then washed three times with phosphate buffered saline (PBS 1X; 13.7 mM NaCl, 0.3 mM KCl, 0.8 mM Na_2HPO_4-$7H_2O$, 0.15 mM KH_2PO_4, pH 7.3) to remove all traces of FBS, and left to rest in complete DMEM for 72 h. The culture medium was then harvested, centrifuged to spin out cells and sodium azide (0.02%) was added. This conditioned medium was concentrated using Centricon plus-20 filters with a 10 kDa cut-off (Amicon, Bedford, MA) and stored at -80 °C. Tumor tissues were pulverized in their frozen state and resuspended in lysis buffer containing 1% (v/v) Triton X-100, 25 mM glycylglycine (Sigma-Aldrich), 15 mM magnesium sulfate (Sigma-Aldrich), and 4 mM EDTA under gentle rotation at 4 °C in a proportion of 30 µl/mg tissue. Then, the tissue lysates were ultracentrifuged at 100,000x g (45 min; 4 °C) and stored at -80 °C. Before use, an aliquot of the supernatant conditioned media and tissue lysate was assayed for protein concentration using the Bradford procedure [24].

Zymographic detection of gelatinase activities: Gelatin zymography was performed using the conditioned media from SP6.5, SP8.0, TP31 and H79 cells cultured in vitro and tissue lysates from the in vivo tumors yielded by these cell lines according to the methods described by Heussen and Dowdle [25] and DeClerck et al. [26]. Molecular weight markers (Rainbow markers, BioRad, Mississauga, Ontario, Canada) were also included on each gel. Gels were dried and proteolysis detected as a colorless band against a purple background. The intensity of each individual band on the gel was determined by scanning densitometry to provide a semiquantitative assay of the enzymatic activity. Serial dilutions of the samples (between 10 and 100 µg) demonstrated that the densitometry values reached a plateau at protein loads of greater than 100 µg per well (data not shown). The relation of proteins load to densitometric values was linear up to and including 25 and 50 µg protein per well. We therefore used either of these amounts for conducting the zymographic experiments appearing in Figure 1 and Figure 2. The MMP-2 activity was measured as the integrated area under the peak bands of the densitometric curves, and the [active MMP-2/(active MMP-2+proMMP-2)] intensity ratio designated as the MMP-2 activation ratio. Within each assay, all samples were assessed simultaneously using the same reagents and batch of gels.

Table 1. Summary of the cases from which tumor cell lines were derived

Cell line	Origin	Age/ sex	Date of enucleation	Date of liver invasion	Follow-up after enucleation (mon)	Tumor histology
SP6.5	PT	77/F	01/26/1998	-	142	Mixed
SP8.0	PT	48/M	04/05/1988	01/01/1996	134*	Mixed
TP31	PT	62/M	10/08/1991	10/13/1994	40*	Mixed
H79	M	76/F	04/23/1996	08/15/1998	28*	Epithelioid

Cell lines were derived from primary uveal tumors (PT) or liver metastases (M) from a patient with uveal melanoma. Age refers to the patient's age in years at diagnosis of the primary tumor. Regarding the first pateint, no liver metastases were detected at the patient's last visit in 1997. After the patient's enucleation, the patient was lost to follow-up. Asterisks indicate follow-up ended when the patient died of metastatic uveal melanoma.

Western blot analysis: Approximately 25 μg total protein prepared from the uveal melanoma cell lines as described [27] were added to one volume of sample buffer and then size fractionated on a 10% SDS-polyacrylamide minigel before they were transferred onto a nitrocellulose filter under conditions previously described [27]. A full set of protein molecular mass markers (Gibco-Invitrogen) was also loaded as a control in order to evaluate protein sizes. The blot was then washed as described [27] and incubated for 2 h at 22 °C with a 1:5000 dilution of a mouse monoclonal Ab raised against MMP-2 MMP-2 (Ab19041; Chemicon International). The blot was then washed and incubated for 1 h at 22 °C in a 1:1000 dilution of a peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratory, West Grove, PA). The membrane was successively washed in TSM [27] and TS [27] buffers before immunoreactive complexes were revealed using western blot chemiluminescence reagents (Renaissance, NEN Dupont, Boston, MA) and autoradiographed.

Statistical analysis: Statistical comparison of the gelatinase activities in the uveal melanoma cell lines (SP6.5, SP8.0, TP31, and H79) compared to the activity from the CAM membrane were performed using a one-way analysis of variance followed by post-hoc analysis with the Fisher probability of least significant difference test.

RESULTS

Tumorigenic properties of the uveal melanoma cell lines: The chick embryo model has long been used for the investigation of angiogenesis and oncogenesis [21,22]. This model provides

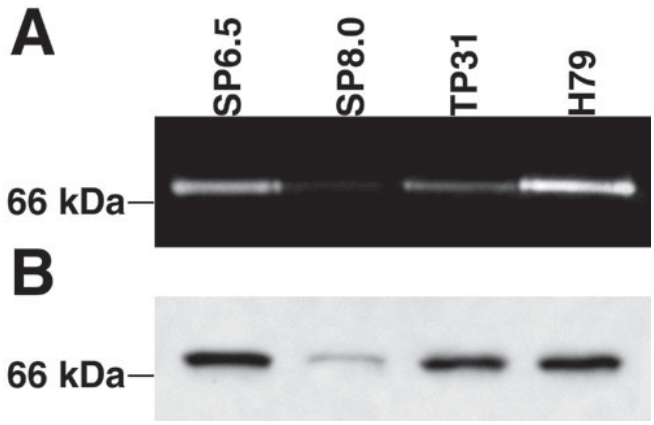

Figure 1. Zymographic and western blot analysis of MMP-2 from in vitro cultured uveal melanoma cell lines. **A**: Representative zymographic profile of the in vitro gelatinolytic activity in uveal melanoma cell lines. The in vitro gelatinolytic activity of MMP-2 was demonstrated in the serum-free conditioned media (25 μg each) from the SP6.5, SP8.0, TP31, and H79 uveal melanoma cell lines. The gelatinolytic activity observed at 72 kDa corresponds to proMMP-2. **B**: Western blot analysis of MMP-2 secreted by in vitro cultured uveal melanoma cell lines. For each sample, equal amounts of proteins (75 μg) were loaded on a 10% SDS-polyacrylamide gel prior to their transfer onto the membrane and further analysis with a monoclonal Ab directed against MMP-2. The position of the 66 kDa molecular mass marker is provided.

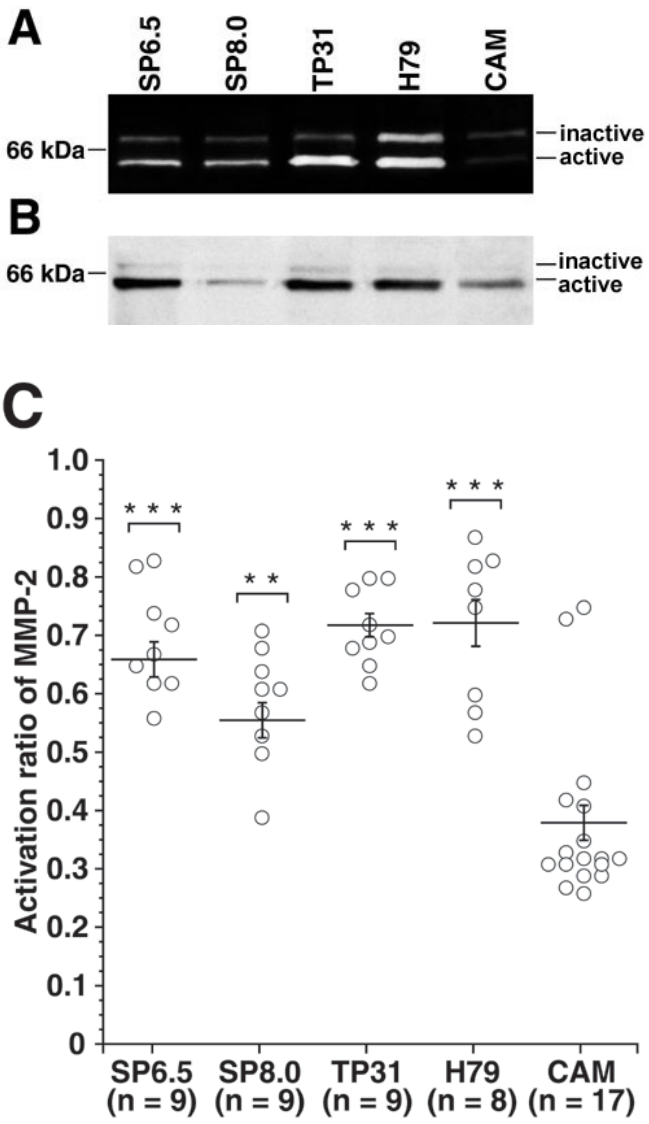

Figure 2. Zymographic and western blot analysis of MMP-2 from the in vivo tumors. **A**: Representative zymographic profiles of gelatinase activities in tumor tissues. The in vivo gelatinolytic activity of MMP-2 was determined by zymographic analysis of pro- and active MMP-2 in tissue extracts (50 μg each) from the tumors generated by the inoculation of the SP6.5, SP8.0, TP31, and H79 uveal melanoma cell lines on the CAM of chick embryos. The 72 kDa proform and the 62 kDa activated form of MMP-2 are indicated (inactive and active forms of MMP-2, respectively). **B**: Western blot analysis of MMP-2 in chick embryo's tumors produced by the uveal melanoma cell lines. For each sample, equal amounts of proteins (50 μg) were loaded on a 10% SDS-polyacrylamide gel prior to their transfer onto the membrane and further analysis with a monoclonal Ab directed against MMP-2. The position of the 66 kDa molecular mass marker is provided. **C**: Activation ratios of MMP-2 in the chick embryo tumor tissues derived from the uveal melanoma cell lines. The activation ratios were measured by computer-assisted image analyses of the gels. Horizontal bars indicate the mean values for each group. The error bars represent the standard error of the mean of the activation ratios of MMP-2. Triple asterisks indicate $p<0.0001$ compared to CAM.

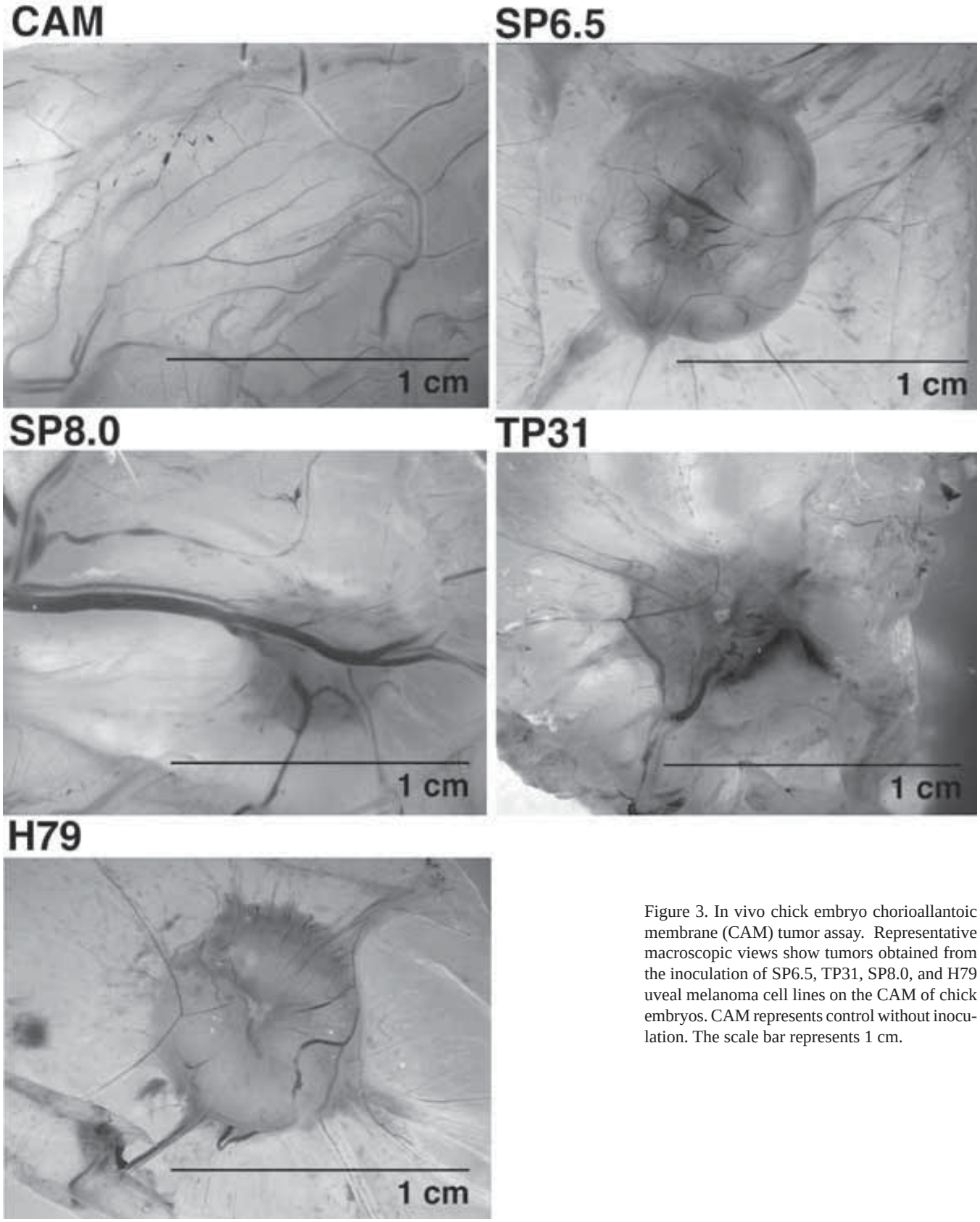

Figure 3. In vivo chick embryo chorioallantoic membrane (CAM) tumor assay. Representative macroscopic views show tumors obtained from the inoculation of SP6.5, TP31, SP8.0, and H79 uveal melanoma cell lines on the CAM of chick embryos. CAM represents control without inoculation. The scale bar represents 1 cm.

a naturally immunodeficient host that accepts transplantation from various tissues and species and extra-embryonic membranes that are connected to the embryo through a continuous circulatory system and that are readily accessible for experimental manipulation and observations. In order to study the tumorigenic ability of the primary tumor-derived SP6.5, SP8.0, and TP31, and the liver metastase-derived H79 uveal melanoma cell lines, a suspension of each of the cell lines was inoculated onto the CAM of chick embryos as described in Material and Methods. The solid tumors formed were harvested from the embryos after 7 days and their wet weight determined. All tumor cell lines derived from primary uveal melanoma yielded tumors in vivo with the SP6.5 cell line consistently yielding the larger tumors (132±12, 68±8 and 62±5 mg for SP6.5, SP8.0, and TP31 cells, respectively; Figure 3). The liver-derived metastatic cell line H79 produced tumors similar in size to those obtained with the SP6.5 cell line (125±7 mg). In vivo observations revealed that the tumors derived from these four cell lines (Figure 3) were vascularized and no gross toxicity on the CAM was noticeable. Therefore, all four uveal

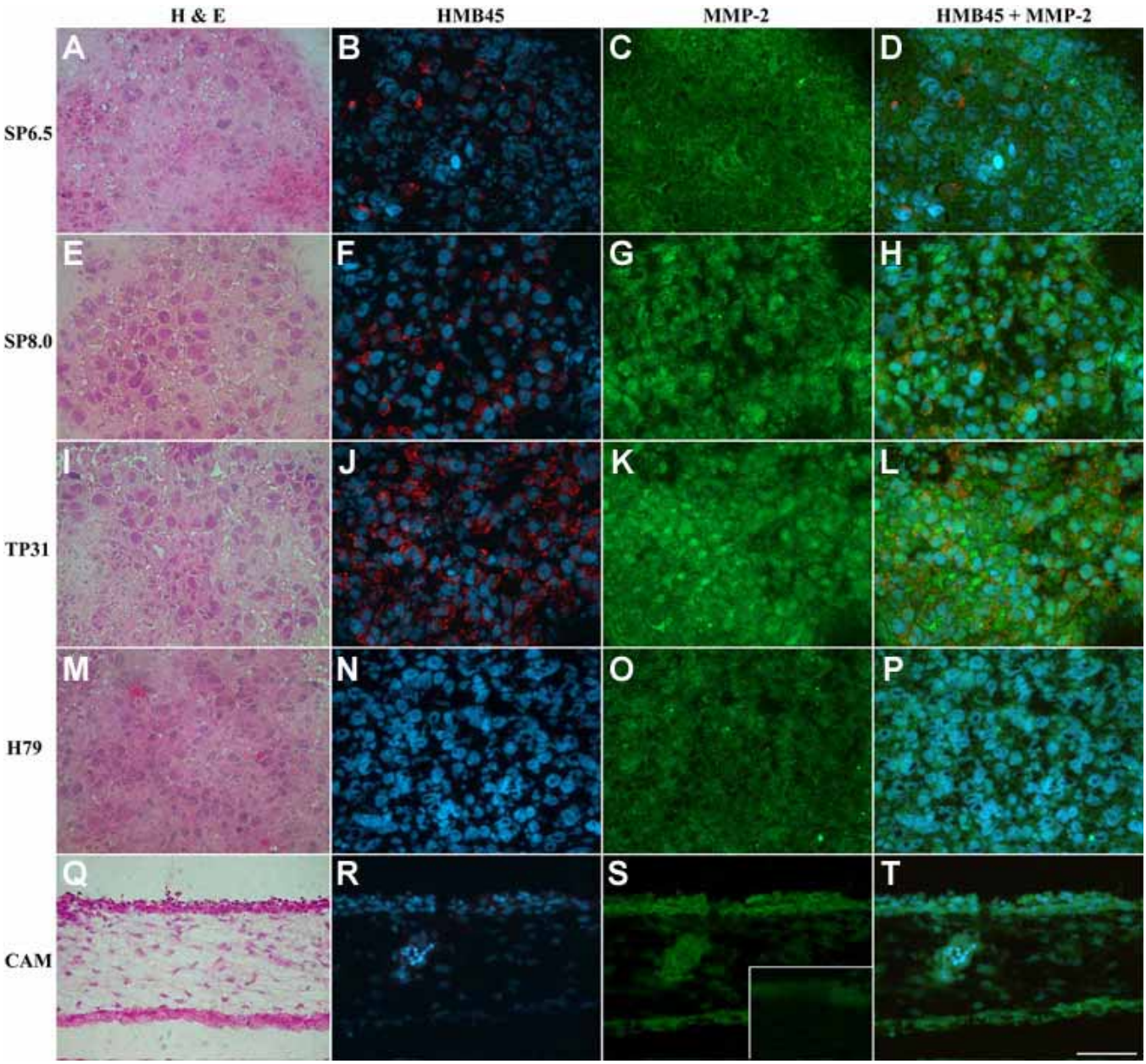

Figure 4. HMB45 and MMP-2 expression in human uveal melanoma-derived tumors. **A**,**E**,**I**,**M**,**Q**: Hematoxylin and eosin staining of 5 μm thick ethanol/formol-fixed cryosections. Panels **B**,**F**,**J**,**N**,**R**: HMB45 staining (red) on consecutive paraformaldehyde-fixed cryosections of the tumors. **C**,**G**,**K**,**O**,**S**: MMP-2 staining (green) of the same cryosections as those stained with the HMB45 Ab (the **S** insert is a negative control for **S**). **D**,**H**,**L**,**P**,**T**: Merge of HMB45 (red) and MMP-2 (green) stainings. Cell nuclei were counterstained with Hoechst (blue). The scale bar represents 50 μm.

melanoma cell lines possess the ability to produce tumors in vivo with those yielded by the SP6.5 and H79 cell lines being consistently larger than those produced by the SP8.0 and TP31 cells.

MMP-2 secretion and activity in uveal melanoma cell lines grown in vitro: As alterations in the level of expression of both MMP-2 and MMP-9 are related to tissue invasion and metastasis in many types of cancers, we next investigated the expression of these enzymes in SP6.5, SP8.0, TP31, and H79 cells through gelatin zymographic analysis using extracts from the serum-free conditioned media in which these cells were grown. Gelatin zymography proved to be the most sensitive technique for the detection of MMP-2 activity and resolves it into its latent and activated forms that migrate at slightly different apparent molecular weights [25]. The latent form does demonstrate some enzymatic activity and so can be detected

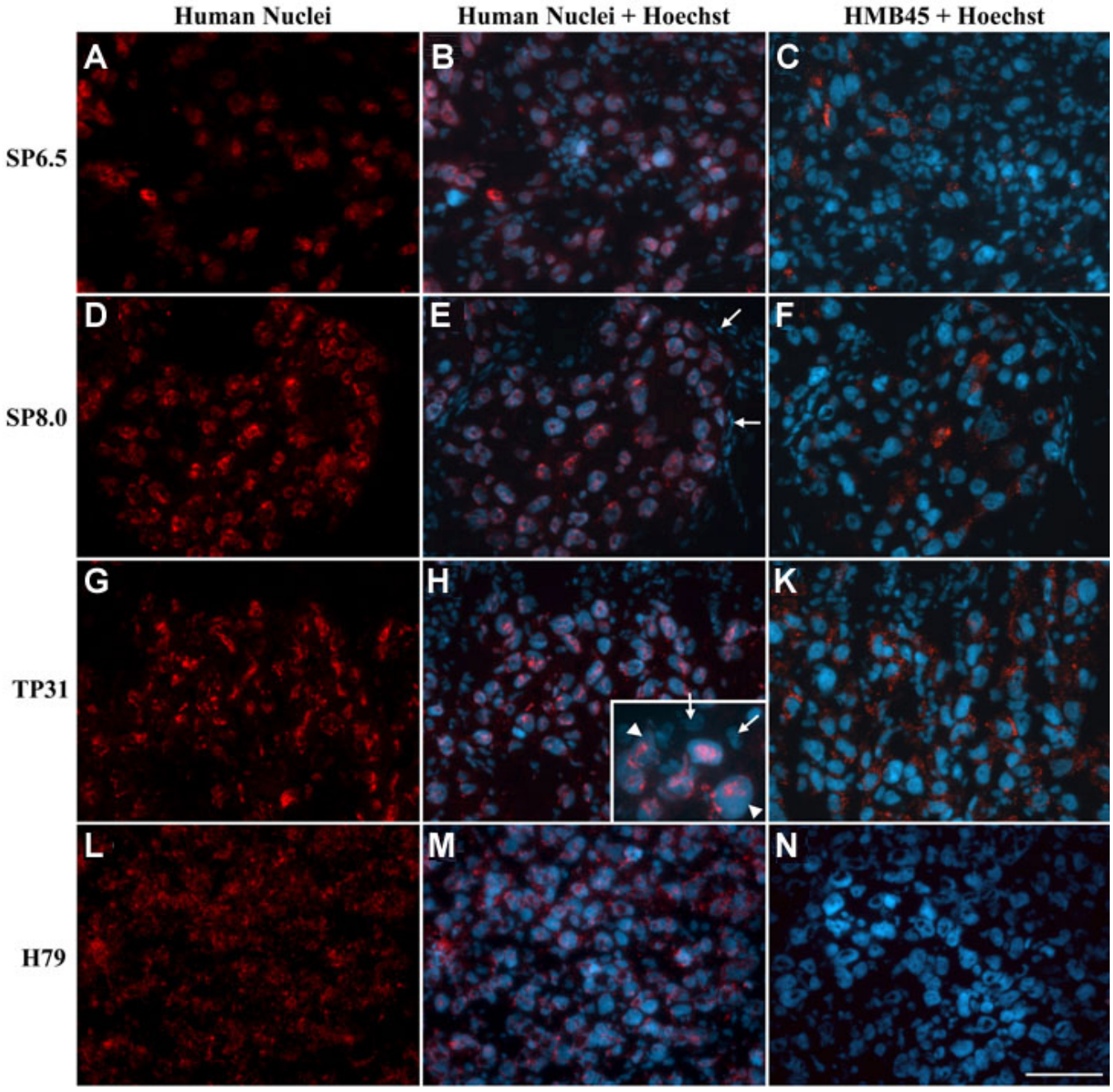

Figure 5. HMB45 expression and human nuclei staining in uveal melanoma-derived tumors. **A**,**D**,**G**,**J**: Human nuclear staining (red) of 5 μm thick paraformaldehyde-fixed cryosections. **B**,**E**,**H**,**K**: Human nuclear staining (red) merged with Hoechst staining (blue) of all cell nuclei (panel **H** insert is a magnification of **H**). **C**,**F**,**I**,**L**: HMB45 staining (red) on consecutive cryosections of the tumors. Cell nuclei were counterstained with Hoechst (blue). Scale bar represents 50 μm. **E**: Arrows identify cells originating from the chick embryos. **H**: Arrows and arrowheads in indicate the position of chicken and human cells, respectively.

by zymography, thereby enabling us to generate an activation ratio. All four uveal melanoma cell lines secreted a gelatinase with an apparent molecular weight (MW) of 72 kDa that corresponds to the inactive form of MMP-2 (Figure 1A). This enzymatic activity was further confirmed to be resulting from the MMPs as incubation of the zymographic gel in the presence of EDTA totally inhibited the gelatinase activity in vitro (data not shown). Gelatin zymography revealed the secretion of the 72 kDa proMMP-2 by all cell lines, with higher levels of secretion observed both in the SP6.5 and the H79 cell lines, suggesting that in vitro, they produce large quantities of proMMP-2 but little, if any, active MMP-2. No gelatinase activity corresponding to MMP-9 was observed under these conditions for any of the uveal melanoma cell line used in this study.

To validate the results from gelatin zymography, a western Blot analysis of MMP-2 was performed (Figure 1B). The proform of MMP-2 could be observed in all cell lines although to different levels. Western blotting of conditioned media from cells cultured in vitro showed that proMMP-2 (72 kDa) was secreted to a lower level in the SP8.0 cell line compared to the other three cell lines SP6.5, TP31 and H79, as also suggested by the zymographic analysis of these protein extracts (Figure 1A).

MMP-2 expression and activity in human uveal melanoma-derived tumors: To determine whether gelatinases are also produced by the tumors generated from the inoculation of the SP6.5, SP8.0, TP31 and H79 into chick embryos, gelatin zymographic analyses were performed using tumor tissue extracts. Two major gelatinolytic activities were observed on zymograms that correspond to both the pro- (72 kDa) and active (62 kDa) forms of MMP-2 (Figure 2A). Both MMP-2 activities were observed in all uveal melanoma cell lines and in CAM. However, whereas CAM expressed very little active but primarily inactive MMP-2, all uveal melanomas expressed predominantly the active form of MMP-2. Both enzymes were entirely inhibited and no gelatinolytic activity was detected following incubation of the gel in EDTA (data not shown). Western blotting of tumor tissues confirmed the expression of both the pro- and active forms of MMP-2 by all the uveal melanoma cell line-generated tumors (Figure 2B). Computer-assisted image analysis of the band intensities from the zymographic data appearing on Figure 2A was reported as a ratio of the active MMP-2 to the sum of proMMP-2 and active MMP-2 (Figure 2C). Examination of these ratios indicated that the gelatinolytic activity of active MMP-2 from the CAM was significantly different from that measured for the four uveal melanoma cell lines ($p<0.0001$).

Staining of the tumor tissues with a monoclonal Ab that recognizes melanoma cells (HMB45) demonstrated that a large proportion of the cells that constitute each tumor produced in chick embryo are of a melanocytic origin (Figure 4B,F,L for the tumors produced by the SP6.5, SP8.0, and TP31 cell lines, respectively) whereas no HMB45 immunoreactivity was observed with either the H79 cell line (Figure 4N) or the CAM (used as a negative control; Figure 4S). A clear immunoreactivity toward a polyclonal Ab that recognizes an epitope shared by both the pro- and active forms of MMP-2 was also observed in the tumors derived from TP31 and SP8.0 cell lines (Figure 4G,K, respectively), whereas those derived from the SP6.5 and H79 cell lines only exhibited a moderate immunopositivity toward this Ab (Figure 4C,O, respectively). The chick embryonic cells were found to be MMP-2 positive (Figure 4S). However, as most of the cells present in the tumors were primarily of a melanocytic origin, it is unlikely that the cells from the chick embryos will contribute to the staining observed, which at the most, might account for the diffuse staining observed within in vivo tumors. Expression of MMP-2 was observed both in the cytosol and outside the cells, where it appears as a diffuse staining. MMP-2 and HMB45 double immunofluorescence micrographs revealed a perfect match between the melanoma and MMP-2 stainings (Figure 4D,H,L for the tumors produced by the SP6.5, SP8.0, and TP31 cell lines, respectively). Although H79 cells showed no reactivity toward the HMB45 Ab, the positive signal for MMP-2 is nevertheless thought to originate from H79 cells, as the tumors derived from this cell line are constituted almost exclusively of human cells, which is revealed from the immunofluorescence staining of the nuclei using a mouse anti-human nuclear Ab (Figure 5K, also refer to the paragraph immediately below). No similar pattern was observed for the CAM (Figure 4R,S,T). The histological examination of the tumors produced in vivo by the inoculation of the uveal melanoma cell lines into chick embryos also show the presence of large cells with larger nuclei and the absence of these cells in the embryonic chick embryo (Figure 4Q). It is also apparent that cells from the tumor produced with the H79 cell line (Figure 4M) were smaller than those from the TP31 and SP8.0 cells (Figure 4I,E, respectively), a characteristic often reported for particularly aggressive types of cancers [28,29].

To confirm that the HMB45 positive cells truly originated from the human uveal tract, we took advantage of an Ab specific for human nuclei [30,31]. Human nuclei and HMB45 stains were performed on consecutive cryosections of tumors produced in vivo as both Abs are mouse monoclonals. All larger, HMB45 positive cells were shown to be human in origin as shown by the positive staining of their nuclei (which appear red on Figure 5). Human cell nuclei were also larger than chick embryonic nuclei (Figure 5H insert; the arrows and arrowheads indicate the position of chicken and human cells, respectively). By taking pictures from the same region on the consecutive cryosections (Figure 5A,D,G,J compared to panels Figure 5C,F,I,L, respectively), cells with the largest nuclei stained positively with both the anti-human nuclear Ab and the HMB45 Ab indicating that the largest cells expressed the melanoma marker. The small cells surrounding the tumors originated from the chick embryos (negative for the anti-human nuclear staining: Figure 5E, arrows).

No positive immunoreactivity toward the HMB45 Ab could be observed for the cells constituting the tumor derived from the H79 cell line. This Ab is specific for the melanosomes present in melanoma cells. However, it is known that not all uveal melanomas stain positively for this marker, as many such cells lose their ability to express melanin as they

are passaged in culture and therefore fail to establish an appropriate melanosomal structure [20].

DISCUSSION

Uveal melanoma is a highly invasive type of cancer with an important capacity for hematogenous dissemination. One particularly critical step in the tumor progression is the ability of the cancer cells to reorganize the ECM through the secretion of enzymes that possess the ability to digest some of its constituting components. MMP-2 is of particular interest in that it can digest collagen type IV, a major structural protein from the basement membrane and ECM [5,9]. By means of the chick embryo tumor model, we investigated the tumorigenic ability of uveal melanoma cell lines isolated from different donors and examined their ability to secrete MMP-2 both in vitro and in vivo. By exploiting this model, we demonstrated that the cell lines of either primary (SP6.5, SP8.0, and TP31) or metastatic (H79) tumoral origins produced tumors in vivo when inoculated onto the CAM of chick embryos, the largest tumors being produced by both the SP6.5 and H79 cell lines. Unlike the intact CAM, all uveal melanoma cell line-derived tumors were found to produce predominantly the active form of MMP-2, which contrasted with the results from cells grown in vitro on plastic, which only secreted the inactive pro-form of MMP-2.

The mechanism of proMMP-2 activation in malignant cells is not yet fully understood. It was recently shown that MMP-2 activation is related to the coordinated expression of MMP-2, MT1-MMP and TIMP-2 in vitro, in which the complex of TIMP-2/MT1-MMP functions as a receptor for MMP-2 at the cell surface [32,33]. Additionally, the integrin $\alpha_v\beta_3$ may be required for activation of proMMP-2 at the cell surface [34]. A recent study conducted by Hofmann and colleagues [11] reported a correlation between the expression of membrane-bound activated MMP-2 and expression of $\alpha_v\beta_3$ in the xenograft model and in human melanoma lesions of the skin. Their findings therefore suggested that a cooperative expression of MMP-2 and $\alpha_v\beta_3$ might contribute to melanoma progression in vivo. However, this may considerably be different for uveal melanoma as both the SP6.5 and TP31 were found to express this integrin in vitro when examined by FACS analyses (data not presented) but yet secreted only the inactive form of MMP-2. The tumors derived in vivo from the H79 and SP6.5 cell lines produced higher amounts of MMP-2 than both SP8.0 and TP31 as revealed by gel zymography. The more diffuse pattern observed for H79 and SP6.5 suggests that MMP-2 is secreted in the vicinity of the tumor cells. Taken together with the in vivo results indicating that these cells generate larger tumors, it suggests that the secretion of MMP-2 may be related to the higher malignity of these tumors. Here, it is likely that the tumor microenvironment generated a proteinase that activated both the tumor cell-derived and the endogenous MMP-2 that was found in the tissue. Blood-born zymogens such as prothrombin (coagulation factor II) or plasminogen might be activated within the tumor stroma, thus being able to further process the proMMP-2 protein [35,36].

In the present study, we showed that all the uveal melanoma cell lines and tumor-derived tissues express MMP-2 to varying levels. Recently, Vaisanen and colleagues [16] provided evidence that MMP-2 may be a prognostic marker in uveal melanoma. An immunohistochemical study of 29 uveal melanomas demonstrated that positive immunostaining for MMP-2 in tumor cells tightly correlated with a worse 5-year survival than patients with melanomas lacking MMP-2 expression. However, these authors exploited immunohistochemical techniques that can detect MMP-2 expression in cancer tissues, but that could not discriminate between the activated and latent forms of MMP-2.

Among the other known prognostic markers for uveal melanoma is cell morphology [37]. Melanomas primarily consisting of spindle cells have the best prognosis, whereas the worst prognoses are seen in those patients with uveal melanomas constituted essentially of epithelioid cells. As with the study of El-Shabrawi [38], Vaisanen and colleagues [16] also demonstrated that all epithelioid cell-containing tumors expressed MMP-2 whereas only 30% of spindle cell tumors were MMP-2 positive. They suggested that differences in the pattern of MMP expression might play a role in the different biological behaviors of epithelioid and spindle cells tumors. Our results are in accordance with these studies as all of our cell lines exhibited a mixed spindle-epithelioid (SP6.5, TP31 and SP8.0) or an epithelioid (H79) morphology. Furthermore, they all expressed MMP-2 at levels that differ from each other in a manner irrespective of the cell's morphology.

The striking difference in the activation of MMP-2 seen between uveal melanoma cells grown in vitro and in vivo points to the need of additional mechanisms in vivo that are lacking in the in vitro culture condition used in this study and which must contribute to shift this enzyme from inactive to active form. Indeed, the newly synthesized ECM that is produced by the growing tumor in vivo may trigger MMP-2 activation, at least in part. Alternatively, cell-to-cell contact between the uveal melanoma cancer cells and the surrounding, non-neoplastic host cells from the invaded tissue might play a role in MMP-2 activation. Finally, one must also consider the possibility that the normal, non-neoplastic tissues surrounding the tumor cells may also secrete components (growth factors and cytokins) that may promote proliferation and migration of the tumor cells. At present we have no evidence as to whether activation of MMP-2 occurs through a single mechanism or whether a combination of mechanisms is required.

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