Investigative Ophthalmology & Visual Science, Vol. 33, No. 6, May 1992

Gelatinolytic Metalloproteinase Secretion Patterns in Ocular Melanoma

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Fifteen posterior uveal melanoma cell lines were analyzed qualitatively for gelatinolytic and caseinolytic proteinase activity after one to five in vitro passages. All 15 cell lines secreted a gelatinolytic metalloproteinase, with an apparent molecular weight of 72 kD, into protein-free culture media; nine of these secreted an additional gelatinolytic metalloproteinase with an apparent molecular weight of 92 kD. Neither species had the ability to degrade casein. This approach may provide insight into the mechanisms of tumor metastasis in uveal melanoma. Invest Ophthalmol Vis Sci 33:1923–1927, 1992

Uveal melanomas are the most common primary malignancy of the eye in adults. They pose a significant threat to survival, and approximately 50% of affected patients die of metastatic disease within 15 years of diagnosis and treatment.[1] It is well recognized that the metastatic potential of uveal melanomas is variable; this is, in part, related to their histologic cell type, with pure spindle cell neoplasms having a better prognosis than epithelioid tumors.[2] Other characteristics, such as large tumor size, anterior location in the eye (excluding iris lesions), and the presence of extrascleral spread, also confer a poor prognosis.[3]

Intraocular tumors, because of the lack of lymphatic drainage, metastasize exclusively through the blood stream, suggesting that tumor cells must be capable of traversing blood vessel walls, including the basement membrane. It is likely that tumor- or host-derived proteinases are major participants in this process. There is evidence suggesting that a proteinase cascade involving serine and/or cysteine proteinase activation of one or more metalloproteinases is pivotal to the breaching of basement membranes, in part caused by the ability of these enzymes to degrade type IV collagen.[4–6]

The metalloproteinase family of enzymes, interstitial collagenase,[7] 72-kD gelatinase (type IV collagenase),[8] 92-kD gelatinase,[9] stromelysin 1,[10] stromelysin 2,[11] stromelysin 3,[12] and PUMP-1,[13] are secreted as zymogens and require modification to their structure before they become active.[14] Several studies suggest that these enzymes, in particular stromelysin and type IV collagenase, are involved in basement membrane degradation at the tumor–host interface. This process represents one of the key events in the invasive process.[4–6] Zymography was used to study the differential expression of two gelatinases (60- and 95-kD forms) secreted by murine fibroblasts, macrophages, and various tumor cell lines; it showed that cell lines with the greatest metastatic potential secreted both gelatinase species.[15,16]

We investigated the expression of gelatinase enzymes in short-term (one to five passages) cultures of human uveal melanomas to determine their enzyme profile in relation to other prognostic indicators. The tumors available to us were derived from patients whose disease was considered severe enough to justify either local resection of the tumor or enucleation of the eye; therefore, they represented the greatest threat to patient survival.

Materials and Methods

Patient Tumor Samples

Tumor samples were obtained after enucleation (12 tumors) or local resection (2 tumors) from 14 patients with uveal melanomas. All tumors were examined by digital B-scan ultrasonography, and their sizes were estimated using the electronic caliper facility (Coopervision). All tumors had a minimum diameter of 15 mm and/or a height of 5 mm. There were nine men (64.3%) and five women (35.7%); their mean age at

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Supported by the Special Trustees for the Former United Sheffield Hospitals, Sheffield, South Yorkshire, and the Yorkshire Cancer Research Campaign, North Yorkshire, United Kingdom.

Submitted for publication: June 19, 1991; accepted December 13, 1991.

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diagnosis was 66.8 yr (standard deviation, ± 12.48 yr). Four tumors were located in the choroid, two in the ciliary body, and the remainder involved both choroid and ciliary body. The duration of follow-up was relatively short (mean, 24 ± 9 months). Three patients died of proven metastases during this period (mean survival, 16.2 ± 13.7 months).

One patient (mel 37), after dissection, was found to have a tumor composed of two morphologically distinct areas (one was deeply pigmented, and the other was amelanotic). Separate tumor samples from the two regions were removed and cultured individually. Patient details are summarized in Table 1.

Cell Culture

Immediately after enucleation, the globe was bisected, and samples of tumor tissue were removed and placed in growth media. The samples obtained from local resection specimens were treated similarly to those from the enucleated eyes. The tumor tissue was minced into fragments of 1 mm^3; the tissue suspension then was digested with a cocktail containing collagenase type II (0.025%), pronase E (0.05%), and dithiothreitol (0.5 μM; Sigma, Poole, England) for 2 hr at 37°C. The tumor cell suspension was washed twice with growth medium, and the suspension was seeded onto vented 25-cm^2 tissue culture flasks (Costar, Cambridge, MA) in Hams F12 medium (Sigma) supplemented with epidermal growth factor (2 ng/ml), glucose (2 g/l), fetal calf serum 15%, and donor horse serum 5% (Sigma). The cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture media was changed twice weekly. The melanoma cell lines were subcultured at subconfluence, using trypsin 0.25% in phosphate-buffered saline at a ratio of 1:2.

Table 1. Gelatinase expression in ocular melanomas: details of patients

Patient	*Sex*	*Age*	*Tumor location*	*Cell type*
14*	M	52	Choroid	Mixed
17	M	64	Choroid	Mixed
22	M	79	Ciliary body + choroid	Mixed‡
30*	M	80	Ciliary body + choroid	Mixed‡
35	M	66	Ciliary body	Epithelioid
37*	F	90	Ciliary body + choroid	Mixed‡
40†	F	48	Choroid	Mixed
44	M	76	Ciliary body + choroid	Mixed
47	M	67	Choroid	Mixed
50	F	48	Ciliary body + choroid	Mixed‡
52	F	72	Ciliary body + choroid	Mixed
55	M	72	Ciliary body + choroid	Epithelioid‡
56†	M	58	Ciliary body	Epithelioid
57	F	63	Ciliary body + choroid	Mixed

* Deceased.
† Local resection.
‡ Evidence of extrascleral spread.

Table 2. Identification of gelatinase activities constitutively expressed by a series of ocular melanoma cell lines

Cell line	*M_r of Gelatinase (kD)*
14	72
17	72 92
22	72 92
30	72 92
35	72 92
37a	72
37b	72 92
40	72
44	72
47	72 92
50	72 92
52	72 92
55	72 92
56	72
57	72 92

Conditioned Media Collection

The cells were seeded at a density of 1×10^6 viable cells per 25-cm^2 tissue culture flask; this cell density ensured that the experiment was done on subconfluent cell monolayers. The cultures were washed twice with phosphate-buffered saline at 1-hr intervals at room temperature to remove trace amounts of serum, and they subsequently were incubated in serum-free Hams F12 media containing 1 mg/ml glucose. After 24 hr, the conditioned media was collected and centrifuged at 15,000 × g for 5 min to remove detached cells and debris; more than 95% of cells remained attached to the plastic after 24 hr. The conditioned media was stored at −80°C. All experiments were completed within 6 weeks of tissue collection on cultures after one to five passages.

Enzyme Activation

Gelatinolytic enzymes were activated by incubation with 1 mmol/l p-amino-phenylmercuric acetate for 4 hr at 37°C.

Zymography

Gelatinolytic and caseinolytic enzyme species were detected using zymography as previously described.[17] Briefly, conditioned medium (20 μl) was subjected to electrophoresis (200 V for 36 min at room temperature) in polyacrylamide 7.5% resolving gels containing either 1 mg/ml gelatin or casein.

The determination of the mechanistic class of gelatinolytic proteinases involved the addition of specific proteinase inhibitors, 10 mmol/l ethylenediaminetetraacetic acid (EDTA), 2 mmol/l phenylmethylsulfonylfluoride (PMSF), 10 mmol/l N-ethylmaleimide (Net-MAL) and 10 mmol/l trans-epoxysuccinyl-l-

leuylamido-(4-guanidino)butane (E-64) in the incubation buffer. Enzyme inhibitor solutions were prepared immediately before use. Apparent molecular weight values were determined by comparison with reduced molecular weight markers prepared by addition of 2.5% v/v 2-mercaptoethanol to the sample buffer followed by boiling for 3 min; these appeared as dark bands against a blue background.

Electrophoresis of both reduced and nonreduced molecular weight markers was done on all zymograms to determine if alteration in apparent molecular weight occurred. A slight increase in apparent molecular weight was observed for nonreduced molecular weight markers, but this increase was typically less than 5 kD. The molecular weight markers used on all zymograms were: carbonic anhydrase (30 kD), egg albumin (45 kD), bovine serum albumin (66 kD), and phosphorylase b (97 kD) at a concentration of 4 mg/ml. As a control, serum-free medium, which had not been in contact with cells, was run on gels to determine the presence of gelatinases in the medium; none were detected.

We loaded 20 μl of supernatant in a sample buffer containing 0.25 mol/l Tris HCl, pH 6.8, sodium dodecyl sulfate 0.4%, and glycerol 34%. The samples were not boiled before electrophoresis. After electrophoresis, the gel was washed with Triton X-100 2% for 1 hr, followed by three 5-min washes in 50 mmol/l Tris HCl, pH 7.4, containing 200 mmol/l NaCl and 5 mmol/l $CaCl_2$. The samples were incubated in the same buffer for 48 hr at 37°C. Bands of degradation could be detected by staining the gel with amido black 0.1% in methanol–acetic acid–water (3:1:6); degradation was visualized as transparent bands against a blue background.

Histologic Examination

The globe (including the rest of the tumor) was fixed in glutaraldehyde 3% in sodium cacodylate buffer at pH 7.4. Sections of the eye were processed into paraffin wax, and 4-μm sections were cut and stained with hematoxylin and eosin. Some sections were bleached before staining to remove melanin. The tumors were assessed using the Armed Forces Institute of Pathology classification.[18]

Results

Histologically, 11 (78.6%) tumors were of mixed cell type, and three were found to be composed of pure epithelioid cells (21.4%). Evidence of overt extrascleral spread was seen in five specimens (33.0%, Table 1). By zymography, all melanoma cell lines released an enzyme of approximately 72-kD molecular weight gelatinase, and ten cell lines secreted an additional 92-kD gelatinase. Figure 1 shows the gelatinolytic profiles of the ocular melanomas. All enzyme activities detected could be activated with p-APMA, suggesting that the enzymes were latent metalloproteinases (Fig. 2). Enzyme activity in supernatants from all tumor cells could be inhibited completely by the inclusion of EDTA in the incubation buffer, but PMSF, Net-MAL, and E-64 had no effect on enzyme activity. This suggests that substrate degradation was not related to serine or cysteine proteinase activity. No caseinolytic activity could be detected in any of the supernatants; therefore, the observed metalloproteinase activity was not caused by stromelysins.

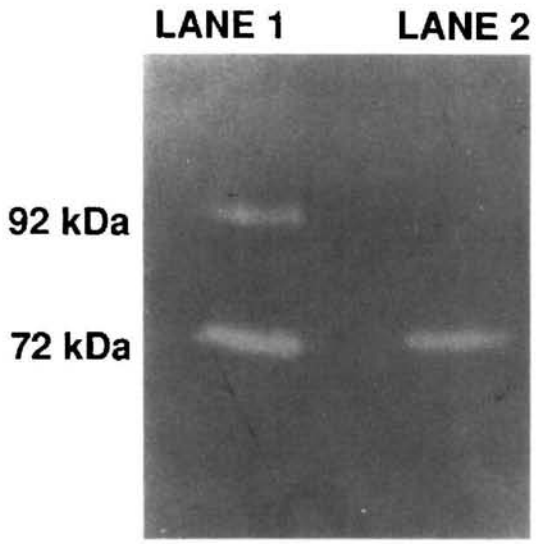

Fig. 1. Activation of progelatinase by p-APMA. Conditioned medium samples containing constitutively expressed progelatinase species were incubated with 1 mM p-APMA for 4 hr at 37°C. The conditioned medium was analysed by zymography on 7.5% w/v polyacrylamide minigels containing 1 mg/ml gelatin. Reduced and nonreduced molecular weight markers were run on each gel (not shown). Lane 1, 72-kD progelatinase alone; lane 2, 72-kD progelatinase following activation with p-APMA.

The pattern of gelatinase secretion was compared with the morphologic and histologic characteristics of the primary tumors. Four of the five tumors derived from women were capable of secreting 92-kD gelatinase. Similarly, all patients with histologic or clinical evidence of extrascleral spread at the time of removal secreted the high molecular weight gelatinase. These factors, however, were not statistically significant. Differences in cell type, location, and the presence of metastases also were not statistically significant.

Discussion

Not all tumors give rise to populations of cells endowed with the ability to metastasize and successfully establish tumors in secondary organs. Genotypic and

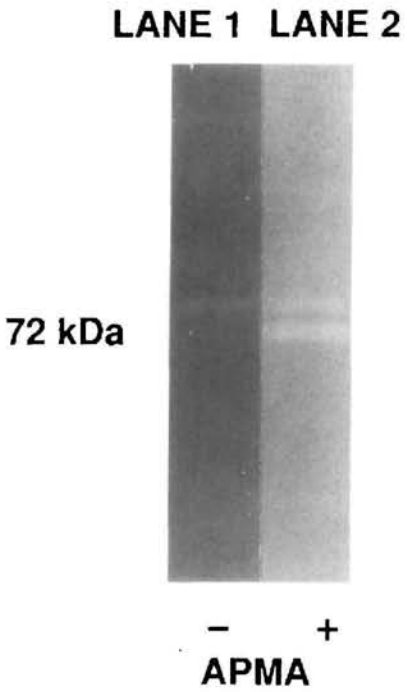

Fig. 2. Examples of 72- and 92-kD progelatinase expression. The conditioned medium was analysed by zymography on 7.5% w/v polyacrylamide minigels containing 1 mg/ml gelatin. Reduced and nonreduced molecular weight markers were run on each gel (not shown). Lane 1, cell line 22 conditioned medium; lane 2, cell line 56 conditioned medium.

phenotypic properties are important in allowing tumor cells to traverse basement membranes and endothelial cell barriers, to survive the hostile environment of the blood (and escape the host immune defense systems), and to colonize and grow at distant sites of the body.[19] Although certain clinical and histologic criteria are associated with tumor progression, currently, there are no definitive markers of the invasive phenotype. One important aspect of tumor cell metastasis is the ability of cells to migrate across basement membranes, a mechanism associated with the production of enzymes with gelatinolytic activity.[4–6] In an attempt to understand the association between such enzymes and tumor cell invasion further, we studied a series of ocular melanomas, propagated in vitro for one to five passages, for their ability to produce differing molecular weight species of enzymes with gelatinolytic activity. Approximately 50% of patients with ocular melanomas removed surgically will die of metastatic disease, commonly liver, within 15 yr. Therefore, any tumor properties that might lead to our understanding the development of metastatic disease in these patients would be valuable.

This study identified two molecular weight forms of gelatinolytic enzymes (72 and 92 kD) produced by ocular melanoma cells in culture; both these enzyme species were able to degrade gelatin but not casein. Of the three major metalloproteinase groups, all are active against gelatin, although the activity of interstitial collagenase and stromelysin is limited. Stromelysin is known to degrade casein under the assay conditions we used,[10] but no caseinolysis was seen. It is unlikely that the active enzymes released from ocular melanoma cell cultures are stromelysins. There are, however, several proteinases capable of degrading basement membrane and intercellular matrix, including the plasminogen activators.[20] It is therefore possible that the synergistic action of these two proteinase groups may contribute to the invasive properties of the tumor. Although not statistically significant, the tumors from four of the five women we studied expressed both species of gelatinase.

Although it was interesting to identify the production of a 90–97-kD gelatinase in uveal melanomas and speculate on its potential role, our relatively small sample size and short follow-up prevented any firm conclusions being drawn as to its clinical significance. Expression of the 92-kD gelatinase may assist in the dissemination of the tumor, potentially decreasing the time required for invasion to occur, but it is not a prerequisite for metastatic disease. The tumor from one patient with metastatic disease did not express the higher molecular weight species. A long-term longitudinal study of the pattern of proteinase secretion and its clinical correlation is currently in progress. Although our study was biased toward large and medium-sized tumors based on our current patient management, it was relevant to ocular melanoma metastasis because it is these tumors that pose the greatest threat to patients.

Key words: ocular melanoma, metalloproteinase, scleral invasion, zymography, metastasis

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