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Comparison of Basement Membrane Matrix Degradation by Purified Proteases and by Metastatic Tumor Cells

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We have examined the nature of biochemical degradation of an isolated basement membrane matrix (bovine lens capsule) using different methodologies. The first strategy was quantitation of the release of surface-bound ^{125}I and a second the documentation by SDS-PAGE of the appearance of putative cleavage products and the loss of high-molecular-weight components from the matrix. Basement membrane matrix bands resolved on SDS-PAGE were identified by their protease sensitivities as well as by Western immunoblots using monoclonal antibodies developed for this study. Radioiodinated components were found predominantly at positions on the gel equivalent to 160–200 kd and 400 kd proteins. Since these labeled moieties were sensitive to bacterial collagenase digestion and stained with anticollagen type IV antibodies, they were determined to represent various configurations of collagen type IV. Several other lower-molecular-weight bands also stained with the anticollagen IV antibodies. Monoclonal antibodies reactive with laminin exhibited a complex staining pattern on the gels, which included the expected 200 and 400 kd components. We confirmed that lens capsule basement membrane contained only a single heparan sulfate glycosaminoglycan species, and tumor cell-induced glycosaminoglycan degradation within the basement membrane matrix was detected using cellulose acetate electrophoresis.

Distinctive putative cleavage products were resolved on SDS-PAGE gels from matrices subjected to digestion by a variety of purified proteases as well as by metastatic tumor cells or their conditioned media. Tumor cells of different histiotypes produced different characteristic cleavage patterns, suggestive of the exis-

Abbreviations used: polyhema, poly(2-hydroxyethyl methacrylate); CMF, calcium- and magnesium-free Tyrode's salt solution; IV, intravenous; IP, intraperitoneal; PBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; UV, ultraviolet light; CM, conditioned medium; TPA, 12-O-tetradecanoyl phorbol 13-acetate; GAG, glycosaminoglycans.

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tence of several pathways of matrix degradation. Overall, primary tumor cells exhibited a greater degradative activity towards the basement membrane matrix than did long-term tissue culture-passaged cells. The same tumor cell line could exhibit considerably different patterns of both protein and glycosaminoglycan degradation depending on recent culture history. The relevance of these biochemical studies to the pathogenesis of malignant neoplasms is shown by: 1) the evaluation of degradative activities of B16 tumor cell populations exhibiting enhanced lung-colonizing phenotypes, and 2) the ability of a known antimetastatic moiety with antiprotease activity (*Haementeria* leech species salivary gland extract) to protect matrix components from degradation by tumor cell-conditioned medium.

Key words: basement membrane, extracellular matrix degradation, metastasis, collagen type IV, laminin, glycosaminoglycan degradation

Basal laminae and subendothelial basement membranes serve as important barriers to invasion and metastasis by malignant cancer cells [1]. Positive correlations exist between the metastatic capacity of a tumor cell line and its ability to degrade either isolated basement membrane components [2,3] or basement membrane matrix itself [4–8]. Furthermore, manipulations of the interactions of tumor cells with the basement membrane components collagen type IV and laminin, correlate very well with predicted results from experimental metastasis assays [9–12]. Although the preponderance of evidence suggests a close dependancy of cellular invasion on degradative activities towards extracellular matrices [2–8,13–17], a small body of evidence also exists suggestive that such degradative activity need not always occur [18–20] or be positively correlated with metastatic ability [21]. There is evidence that activated N-*ras* and Harvey-*ras* oncogenes can confer metastatic ability [22,23] and that degradative activity towards collagen IV can be proportionally increased in such *ras*-transfected metastatic cells [22]. However, cellular p21 levels do not correlate with metastatic ability [24], correlations with *ras* oncogene expression may become less obvious with continuing animal passage [25], metastasis in immunocompetent hosts may require expression of additional oncogenes [23], and cotransfection of the *ras* oncogene with the E1a oncogene can interfere with *ras* induction of metastatic ability [26], all indicating a rather complex relationship. Clearly, basement membrane invasion is a highly integrated activity involving extracellular degradative activity, cellular motility, and deformability and adhesive phenomena. An isolated basement membrane matrix, the bovine lens capsule, has been utilized as a tissue culture substrate for several types of normal cells [27–28], and we have demonstrated that it also provides a singularly useful substrate for examining the interactions of metastatic tumor cells with basement membrane matrix [5]. In an earlier study, although we did show increased overall degradation of the matrix by highly metastatic tumor variants, biochemical analysis of the matrix degradation was not attempted because we lacked a suitably defined experimental system. In this paper, we describe the initial development of such a system to analyze matrix degradation and evaluate its potential usefulness.

MATERIALS AND METHODS

Animals and Cell Lines

C57BL/6 and BALB/cByJ mice were obtained from the Montana State University animal facility from colonies originating with animals purchased from The

Jackson Laboratory (Bar Harbor, ME). BD-IV rats were originally donated by Prof. Rajewsky, Institute for Cell Biology, University of Essen, and by Dr. R. Montesano, International Agency for Research on Cancer, Lyons. These animals were cesarian-derived and were maintained as a specific pathogen free (SPF) colony at the Montana State University animal facility.

The Englebreth-Holm-Swarm (EHS) sarcoma [29] was kindly donated by Dr. G. Martin, National Institutes of Health, and was maintained by subcutaneous growth in the flank area of C57BL/6 mice. The highly invasive and metastatic B16 melanoma variant B16BL6 [30] was obtained from the Mason Research Institute (Worcester, MA). The poorly lung-colonizing and highly lung-colonizing B16 melanoma variants B16-F1 and B16-F10 were obtained from Dr. I.J. Fidler, Frederick Cancer Research Facility. All three B16 variants were animal-passaged subcutaneously in the flank area of C57BL/6 mice. Experiments were initiated from frozen tumor cell stocks, and all cells used were fewer than 10 tissue culture passages from the receipt of the cell line in the laboratory. −SA, an anchorage-dependent mammary adenocarcinoma of the BALB/c mouse, and +SA, a more highly lung–colonizing anchorage-independent variant, were derived at Washington State University [31] and were originally provided by Dr. H.L. Hosick, Department of Zoology, Washington State University. +SA and −SA cell lines were animal passaged in the mammary fat pad of BALB/c mice. The highly metastatic RT7-4bs hepatocarcinoma line and its even more invasive and metastatic variant, RT7-4bLs [5,32], were both animal passaged subcutaneously in the flank area of BD-IV rats.

Nonadhesive Tissue Culture Substrates

Nonadhesive polyhema (Interferon Sciences Inc., New Brunswick, NJ) substrates were prepared by dissolving polyhema to 0.12% and 0.6% w/v in 70% ethanol, spreading these solutions over the surface of tissue culture flasks, and evaporating to dryness under a laminar flow hood.

Lung Colony (Experimental Metastasis) Assay

The lung colony assay for experimental metastasis was conducted essentially as described by Talmadge et al [32]. A subpopulation of B16BL6 cells that had invaded across the full thickness of the lens capsule [5] was compared for its lung-colonizing ability with the unselected parent population. For each tumor cell population, 10^4 monodispersed cells in 0.2 ml CMF were inoculated IV into 10 C57BL/6 mice via the tail vein. Twenty-one days later, the mice were killed and autopsied. The lungs were fixed in Bouin's fixative, and the number of superficial lung colonies was determined with the aid of a dissecting microscope. The antimetastatic activity of the leech salivary gland extract [33] was also determined using the lung colony assay. C57BL/6 mice were inoculated with 5×10^4 monodispersed B16BL6 tumor cells in 0.2 ml CMF. Ten mice were also injected with 3×0.1 ml aliquots of the leech salivary gland extract (~8 mg/ml) IP at −1 hr, at +1 hr, and at +3 hr relative to the tumor cell inoculation. Control mice received a similar series of injections substituting saline buffer for the leech extract. Twenty–one days later, the mice were killed, and the lung colonies were enumerated.

Isolation and Radioiodination of Bovine Lens Capsule Basement Membrane

Bovine eyes were obtained from local packing plants, and the anterior lens capsules were obtained and prepared for use as previously described [5]. Lens

capsules, spread out and attached to "Lux" coverslips, were radioiodinated using "enzymobeads" (Biorad) as reported earlier [5].

Solubilization and SDS-PAGE of Isolated Basement Membrane Matrix

Lyophilization was used routinely as the initial step in solubilization of the basement membrane matrix. The lyophilized lens capsule was cut into pieces ~1.5 × 1.5 mm and then dissolved at 80°C in Laemmli buffer [34] containing 5% SDS and 12% 2-mercaptoethanol. The matrix dissolved slowly over 2 hr and was subjected to at least three freeze/thaw cycles during this time. Dissolved matrix was electrophoresed on 32 cm 4–18% or 5–15% polyacrylamide gels using a 4% stacking gel. Following electrophoresis, the gels were silver stained [35] or electroblotted [36]. Autoradiographs were processed using Kodak X-omat film in X-ray cassettes with intensifying screens.

Degradation of Basement Membrane Matrix by Isolated Proteases

Unlyophilized lens capsule (one-half of an anterior lens capsule per enzyme assay) was exposed to digestion by: 1) 10 μg bacterial collagenase (clostridial, Worthington, Freehold, NJ), 2) 20 μg trypsin (three times recrystallized, Worthington), 3) 20 μg chymotrypsin (Sigma, St. Louis, MO), and 4) 25 μg V8 protease (Pierce, Rockford, IL). The matrix was incubated with enzyme in 200 μl buffer for 4 hr at 37°C, was extensively washed in PBS, and then was lyophilized prior to being dissolved in Laemmli sample buffer for SDS-PAGE. The following digestion buffers were used: for collagenase, 10 mM Tris, 10 mM $CaCl_2$, 5 mM N-ethylmaleimide, pH 6.8; for trypsin and chymotrypsin, 10 mM Tris, pH 6.8; and for V8 protease, 10 mM Tris, 0.1% SDS, pH 6.8.

Under the conditions used, the bacterial collagenase failed to degrade noncollagenous proteins such as bovine serum albumin. Also, similar collagenase degradation resulted from the use of collagenase III (from Advance Biofactures, Lynbrook, NY), a preparation reputed to be substantially free of contaminating proteases. For SDS-PAGE, low-molecular-weight globular protein standards (Pharmacia, Piscataway, NJ), as well as collagen standards (collagen type I, from Collagen Corp. Palo Alto, CA, collagen type IV and collagen type V, both kindly provided by Dr. Helene Sage, University of Washington, Seattle) were routinely used. To observe losses of high-molecular-weight materials, lens capsules were subjected to limit digests. For each digest, one capsule (1.0–1.5 mg dry weight) was incubated in 1 ml of enzyme solution for 16 hr at 28°C. The following enzyme amounts and buffers were used: 1) 200 μg clostridial collagenase in 10 mM Tris, 10 mM $CaCl_2$, 5 mM N-ethylmaleimide, pH 6.8; 2) 50 NIH units thrombin (Sigma) in PBS; and 3) 30 μg plasmin (Sigma) in PBS.

At the end of the incubation period, the collagenase digests were stopped with addition of EDTA, the thrombin digests with addition of Huridin (Sigma), and the plasmin digests with addition of Leupeptin (Calbiochem). The residual lens capsules were then washed extensively in CMF, were lyophilized, and were analyzed using SDS-PAGE and autoradiography. Pepsin (Worthington) digestion was carried out at 28°C in 0.1 M acetic acid, pH 2.8, using 50 μg enzyme/ml for various times up to 6.5 hr.

Degradation of Basement Membrane Matrix by Metastatic Tumor Cells and by Tumor Cell-Conditioned Media

Degradative activity of metastatic tumor cells towards lens capsule basement membrane matrix was assessed using cell-associated digests as well as digestion by

tumor cell-conditioned media. For the cell-associated digests, one-fourth of an anterior lens capsule was incubated with 1.5×10^6 tumor cells at 37°C as described in Starkey et al. [5] for time intervals from 48 to 72 hr. RPMI 1640 medium containing 2.5% acid-treated FBS or serum-free RPMI 1640 medium was used to avoid effects from inhibitors in serum [5]. At the end of the incubation period, the residual lens capsule was retrieved, washed extensively in CMF, and subjected to SDS-PAGE as described earlier. Resolved proteins and peptides were visualized using silver staining and autoradiography as appropriate. Tumor cell-conditioned medium was generated and concentrated, and aliquots were subjected to trypsin activation as previously described [5]. A single anterior lens capsule (~ 1 mg dry weight) was incubated at 37°C with 2 ml of conditioned medium for time periods varying from 48 to 72 hr. Primary tumor cells were used to assess degradative activity towards the basement membrane matrix, and, in a limited number of cases, the activity of long-term tissue culture-adapted (more than 10 passages from the animal) cells was compared with the activity of primary cells.

Assay for the Effects of the Presence of Leech Salivary Gland Extract on Lens Capsule Matrix Degradation

Leech salivary gland extract at 1:2, 1:10, and 1:100 final dilutions were added to concentrated B16BL6-conditioned medium digests of lens capsule matrix. The digests were incubated for 48 hr at 37°C. Controls consisted of 1) lens capsule matrix incubated with concentrated conditioned medium diluted with the saline buffer used to dissolve the leech salivary gland extract, and 2) lens capsule matrix incubated with dilutions of leech salivary gland extract alone. The results of the digests were assessed by SDS-PAGE.

Production of Monoclonal Antibodies to Basement Membrane Matrix Components and Their Initial Characterization

To maximize the yield of monoclonal antibodies to basement membrane matrix components that would retain a strong reaction towards denatured proteins on Western immunoblots, we used bovine lens capsule matrix previously denatured in Laemmli buffer as the immunizing antigen for a group of four male BALB/cByJ mice. Spleen cells from the immunized mice were fused with X63-Ag8.653 myeloma cells (obtained from Dr. W.C. Davis, Department of Veterinary Microbiology/Pathology, Washington State University) using standard protocols, and hybridoma lines were isolated using HAT selective medium. Primary screening of hybridoma cell lines was carried out by incubating tissue culture supernatants in a 96-well pattern manifold (Biorad, Richmond, CA) with lens capsule matrix dissolved in Laemmli buffer and subsequently bound to 0.45 μm pore nitrocellulose sheets (Schleicher and Schuell, Keene, NH). The nitrocellulose sheets were transferred to a large petri dish and were further reacted with peroxidase-conjugated goat antimouse Ig (Biorad), followed by a 4-chloro-1-napthol containing substrate. A variety of purified basement membrane components were similarly bound to nitocellulose sheets, and these were used in a secondary screen to obtain further information on the specificity of the monoclonal antibodies produced. Basement membrane components used were: human placental collagen IV (Sigma), at 1 mg/ml; EHS murine collagen IV (prepared by us following Timpl et al [37], at 0.2 mg/ml; EHS murine laminin (E-Y Laboratories), at 0.3 mg/ml; EHS heparan sulfate proteoglycan (prepared by us following Hassell et al [38],

at 0.7 mg/ml; and human plasma fibronectin, (Sigma), at 0.1 mg/ml. The purity of these preparations was assessed using SDS-PAGE or cellulose acetate electrophoresis as appropriate.

Western Immunoblots

Lens capsule matrix components resolved on SDS-PAGE were electroblotted onto nitrocellulose sheets following the method of Towbin et al [36]. Transfer of high-molecular-weight components (up to 400 kd) was satisfactory when electroblotting was carried out for 16 hr at 150 mA. The transfers were reacted with antibodies and stained following the procedure outlined earlier for ELISA tests.

Glycosaminoglycan Analysis

GAGs were purified from lens capsule matrix using a protracted pronase digestion [39] over 48 hr and ethanol precipitation [40], followed by pelleting at 17,000g. Any undigested protein was eliminated by TCA precipitation prior to ethanol precipitation [40], and residual protein was found to be negligible (0.22% w/w). Lens capsule GAG was identified by means of specific degrading enzymes [chondroitin sulfate lyases ABC and AC, keratanase, *Streptomyces* hyaluronidase, heparitinase (Sigma), and heparinase (Miles, Naperville, IL) using published procedures [41–45]. GAG preparations were quantified by uronic acid determination [46], with D-glucuronolactone as a standard. The controls and the digestion products were resolved by cellulose acetate electrophoresis using 2.5 × 17 cm Sepraphore III cellulose acetate strips. The electrophoresis was performed at room temperature and 220 V in 0.1 M barium acetate, pH 8.3 [47], or 0.1 M HCl [48], each for 1 hr, or in 0.3 M calcium acetate, pH 10, for 3 hr [49]. The GAGs were visualized using the toluidine blue stain of Seno et al [50]. To determine whether tumor cells could degrade lens capsule GAG within the matrix, capsules were incubated in aliquots of concentrated CM at 37°C. Capsules were also similarly incubated in concentrated medium that had been incubated without cells (mock CM) as controls. At 24 hr intervals, the media were replaced with fresh aliquots in the event that CM might contain proteases destructive of endoglycosidase activity. After 96 hr the incubation was terminated by boiling for 5 min. The capsules were rinsed three times in 0.2 M Tris buffer, pH 8.0, and the GAG was isolated and resolved by cellulose acetate electrophoresis as described above. As controls, and as probes for the specificity of the CM endoglycosidase revealed in these experiments, authentic bovine lung heparin and bovine kidney heparan sulfate (Sigma) were incubated in CM or mock CM. Fresh aliquots of media were added at each 24 hr time point, with four such sequential aliquots being employed per assay. The undigested GAG were ethanol precipitated, centrifuged, made to small volumes in H_2O, and analyzed by cellulose acetate electrophoresis, as described above. Positive transparencies from photographic negatives of the electrophoretograms were made by projection onto Kodak 4498 electron microscope sheet film. The enlarged transparencies were scanned on a Hoefer GS 300 densitometer. Relative peak heights for the migratory GAG and the GAG fraction remaining at the origin were calculated.

RESULTS

Analysis of ^{125}I Release Assays

As we have shown in an earlier study [5], degradation of lens capsule basement membrane matrix can be measured using the release of ^{125}I from iodinated capsule

substrates (Table I). Autoradiograms of SDS-PAGE-resolved capsules reveal heavily labeled material in regions equivalent to the positions of 400 kd and 160–200 kd globular proteins (Fig. 1B, lane 2). Furthermore, predigestion of labeled lens capsule matrices with bacterial collagenase results in a selective loss of this labeled material (Fig. 1B, lane 3) as well as loss of bands reactive with anticollagen IV monoclonal antibodies (Fig. 1A, lane 3; Fig. 1B, lane 1). Pepsin digestion of iodinated capsules results in fragment distribution patterns on autoradiograms from SDS-PAGE consistent with those obtained for isolated type IV collagen (Fig. 1B, lanes 4–6). We conclude that the majority of the ^{125}I is bound to collagen IV and therefore that ^{125}I release will measure predominantly the degradation of this matrix component. ^{125}I is slowly released from iodinated capsules incubated in the absence of any degradative enzymes. The ^{125}I release occurs at 4°C as well as 37°C and is observed regardless of the iodination methods used. The released isotope is not precipitable by TCA, nor are labeled products found on SDS-PAGE. Thus we doubt that the ^{125}I originates with isotope bound to the capsule via the iodination reaction, and we suspect that it originates from isotope loosely associated with sugar groups. Whatever its nature, this background release is too low to interfere with assays using purified enzymes when incubation times are relatively short (up to 6 hr) but results in a high nonspecific release in the long-term assays needed with cell-associated or CM digests. This requires the use of very large cell numbers to ensure significant results. The amount of ^{125}I release obtained with various tumor cell lines was affected by the conditions of assay incubation and the origin and culture history of the cells. Primary cultures of B16BL6 cells assayed using a serum-free CM protocol exhibited nearly three times the degradative activity of long-term tissue culture-adapted B16BL6 cells (Table I). However, the same cells compared using the cell-associated protocol demonstrated equivalent levels of degradation (Table I). B16BL6 cells grown in a rounded state on polyhema substrates produced CM with either no degradative activity (completely rounded in a 0.6% polyhema substrate) or very little degradative activity (partially rounded on a 0.12% polyhema substrate) compared to the same cells grown on tissue culture plastic (Table I). For B16Bl6 cells, most of the ^{125}I release took place over the first 24 hr of incubation (Table I). The use of serum-free protocols led to greater levels of detectable degradative activity for the B16BL6 cells than the use of protocols using 2.5% acid-treated serum-containing medium (Table I). However, this difference was marginal for the subpopulation of B16BL6 cells, which was selected for the ability to invade the lens capsule basement membrane matrix (Table I). Cell-associated digests using +SA cells exhibited more activity when protocols using medium containing 2.5% acid-treated serum were utilized (Table I).

Biochemical Evaluation of Lens Capsule Matrix Degradation

To obtain more specific information on the biochemistry involved, we assessed lens capsule basement membrane matrix degradation using SDS-PAGE and cellulose acetate electrophoresis. Lens capsule matrix was subjected to partial digests using a variety of purified enzymes (data not shown). Distinctive putative cleavage fragments were detected by SDS-PAGE. Digestion with V8 protease resulted in the appearance of new bands at 31, 91, and 104 kd; digestion with chymotrypsin resulted in the appearance of new bands at 33, 36, and 67 kd; digestion with bacterial collagenase resulted in the appearance of a new band at 51 kg; and digestion with trypsin resulted in new bands at 33 and 91 kd. Even in this relatively limited 4 hr digest, some loss of

TABLE I. Degradation of Lens Capsule Basement Membrane Matrix Measured Using ^{125}I Release*

Tumor cell line	Regimen		Specific release of surface-bound ^{125}I from lens capsule matrix (%)	
	Conditioned medium digest	Cell-associated digest	At intermediate time points (hr)	At end of assay—total release (hr)
Primary culture of B16BL6 parental population		Medium containing 2.5% acid treated serum	ND[a]	7.8 (48)
Primary culture of B16BL6 parental population		Serum-free medium	ND	23.6 (48)
Primary culture of B16BL6 population selected for lens capsule invasion		Medium containing 2.5% acid treated serum	ND	8.5 (48)
Primary culture of B16BL6 population selected for lens capsule invasion		Serum-free medium	ND	9.4 (48)
Primary culture of B16BL6 parental population	Serum-free medium		ND	27.0 (48)
Primary culture of B16BL6 population selected for lens capsule invasion	Serum-free medium		ND	22.5 (48)
Primary culture of +SA cells		Medium containing 2.5% acid treated serum	ND	9.2 (48)
Primary culture of +SA cells		Serum-free medium	ND	2.1 (48)
Primary culture of +SA cells	Serum-free medium		ND	23.5 (48)
B16BL6 tissue culture-adapted cells		Serum-free medium	18.9 (24), 5.4 (48)	24.3 (72)
B16BL6 tissue culture-adapted cells	Serum-free medium and tissue culture plastic substrate		9.3 (24), 1.0 (48)	10.3 (72)
B16BL6 tissue culture-adapted cells	Serum-free medium and 0.12% polyhema substrate		1.5 (24), 1.6 (48)	3.1 (72)
B16BL6 tissue culture-adapted cells	Serum-free medium and 0.6% polyhema substrate		0.0 (24), 0.0 (48)	0.0 (72)

*^{125}Iodinated lens capsule was incubated with 1.5×10^6 tumor cells for various time intervals at 37°C or incubated for various time intervals with 2 ml concentrated tumor cell-conditioned medium as described in Methods. Lens capsule basement membrane matrix-associated ^{125}I counts were measured at the start and the end of the assay as was the radioactivity released into the supernatant. All assays were conducted at least three times, with similar results. Representative results are shown. Because of the use of multiple aliquots of conditioned media, insufficient material was available to run multiple repeats of experiments using polyhema substrates.

[a]ND, not done.

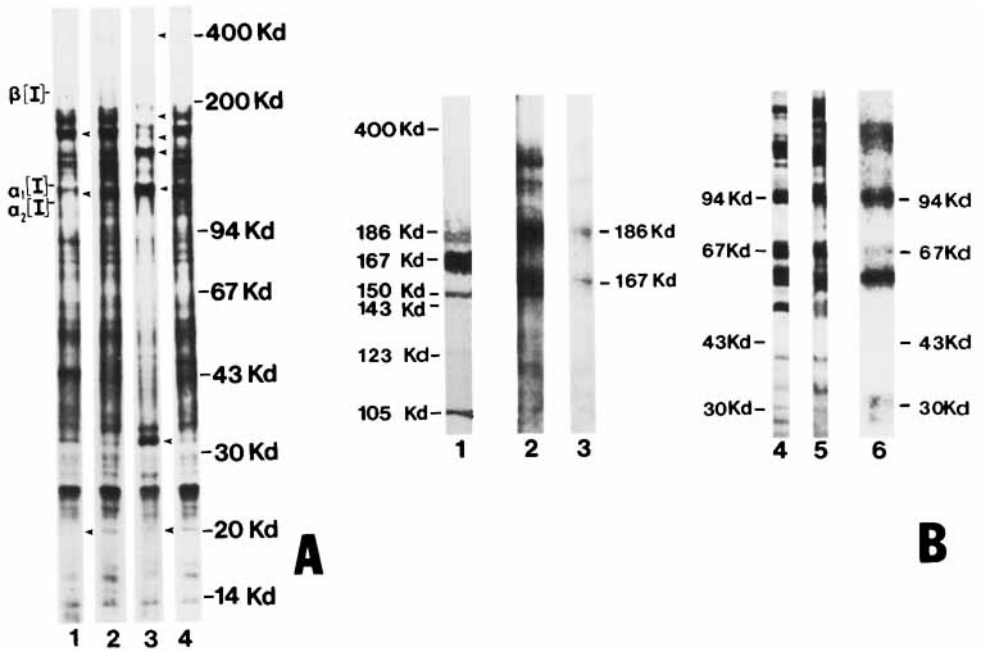

Fig. 1. **A)** SDS-PAGE analysis of limit digests of lens capsule matrix with plasmin **(lane 1)**, thrombin **(lane 2)** and collagenase **(lane 3). Lane 4**, undigested material. All lanes are silver stained; arrowheads indicate enzyme-induced changes. **B)** Immunoblot of whole lens capsule matrix separated by SDS-PAGE and reacted with a representative hybridoma supernatant specific for collagen IV **(lane 1)**. Also shown are results of SDS-PAGE analysis of limit digests of ^{125}I surface radiolabeled lens capsule matrix. **Lane 2**, autoradiogram of undigested matrix; **lane 3**, autoradiogram of the same matrix subjected to collagenase digestion. **Lanes 4** and **6**, SDS-PAGE—resolved lens capsule matrix previously subjected to pepsin digestion. **Lane 5**, similar digest of collagen IV isolated from human placenta. Lanes 4 and 5 are silver stained; lane 6 is an autoradiogram.

high-molecular-weight material at about 200 kd was apparent in the chymotrypsin digest. Such loss of material is, however, more easily appreciated in limit digests. Limit digestion with plasmin resulted in a partial loss of the 167 kd collagen band, loss of a 117 kd band, and loss of a 22 kd band (Fig. 1A). Western immunoblots demonstate staining for collagen IV by the 167 kd band and for laminin by the 117 kd band. At this time, the 22 kd band is undefined. Limit digestion with bacterial collagenase resulted in a loss of material at 400, 186, 167, and 22 kd (Fig. 1A). Western immunoblots show staining for collagen IV by material at the 400, 186, and 167 kd positions. Again, the 22 kd band is undefined at this time. Limit collagenase digestion also resulted in intensification of bands at 150, 120, and 34 kd (Fig. 1A).

Distinct putative cleavage fragments were found in SDS-PAGE preparations of lens capsules that had previously been subjected to incubation with concentrated tumor cell-conditioned media or cell-associated digestion (Fig. 2, Table II). Figure 2 shows the result of concentrated CM digests of lens capsule matrix resolved on SDS-PAGE. The most obvious putative cleavage fragments are indicated by arrowheads. Digestion with −SA-conditioned medium results in the appearance of new bands at 23 and 69 kd; digestion with +SA-conditioned medium results in the appearance of a new band at 69 kd; and digestion with RT7-4bL-conditioned medium results in the

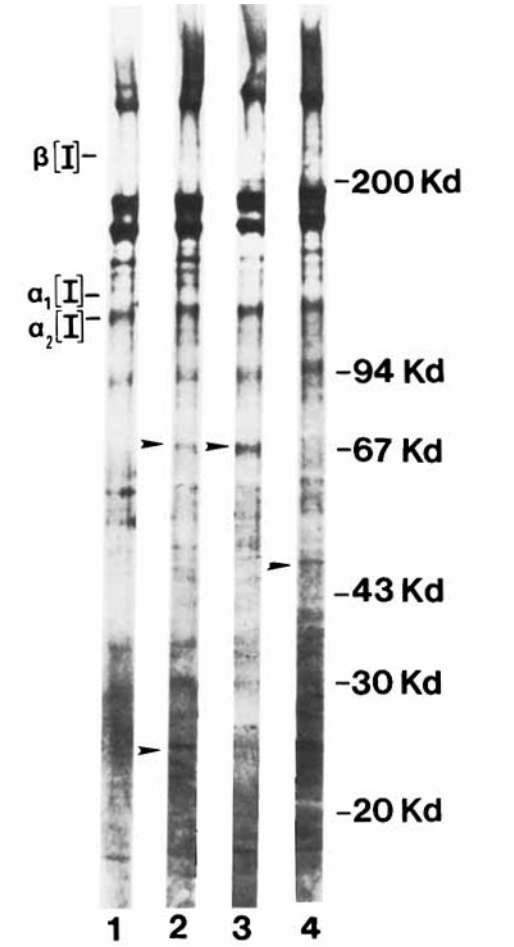

Fig. 2. SDS-PAGE analysis of lens capsule matrix subjected to digestion with various tumor cell-conditioned media. **Lane 1**, undigested material; **lane 2**, lens capsule matrix incubated with −SA-conditioned medium; **lane 3**, lens capsule matrix incubated with +SA–conditioned medium; **lane 4**, lens capsule matrix incubated with RT7-4bL—conditioned medium. Arrowheads indicate the positions of obvious putative cleavage fragments. All lanes are silver stained.

appearance of a new band at 45 kd. Table II summarizes all the changes seen using SDS-PAGE to evaluate tumor cell degradation of lens capsule. Related cell lines, ie, +SA and −SA or RT7-4b and RT7-4bL or B16-F1 and B16-F10 appear to produce similar but not always identical putative cleavage fragments. The patterns are different between cell lines of different histiotypes and are often different depending on whether a cell-associated or concentrated CM digest was carried out. The most invasive of the B16 variants, B16BL6, clearly shows losses of high-molecular-weight material at 400 and 200 kd. Such losses are hard to detect with any of the other cell lines examined. Also, with this cell line, no putative cleavage fragments were found (Table II). These two results may indicate a more complete degradation in the case of B16BL6. Long-term tissue culture-adapted +SA cells and the long-term tissue culture-adapted B16BL6 parental cell population fail to demonstrate degradation, whereas degradative changes are easily found for their recently animal passaged counterparts.

TABLE II. Degradation of Lens Capsule Basement Membrane Assessed Using Silver-Stained SDS-PAGE of Lens Capsule*

Tumor cell line	Origin of cells used in assay	Changes noted in the SDS-PAGE profile of lens capsule basement membrane matrix after digestion: Cell-associated digest	Changes noted in the SDS-PAGE profile of lens capsule basement membrane matrix after digestion: Conditioned medium digest
−SA	Primary cell culture	New band at 17 kd New material at 48 kd	New bands at 23, 34, and 69 kd
+SA	Primary cell culture	New band at 17 kd New material at 48 kd	New bands at 23, 34, and 69 kd
+SA	Tissue culture-adapted[a]	No changes	ND
RT7-4b	Primary cell culture	New material at 28 kd	New bands at 13, 16, and 69 kd
RT7-4bL	Primary cell culture	New material at 28 kd	New bands at 45 kd
B16F1	Primary cell culture	ND[b]	New bands at 13 and 16 kd
B16F10	Primary cell culture	ND	New bands at 13 and 16 kd
B16BL6	Primary cell culture	Loss of material at 400, 200, and 140 kd	Loss of material at 400 and 200 kd
B16BL6 parental population	Tissue culture-adapted	No changes	No changes
B16BL6 population selected for lens capsule invasion	Tissue culture-adapted	Loss of material at 400 kd	Loss of material at 400 and 200 kd

*Lens capsule matrix was incubated with 1.5×10^5 tumor cells for various time intervals from 48 to 72 hr at 37°C as described in Methods. The digested capsules were washed exhaustively in CMF, were lyophilized, and then were subjected to SDS-PAGE as described in Methods. This table presents cumulative data from multiple repeat assays, and changes noted were seen in all repeats.
[a]Tissue culture-adapted = greater than 10 consecutive passages in vitro.
[b]ND, not done.

Available information on lens capsule GAG indicates the presence of a heparin/heparan sulfate species [51–53] but does not further analyze the GAG. Following digestion of lens capsule matrix with pronase, we found only one band on cellulose acetate electrophoresis regardless of the buffer system used. Enzymatic digestion of lens capsule polysaccharide confirmed the absence of hyaluronic acid, chondroitin sulfate, chondroitin, keratan sulfate, and dermatan sulfate (data not shown). Lens capsule GAG was degraded by heparitinase at 30°C and at 42°C [45] but was refractory to heparinase (Fig. 3A, data not shown). These findings confirm the identification of lens capsule GAG as heparan sulfate. Uronic acid was present at 16 μg/mg freeze-dried lens capsule, which extrapolates to 32 μg GAG/mg lens capsule.

GAG from lens capsule incubated with CM from primary cultures and resolved in barium acetate buffer exhibited two alterations. 1) When lens capsules were treated with CM, two migrating GAG bands could be seen (Fig. 3B, large arrowhead). This is in contrast to controls, which always exhibited a single migrating band. 2) GAG from control lens capsules always exhibited more toluidine blue-positive material at the origin than did GAG isolated from CM-treated capsules. This strongly suggests a

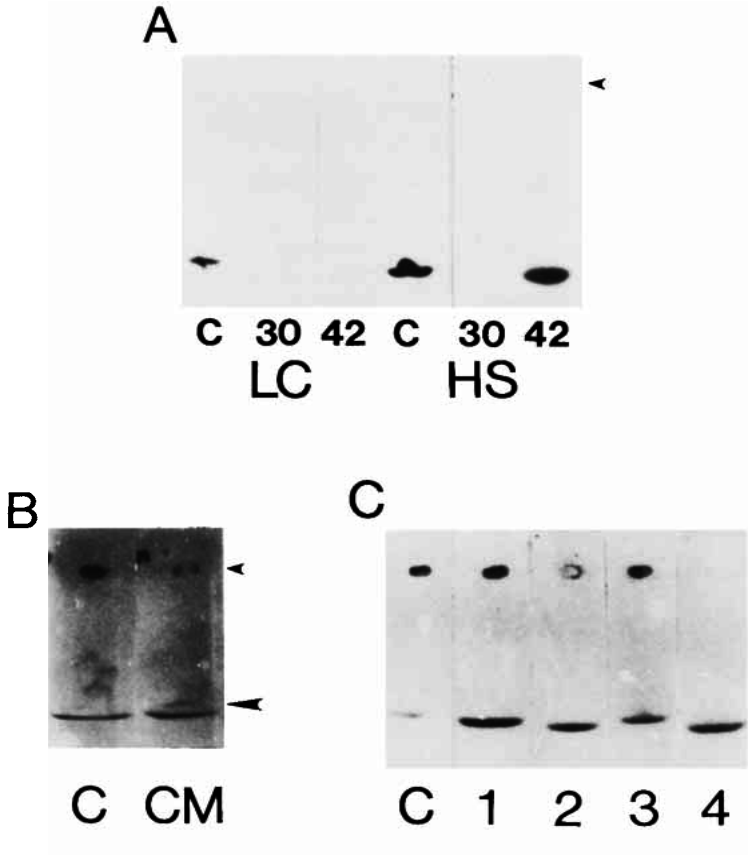

Fig. 3. Cellulose acetate electrophoresis of lens capsule heparan sulfate in 0.1 M barium acetate, pH 8.3, 1 hr at room temperature. **A**) Lens capsule heparan sulfate (LC) and authentic bovine kidney heparan sulfate (HS) treated with purified heparitinase for 24 hr at 30°C (30) and at 42°C (42). C, control. Small arrowhead points to the origin. The anode is at the bottom of the figure. **B**) Lens capsule heparan sulfate treated with conditioned medium (CM) from primary cultures of B16BL6 cells for 96 hr at 37°C. Controls (C) are lens capsule heparan sulfate treated with "mock" CM. Large arrowhead points to the secondary band. **C**) lens capsule GAG treated with B16BL6—conditioned media from long-term tissue culture—adapted cells and resolved in barium acetate buffer. **Lanes 1** and **3**) 0.6% and 0.12% polyhema substrates, respectively; **lanes 2** and **4**) tissue culture plastic substrate.

degradation of origin-bound GAG by CM. Differences in the GAG patterns on electrophoresis between control capsules and CM-treated capsules were also demonstrable when run in HCl (data not shown).

The effect of CM from long-term tissue culture-adapted cells was not as dramatic as the effect of CM from primary cells. When resolved in barium acetate, GAG from control capsules showed densely staining origin-bound material, and the single migrating band was relatively pale (Fig. 3C). Lens capsule GAG following incubation of the capsule with CM from polyhema-grown B16 cells exhibited densely staining origin-bound material and a migratory band of R_f value similar to that from control capsules (Fig. 3C, lanes 1 and 3). Incubation of the capsule with CM from B16 cells grown on tissue culture plastic caused a reduction in origin-bound toluidine blue-positive material as well as an increase in the R_f of the migratory band (Fig. 3C, lanes 2 and 4). These differences were also seen when the GAG was resolved in

calcium acetate buffer (data not shown). With long-term tissue culture-adapted cells, double migratory bands were never observed. The ratios of the peak heights (origin/migratory) calculated from scans of the GAG electrophoreses gave an indication of the amount of degradation observed. The ratios for GAG isolated from control capsules incubated with unconditioned media were 5.8 $\pm$ 3.0 and for GAG isolated from capsules incubated with B16BL6 CM 0.07 $\pm$ 0.03. Ratios for GAG isolated from capsules treated with media conditioned by long-term tissue culture-adapted B16BL6 cells lay between these two values. Authentic heparin was not degraded by CM or mock CM (data not shown), indicating that this GAG is not a substrate for the endoglycosidase released by B16BL6 cells into the medium. However, authentic heparan sulfate isolated from bovine kidney, like the lens capsule heparan sulfate, was degraded by CM to give two new bands on cellulose acetate electrophoresis (data not shown). Also, like the lens capsule GAG, origin-bound material was lost in CM-treated samples.

Western Immunoblot Analysis

Preliminary studies with available polyclonal antibodies to basement membrane components, which were produced using native antigens from nonbovine materials, indicated that such reagents reacted poorly or not at all in Western immunoblots from lens capsule matrix. We therefore prepared monoclonal antibodies to denatured bovine lens capsule. All anticollagen IV antibodies stained identical bands, with the exception that only one efficiently stained the 400 kd band. The two antilaminin antibodies gave identical patterns of staining. Seven bands were stained by the anticollagen IV antibodies at positions on the SDS-PAGE gel equivalent to 400, 186, 167, 150, 143, 123, and 105 kd proteins (Fig. 1B, lane 1). The high-molecular-weight bands at 167, 186, and 400 kd correspond well with bands that we also demonstrated to be sensitive to digestion with clostridial collagenase. Eight bands reacted with the antilaminin monoclonal antibodies. These were at 400, 260, 193, 173, 160, 140, 129, and 117 kd. We used representative anticollagen IV and antilaminin monoclonal antibodies from this set to verify the identity of matrix bands of interest on the SDS-PAGE studies and to clarify results affecting close or overlapping bands.

Matrix Degradation/Invasion and Modulation of Tumor Metastasis

The population of B16BL6 cells previously selected for the ability to invade across the lens capsule [5] was examined to determine its metastatic potential. This cell population exhibited three times the metastatic ability in the lung colony assay compared to the parental population (Table III). A lung colony assay was also carried out to evaluate the antimetastatic activity of the batch of leech salivary gland extract used in these experiments. Treatment of mice shortly before and shortly after IV injection of B16BL6 cells results in a greater than tenfold reduction of tumor lung colonization (Table IV). When this same leech salivary gland extract was included in lens capsule basement membrane digests using B16BL6 CM and the results were analyzed using SDS-PAGE, it was found to reduce degradation of high-molecular-weight capsule material (Fig. 4). Protected bands occurred in the region equivalent to 160 to 400 kd globular proteins. Incubation with leech salivary gland extract alone caused no detectable changes in the high-molecular-weight portion of the gel. The leech salivary gland extract was only partially purified and itself contained proteolytic

TABLE III. Lung-Colonizing Ability of a Subpopulation of B16BL6 Cells That Invaded Across the Full Thickness of the Lens Capsule Compared to the Lung-Colonizing Ability of the Parent Cell Population*

	No. of tumor lung colonies present at autopsy		
Tumor cell line	Mean	(SD)	Range
B16BL6 parental cell line (passage 3)	95	(31)	51–138
B16BL6 population selected for matrix invasion	303	(46)	222–381

*C57BL6 mice were injected IV with 10^4 tumor cells (10 mice per cell line) as described in Methods; 21 days later, the mice were killed and autopsied, and the lung-colonizing ability of each tumor cell population was evaluated. P value for differences between the two data sets = 0.001 (Mann-Whitney two-tailed U test).

TABLE IV. Effect of Leech Salivary Gland Extract on the Lung-Colonizing Ability of B16BL6 Tumor Cells*

	No. of tumor lung colonies present at autopsy		
Regimen	Mean	(SD)	Range
Mice injected with tumor cells plus leech salivary gland extract	17	7	8–27
Mice injected with tumor cells plus saline	243	(56)	182–341

*C57BL/6 mice were injected IV with 5×10^4 tumor cells as described in Methods; 21 days later, the mice were killed and autopsied, and the lung-colonizing ability of the tumor cells in leech salivary gland extract-treated and mock treated mice (10 mice per group) were evaluated. P value for differences between the two data sets = 0.0001 (Mann-Whitney two-tailed U test).

activities, which precluded its evaluation for protection against degradation using ^{125}I release.

DISCUSSION

In an earlier preliminary study, we showed that release of surface-bound ^{125}I from radiolabeled lens capsule could be used to detect basement membrane matrix degradation by several different purified enzymes as well as by metastatic tumor cells [5]. In this study, we have shown by autoradiography, collagenase sensitivity, and Western immunoblots that the great majority of the radiolabel is bound to collagen IV. This contrasts with the basement membrane radiolabeling procedure used by Sheela and Barrett [54], in which only 14–20% of the isotope was bound to collagen IV. Degradation of collagen IV appears to be particularly well correlated with metastatic ability [2]. Thus the radiolabeled lens capsule may represent a particularly appropriate substrate for examining the relationship of basement membrane matrix degradation to metastatic processes. Because of the background ^{125}I release, rather large numbers of tumor cells are required in the long-term cell-associated or CM digests. Similar problems with background release of isotope have been reported in studies on tumor cell degradation of HR9 extracellular matrix [7]. Despite the above-mentioned restrictions, we conclude that the ^{125}I release assay is useful for measuring overall degradative activity of tumor cells towards basement membrane matrix.

Using the ^{125}I release assay, we examined further the degradation of basement membrane matrix by a number of metastatic tumor cell lines. Overall, primary cells

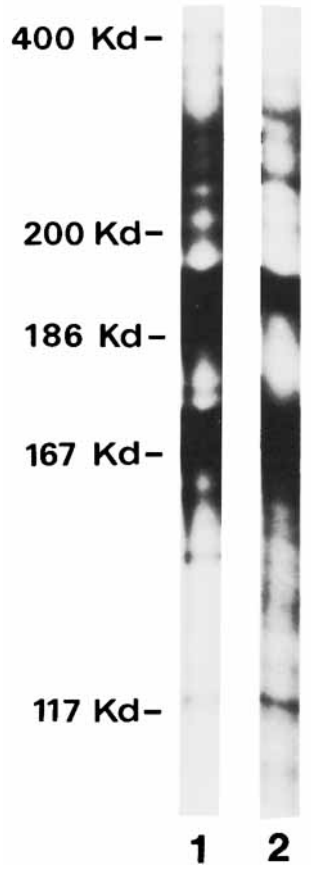

Fig. 4. Effect of the presence of leech salivary gland extract on the digestion of lens capsule matrix by CM prepared from primary B16BL6 cultures. **Lane 1**), matrix digested by B16BL6—conditioned medium in the presence of leech salivary gland extract at 1:10 final dilution; **lane 2**) the same digest without the leech salivary gland extract. The digested matrices were analyzed by SDS-PAGE; all lanes are silver stained.

exhibited greater degradative activity than their long-term tissue culture-adapted counterparts. Indeed, only in the case of the most highly invasive B16 variant tested, B16BL6, was degradation measurable using tissue culture-adapted cells. Two strategies were utilized to avoid interference from serum antiproteases. These were the use of low levels of acid-treated serum [5] and the use of serum-free medium. The different cell lines responded differently to the two culture conditions so that, in a cell-associated digest, B16BL6 cells exhibited more activity in serum-free medium, +SA cells more in medium containing acid-treated serum. This may indicate differences in enzyme:inhibitor relationships, in proenzyme:enzyme relationships, or in the secretory activity of the cells. Surprisingly, we did not find the B16BL6 subpopulation selected for lens capsule invasion to exhibit greater degradative activity than the parental cell population. However, this subpopulation was found to be much more lung-colonizing. We have no direct evidence bearing on these results, although it is possible that the subpopulation differs in nondegradative aspects of cell:matrix interaction such as adhesion, response to chemotactic agents, or deformability. Recently,

Sas et al [21] have reported a lack of positive correlation between metastatic ability and release of substrate matrix components. Our results, as well as those of Sas et al, underscore the inherently complex nature of cellular invasion of extracellular matrices. B16 cells grown in a rounded state on polyhema substrates have been reported to exhibit a greater lung-colonizing ability than their tissue culture plastic-grown counterparts [55]. More recently, this finding has been questioned [56]. Nonetheless, we postulated that such rounded cells might exhibit increased degradative activity, since it has been reported that collagenase activity of similarly rounded synovial cells is increased over that of their "flat" counterparts [57]. Neither protein nor GAG degradation was increased in polyhema-grown B16BL6 cells. The apparent difference between the response in normal synovial cells and these tumor cells may reflect differences in control mechanisms operating in normal and malignant cells. Also, a study of the induction of the collagen IV-degrading metalloproteinase in normal fibroblasts by TPA has indicated that shape change need not be a determining factor in induction of collagen-degrading enzymes [59].

To evaluate changes seen in lens capsule matrix digests resolved on SDS-PAGE, we first partially elucidated the composition of the bands obtained after silver staining. Using combinations of protease sensitivity, Western immunoblots, and protease cleavage patterns, we concluded that collagen IV bands could be identified at positions on the gel equivalent to 400, 186, 167, 150, 143, 123, and 105 kd globular proteins. The 400 kd component probably represents an aggregated matrix form of collagen IV. The 186 and 167 kd bands lie between the positions of the α- and β-chains of collagen I on the gel, and the calculated molecular weights correspond closely to those published for unpepsinized α_1 and α_2-chains of collagen IV [59]. The 150, 143, 123, and 105 kd bands probably represent degradation products from solubilization procedures. Cammarata and Spiro [60] have examined the collagenous components of lens capsule by SDS-PAGE and obtain high-molecular-weight collagen bands similar to ours. Although the 400, 186, and 167 kd bands were all sensitive to digestion with bacterial collagenase, only a very slight reduction of the 167 kd band was noted after plasmin digestion (Fig. 1A). This is consistent with findings by Liotta et al [61], who found only the α_2-collagen IV chain to be slightly sensitive to plasmin and attributed this to cleavage of globular portions of the molecule. Protease digests yielded no information relevent to identification of laminin bands. Western immunoblots, however, identified bands reactive with antilaminin antibodies at 400, 260, 193, 173, 160, 140, 129, and 117 kd. The 400 and 193 kd bands presumably represent the expected 200 and 400 kd bands obtained with isolated laminin on reduced gels [61,62]. The laminin-reactive bands at 260, 160, and 140 kd are not readily visualized using silver staining and must therefore represent a very small proportion indeed of the solubilized matrix. Following thrombin digestion, we failed to detect changes in the laminin bands visualized by silver staining or using immunoblots. Thrombin would be expected to cleave only the 400 kd band and effects at this portion of the gel might be obscured on the silver stained gels by the collagen IV species running at the same position. Although we did not find a reduction in intensity for bands reactive with antilaminin antibodies in the region of the gel corresponding to 200 kd proteins after plasmin digestion, we did note a loss of the 117 kd laminin-reactive band. The relative paucity of laminin degradation that we note after matrix digestion may indicate that laminin is quite protected within the matrix. We have not examined our gels for fibronectin, entactin, or nidogen. Cammarata and colleagues [60,63], however, have demonstated the presence of fibronectin and entactin in the bovine lens capsule.

From our work, it appears that the mouse tumor line B16BL6 has an endoglycosidase(s) capable of cleaving lens capsule GAG. This finding is similar to the findings of Kramer [8] and Nakajima et al [64] showing that variants of the B16 melanoma possess an endoglycosidase that degrades heparan sulfate from bovine endothelial cell extracellular matrix or from bovine lung. Nakajima et al [64] found, however, that commercial heparin, isolated from porcine mucosa, was not degraded by this enzyme, although it did interact with the enzyme. This should not be taken to mean that the tumor enzyme is not capable of degrading all heparins; these GAGs vary in size and sulfation, depending on the source of the GAG (see, for example, Jaques et al [65]. Our results confirm those of Nakajima et al and extend them, demonstrating that the B16BL6 enzyme also degrades lens capsule and bovine kidney heparan sulfates. Our results (Fig. 3) also indicate that there are variations in lens capsule GAG degradation depending on the recent culture history of the cells. Variations in the electrophoretic pattern of GAG from capsules treated with CM from primary cultures, from tissue culture-adapted cells, and from cells grown on polyhema substrates support this contention. These differences in GAG electrophoresis patterns may reflect differences in the types of glycosidic linkages cleaved, ie, linkages in poorly sulfated regions rich in glucuronic acid as opposed to linkages in highly sulfated, iduronic-rich regions.

Putative cleavage fragments were easily demonstrated by SDS-PAGE following purified protease digests or tumor cell digestion of the lens capsule matrix. Changes in the SDS-PAGE profiles were rare for capsules subjected to digestion by long-term tissue culture-adapted cells. Similar changes in the SDS-PAGE profiles were found for related tumor cell lines, although these differed between cell-associated and CM digests, suggesting differences in enzymatic content, activation, release, or inhibitor profiles. Differences in SDS-PAGE profiles were also noted for tumor cell digests by cells of different histiotypes.

The general usefulness of SDS-PAGE analysis of lens capsule matrix degradation is shown by our results incorporating the leech salivary gland extract, in which we were able to show protection of high-molecular-weight matrix components from tumor cell degradation. The leech salivary gland extract is known to contain several diverse protease inhibitors and to interfere with digestion of isolated collagen IV by tumor cell metalloproteinase [33,66], and these inhibitory properties are consistent with our SDS-PAGE results. In this case, the lens capsule assay system provided evidence for an antimetastatic effect correlating with protection from degradation of basement membrane components. On the other hand, use of the lens capsule to analyze basement membrane matrix degradation failed to provide evidence for an increased degradative activity of the highly lung-colonizing B16BL6 subpopulation selected for matrix invasion.

Our electrophoretic analyses of tumor degradation of the lens capsule basement membrane matrix strongly indicate the existence of several pathways of matrix dissolution. This was seen both for GAG and for protein components. We do not know the full implications of this, although it does suggest a potentially highly regulated system responsive perhaps to the different matrices with which metastatic/invasive tumor cells must interact. This type of control is suggested by recent experiments showing that transfected *ras* oncogenes have the ability to lead to both increased tumor cell degradation of collagen IV [22] and increased expression of the cellular laminin receptor [67]. Analysis of multienzyme systems such as cellular

matrix degradation is difficult but can be approached using systems such as that reported in this paper. Inclusion of specific protease inhibitors, for instance, would yield considerable additional information, as would modulation of cellular expression via growth on or in more appropriate biological matrices.

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