Invasion of Reconstituted Basement Membrane Matrix by Metastatic Human Tumor Cells

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ABSTRACT

A gel-like reconstituted basement membrane matrix containing type IV collagen, laminin, entactin, nidogen, and heparan sulfate proteoglycan was used to examine the invasive properties of human HT1080 fibrosarcoma cells. Within several hours after seeding, the tumor cells initiated a random migration, leaving behind channels etched in the surface of the matrix. Eventually the channels became interconnected into a complex network. As the tumor cells proliferated, the channels became filled until the surrounding matrix was gradually dissolved. Cells then migrated outward, forming the typical disorganized cell monolayer normally observed when fibrosarcoma cells are cultured on plastic surfaces. In contrast to the fibrosarcoma cells, normal skin fibroblasts, while able to attach to the matrix, exhibited minimal migration, tracking, and invasion during the same time period. When tumor cells were seeded onto thick layers of matrix, the cells ultimately invaded downward into the matrix, leaving behind open tunnels. At the front of the invading cells, long irregular pseudopodia projected in the direction of movement. Electron microscopy demonstrated these filopodial and lamellopodial projections to directly extend into the surrounding matrix, with focal clearing of the matrix in the immediate vicinity of these invading pseudopodia. Thus, tumor cell invasion of extracellular matrices, including basal lamina, may proceed by the formation of specialized pseudopodia that not only form adhesion contacts with the matrix but also provide an efficient mechanism for the focal hydrolysis of the matrix at the site of directed cell movement.

INTRODUCTION

A characteristic of malignancy is the ability of cancer cells to invade neighboring tissues and vessels and eventually form distant metastases. These processes require penetration of host tissue layers and associated extracellular matrix. Basement membranes, the ubiquitous, specialized extracellular matrix structures found at the interface between epithelia and the adjacent mesenchymal tissue, can act as an important barrier to tumor invasion. Only by a complex process that appears to involve the elaboration of hydrolytic enzymes can invading tumor cells penetrate a basement membrane (1). Thus, it has been demonstrated that highly metastatic tumor cells secrete a collagenase specific for type IV collagen, the major structural component of basement membranes (2).

The exact sequence of events in the invasion of this extremely thin sheet of basement membrane matrix is not clearly understood. Ultrastructural studies have used fixed tissues and consequently cannot fully describe a dynamic event that may be completed within minutes. Nevertheless, current information suggests that tumor cell invasion of basement membranes (and other types of extracellular matrix) proceeds in three distinct stages (1, 3). In the first step, the tumor cell attaches to the basement membrane, a process that appears to be mediated by specific cell surface receptors for matrix glycoproteins such as laminin (1) or type IV collagen (4). Next, the basement membrane is locally hydrolyzed by the elaboration of cell-surface-bound or locally secreted enzymes, such as specific proteinases, collagenases, and glycosidases. Finally, the tumor cell migrates into the digested region of the matrix and attaches to the newly exposed matrix. This cycle of attachment, degradation, and migration is repeated indefinitely and permits extensive invasion into neighboring tissues.

Because of the inherent limitations in studying tumor cell invasion of basement membranes in vivo, we have used a reconstituted matrix that contains a mixture of all known constituents of authentic basement membranes, including type IV collagen, laminin, nidogen, entactin, and heparan sulfate proteoglycan (5–7). Using this model system, it has been possible to describe in detail the morphological progression of the invasion process. We find that tumor cell invasion occurs initially with the generation of unique pseudopodial projections, and that such processes may maximize the cell membrane surface area from which surface-bound or secreted proteinases can induce a rapid but local lysis of the apposed matrix.

MATERIALS AND METHODS

Cell Culture. Human HT1080 cells were originally isolated from a metastasis to the mediastinum (8). The cells were obtained from the American Type Culture Collection and were maintained as monolayer cultures in DMEM containing 10% fetal bovine serum and gentamycin (50 μg/ml). Cells were routinely passaged at preconfluence using 0.25% trypsin-2 mM EDTA.

For invasion assays, the HT1080 cells or normal skin fibroblasts were harvested with trypsin-EDTA and washed twice with medium containing serum. Cells were adjusted to a density of 1–5 x 10^5/ml and seeded onto the preformed matrices in DMEM plus 10% fetal bovine serum and antibiotics. Every 24–48 h the culture medium was replaced.

Low passage dermal fibroblasts were isolated from human foreskin as follows. Briefly, the dermis was cut into 5-mm-square sections and incubated with 0.25% trypsin-2 mM EDTA in calcium- and magnesium-free PBS for 40 min at 37°C. The tissue was rinsed three times with PBS, and the cells were then expressed from the tissue squares with a scalpel blade. The cells were collected by centrifugation and seeded onto fibronectin-coated plastic culture dishes in DMEM containing 10% fetal bovine serum plus antibiotics. After confluence, the monolayers were subcultured with trypsin-EDTA at a ratio of 1:3. For invasion assays, only passages 3–4 were used.

Preparation of the Reconstituted Basement Membrane Matrix. The procedure described by Kleinman et al. (5) was followed. Mice (C57BL/6) were inoculated s.c. on their posterior flanks with EHS tumor fragments. To inhibit collagen cross-linking, animals received 0.1% ethoxylated n-propanol in their water and food pellets as lathrogen. After 3–4 weeks the tumors were harvested, washed with PBS, and minced finely with scissors. The tumor fragments were passed through a 40 mesh screen, then washed by homogenization at 4°C in 3.4 M NaCl, 50 mM Tris-HCl, 10 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.5). After centrifugation at 300 g for 10 min, the supernatant was discarded, and the washing procedure was repeated two more times. The final centrifugation was at 30,000 x g for 10 min. The pellet was used to examine the invasive properties of human HT1080 fibrosarcoma cells.
was resuspended in one volume of freshly prepared 2 m urea, 50 mM Tris-HCl, 10 mM EDTA, N-ethylmaleimide (10 µg/ml), and 1 mM phenylmethylsulfonyl fluoride. The suspension was stirred overnight at 4°C, and the insoluble material was removed by centrifugation at 30,000 × g for 1 h. The supernatant was dialyzed 24–48 h at 4°C against calcium- and magnesium-free PBS containing 0.5 mM EDTA and then frozen in small samples at −20°C.

For reconstitution, the matrix extract was kept on ice at all times and adjusted to 1 mM CaCl₂ and MgCl₂. Type IV collagen, purified from the EHS tumor according to the procedure of Kleinman et al. (9), was added at a final concentration of 10–20 µg/ml. The extract was layered onto culture dishes (1 ml/35-mm-diameter dish) and incubated at 35°C in a humidified chamber for 30 min. For invasion assays, the gelled matrix was then carefully overlaid with culture medium. In certain studies, smaller amounts of matrix extract was layered onto culture dishes to produce a thin coating (~100 µm thick).

SDS-Polyacrylamide Gel Electrophoresis. After gelation, the reconstituted matrix was washed extensively with PBS and solubilized with SDS-solubilization buffer containing 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 10 mM EDTA, and 50 mM Tris-P0₄, pH 6.7, at 100°C for 3 min in a boiling water bath. Samples of solubilized matrix were loaded onto a 4%/10% polyacrylamide gel with a 3% stacking gel as described previously (10). Electrophoresis was performed at 10 mA for 12–17 h, and gels were stained with Coomassie brilliant blue R250.

Immunoblotting. The method of Towbin et al. (11) was followed, with modifications. After electrophoresis, the gel was positioned on a sheet of nitrocellulose (Bio-Rad Laboratories) and subjected to a 250 mA current for 12 h. The nitrocellulose sheet was then processed for detection of laminin, type IV collagen, and entactin by means of specific antisera. First, the nitrocellulose sheets were blocked with a 3% solution of bovine serum albumin in 0.1% Nonidet P-40 non-ionic detergent, 100 mM Tris-HCl, pH 7.5. Dilutions of immune sera (1:500 to 1:2000) in the same buffer were then incubated with the nitrocellulose sheets for 1 h at room temperature. After extensive washing, the sheets were reacted with goat anti-rabbit IgG (Boehringer-Mannheim Biochemicals) conjugated with horseradish peroxidase at a dilution of 1:5000 for 1 h at room temperature. After washing, the sheets were exposed to chromogen substrate (4-chloro-1-naphthol, 0.5 mg/ml) in the presence of hydrogen peroxide (0.015%) until maximum reaction product was obtained, usually 30–45 min. Affinity-purified antibodies to laminin and type IV collagen were generously supplied by Drs. Hynda Kleinman and George Martin (NIH), and antibody to entactin was a gift of Dr. Albert Chung (University of Pennsylvania).

Microscopy. During the progress of the experiments, tumor cell cultures were photographed with a Nikon Diaphot inverted microscope equipped with phase-contrast optics. For embedding in plastic, cultures were fixed with 1% glutaraldehyde in Dulbecco’s PBS at 37°C for 15 min. After dehydration in a series of graded ethanol solutions, the samples were infiltrated with GMA plastic resin according to manufacturer’s instructions. Semi-thin sections 1–3 µm in thickness were cut on a Sorval microtome equipped with glass knives and stained with Touluidine blue.

For electron microscopy, the cultures were fixed with glutaraldehyde as above but were post-fixed with 1% OsO₄ in cacodylate buffer followed by staining en bloc with uranyl acetate. Dehydration was performed with ethanol, and the samples were embedded in Araldite resin. Ultrathin sections were cut with a diamond knife, stained with lead and uranyl salts, and viewed in an Elmiskop 101 electron microscope.

RESULTS

Analysis of the Reconstituted Matrix. When viewed by phase-contrast microscopy the reconstituted EHS matrix appeared nearly transparent but did develop some granularity, especially after incubation for several days at 37°C. As reported previously by Kleinman et al. (5) and Martin et al. (6), examination of the reconstituted EHS matrix by electron microscopy revealed a dense meshwork of fine laminae that frequently formed anastomoses. The interconnecting laminae were frequently interspersed with granular material that stained much more intensely than the filaments and may represent condensed arrays of proteoglycan. In fact, preliminary results using matrices stained with ruthenium red indicate discrete proteoglycan granules similar to those normally found in basal lamina.

Analysis of the reconstituted matrix by SDS-polyacrylamide gel electrophoresis revealed several polypeptides with molecular weights ranging between 100,000 to 450,000 (Fig. 1, lane 1). Along with some extremely high molecular weight material at the top of the overloaded gel, two major bands were present that had molecular weights of approximately 200,000 and 400,000. A minor band was detected at approximately 160,000. Purified laminin and type IV collagen isolated from the EHS tumor were run in parallel on the same gel (Fig. 1, lanes 2 and 3, respectively). The 200,000 and 400,000 subunits of laminin comigrated with the two major bands present in the reconstituted basement membrane matrix. The α-1 and α-2 chains of type IV collagen migrated in the same region of the gel as did the laminin 200,000 subunit. The β-chain, which represents the cross-linked dimers of type IV collagen, migrated with the expected molecular weight of approximately 400,000. Immunoblotting with monospecific antibodies to laminin, type IV collagen, and entactin resulted in detection of all three antigens in the reconstituted matrix (Fig. 1, lanes 4–6). As expected, the anti-laminin antibodies stained the two subunits at 200,000 and 400,000. Antibodies to type IV collagen stained the α-1, α-2, and β-chains at 160,000, 180,000, and 400,000, respectively. Finally, the anti-entactin antibodies stained a principal band at about 160,000 and a minor band at 145,000 which is presumably a minor degradation product of the 160,000 band. Based on the work of Kleinman et al. (5) and Martin et al. (6), nidogen is also expected to be found in the same molecular weight region of the SDS-gel as entactin. Therefore, some of the Coomassie-blue-stained protein in this region may correspond

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to this newly discovered protein (2). By immunostaining and enzyme-linked immunosorbent assay, we have detected the presence of basement membrane-specific heparan sulfate proteoglycan in the matrix, which confirms the finding by Kleinman et al. (5). In summary, the reconstituted EHS matrix contains laminin, type IV collagen, entactin, and proteoglycan as major constituents, and thus the composition of the matrix obtained in our hands appears similar to that studied by Kleinman et al. (5) and Martin et al. (6).

Morphology and Behavior of Tumor Cells Cultured on Matrix Gel. Time-lapse photomicroscopy was used to study the interaction of HT1080 and fibroblasts with the EHS-derived matrix. Single-cell suspensions of HT1080 cells were plated on the surface of preformed matrix, and within 60 min nearly all cells became firmly adherent. Within a few hours, cells had spread and begun to migrate on the matrix substratum. The motile tumor cells exhibited the typical elongated, polarized morphology of locomotive cells, having a ruffled leading edge with an extensive array of lamellipodia and a knoblike tail (Fig. 2E). Within 12 hours, most cells had migrated large distances, ranging between 0.1–0.5 mm. While most cells were actively migratory, some apparently demonstrated little or no migration even after 12 h of incubation (Fig. 2A), suggesting heterogeneity with respect to this property.

As they migrated, the tumor cells formed tracks—depressions or channels—in the matrix substratum (Fig. 2A). Depending on the initial cell density, the tracks eventually (12–24 h) merged into complex interconnected meshworks in the gel (Fig. 2, B–D). The channels were linear but random in direction and approximately as wide as the cell diameter. It was common to observe a single tumor cell migrate in one direction, creating a channel, then suddenly reverse direction and move back along the original migration track. Frequently, solitary cells were seen to diverge from the established paths and form new channels. After 2–4 days the network of channels covered the entire surface of the matrix substratum (Fig. 2G). During this period the tumor cells proliferated, and the daughter cells apparently remained in the migratory tracks of their progenitors (Fig. 2B).

Fig. 2, E and F shows two daughter cells which migrated in opposite directions and, as expected, began to display mirror image morphologies. However, one of the daughter cells subsequently encountered a matrix channel formed by another cell and began to spread along this track (Fig. 2F).

After 2–3 days of culture, cell density increased to the point that most of the space in the migratory tracks was continuously lined with tumor cells (Fig. 2C). At this time, areas of matrix adjacent to the channels began to show evidence of deterioration and took on a granular appearance (Fig. 2C). Immunofluorescent staining with anti-laminin or anti-type IV collagen antibodies revealed that the tumor cells had cleared away much of the matrix in the etched channels (not shown). By 5 days of culture, much of the surrounding matrix had disappeared, and tumor cells occupied most of the available surface area (Fig. 2D). The morphology of the cells had also reverted to the more disorganized form typically displayed by the HT1080 cells when cultured on regular tissue culture dishes. Both tracking and networking also occurred on thick gels.

As a comparison to the HT1080 cells, we examined the behavior of normal low passage cultured human skin fibroblasts when seeded on the EHS matrix. Fibroblast viability approached nearly 100% during the time period studied (up to 2 weeks). In contrast to the fibrosarcoma cells, which exhibited tracking and matrix clearing within several hours, most of the fibroblasts attached but spread poorly on the matrix after 24 h incubation (Fig. 4A). By 3 days cell spreading had occurred, and some cells emitted small feather-like pseudopodia (Fig. 4B). Evidence of matrix distortion by the contraction of cellular processes was occasionally observed. After 10 days, there were colonies of fibroblasts extending some distance from their center, with more prominent distortion of the matrix and some evidence of matrix clearance. Although not common, thin phase-dense tracks similar to those produced by the tumor cells were sometimes observed at this time (not shown).

Tumor Cell Invasion of the Matrix. When HT1080 cells were plated onto thick layers of reconstituted EHS matrix and cultured for several days, they were observed to invade deeply into the underlying matrix gel (Fig. 3, B and C; Fig. 5, A and B). Unusual pseudopodia were observed at the leading tip of the invading cells. Although variable in shape, length, and diameter, these cellular extensions were spearheading the direction of cell movement and seemed to represent the active site of penetration into the matrix. These unusual pseudopodia were not present when the HT1080 cells were cultured on regular tissue-culture plastic surfaces (Fig. 2D). As migration continued into the matrix, empty tunnels were frequently visible (Figs. 3C and 5B). With time, interconnecting tunnels were formed, much like those seen when tumor cells were seeded onto thin layers of matrix, except that the latter were in a three-dimensional array. Eventually, after prolonged incubation these channels expanded into large colonies of cells that in many areas resulted in complete lysis of matrix.

Ultrastructural Studies of Matrix Invasion. The invasion of the matrix was examined by electron microscopy after 7-day incubation of tumor cells initially seeded on the surface of the gel. At low magnification, cords of cells were seen to have infiltrated deeply into the interior of the matrix (Fig. 6). At the surface was a layer of cells that in places was several cells thick. Not infrequently, these cells were adherent to other tumor cells that had invaded the matrix. There were areas, however, where tumor cells were in direct contact with the matrix but showed no evidence of having penetrated it.

On closer examination it was seen that in many cases the invading tumor cells were in direct contact with the matrix and that tumor cell invasion had occurred in roughly linear tracks. The diameter of the channels, which were usually packed with tumor cells, was fairly constant, averaging about 15 μm. Typically, the tumor cells in these channels were elongated and had established complex interdigitating forms of cell-cell adhesion contacts. Empty channels lacking cells were occasionally visible.

Of particular interest in the electron micrographs was the frequent direct invasion of the matrix by tumor cell processes. These pseudopodia were observed penetrating into the matrix at what appeared to be the leading edge of tumor cell invasion. In some cases these projections were seen only at one pole of the cell surface, but in others the projections were found at several sites on a cell's periphery (Figs. 6 and 7). At high magnification these cytoplasmic projections were seen penetrating into the dense matrix, at times to distances of several micrometers. Usually the very tip of a cytoplasmic projection was in direct contact with the matrix, and at areas proximal to the invading tip discrete zones of matrix clearing were visible (Fig. 7). In some cases, a uniform zone of matrix clearance was found to surround the pseudopod. The cytoplasmic interior of these processes contained an easily discernable meshwork of microfilaments. It is unclear if the zones of matrix removal were caused by a change in the diameter of the projection, by extended hydrolysis, or both.
INVASION OF BASEMENT MEMBRANE MATRIX BY TUMOR CELLS

Fig. 2. Morphology and behavior of HT1080 fibrosarcoma cells cultured on the reconstituted basement membrane matrix. Fibrosarcoma cells were plated onto 22-mm-diameter culture dishes coated with a thin layer of matrix (approximately 100 μm thick) and examined at selected time intervals by time-lapse photomicroscopy using phase-contrast (A–F) or darkfield optics (G). In A, after 12 h, the phase-bright tumor cells produced migratory channels in the matrix (phase-dark tracks) (×100). In B, at 24 h, the same field as in A reveals the continued formation of interconnecting channels and an increase in the number of tumor cells (×100). An arrowhead marks the identical area in both A and B. In C, by 72 h, an adjacent area shows a nearly continuous network of tumor cells. The neighboring matrix has become granular, and several solitary tumor cells have begun to infiltrate virgin portions of the matrix substrate (×100). In D, after 5 days of culture, the tumor cells have invaded nearly all of the available surface area, and the matrix appears to be completely dissolved (×100). The tumor cells have taken on their normal morphology, similar to that seen when they are cultured on plastic or glass substrates. E and F show a high magnification of the culture at 12 h after seeding but photographed 1 h apart (×340). In E, a tumor cell has divided, and the two daughter cells have initiated movement in opposite directions. One of the daughter cells (arrowhead) has collided with a migration channel (arrows) previously created by another cell. In F, the tumor cell (arrowhead) has begun to migrate along that channel; the other daughter cell is migrating in an independent pattern. G, a low magnification of the field in B, taken with darkfield optics to demonstrate the extensive network of interconnecting tracks of cells (×35).
Fig. 3. Invasion of the reconstituted basement membrane matrix by the HT1080 fibrosarcoma cells. A, high-power view of the field in Fig. 2A after 12 h of culture (x325). Note the general confinement of tumor cells to the migration tracks and the presence of fine pseudopodia extending from cells at sites of new invasion (arrows). B, invasion of fibrosarcoma cells into thick gels of the reconstituted matrix after 5 days of culture (x1100). The outwardly invading cells exhibit unusual and irregular pseudopodial extensions (arrows) at the site of tumor cell penetration into the matrix. These extensions mark the direction of cell movement. C, solitary tumor cell invading the matrix with the characteristic pseudopodia (arrow) (x1100). Note the presence of a clear channel directly behind the migrating cell.
Fig. 4. Behavior of normal human skin fibroblasts on basement membrane gel. Low passage fibroblasts isolated from foreskin dermis were seeded onto the matrix as described in “Materials and Methods.” Left, after 24 h of incubation, cells are attached but only limited spreading has occurred. There is no evidence of cell migration or tracking (×500). Right, typical morphology of fibroblasts seeded onto tissue culture dishes after 72 h of culture. There is some cell spreading on the matrix, but migration is limited (×500).

Fig. 5. Semi-thin sections of fibrosarcoma cells invading the basement membrane matrix. Tumor cells were plated onto the matrix and incubated for 2 (A) or 7 (B) days. The samples were fixed and embedded in plastic, and 1-μm sections were prepared. At 2 days, tumor cell invasion into the matrix was beginning (×925). By 7 days, the invasion by long cords of cells was extensive (×925). Note the presence of long pseudopodial projections (arrow) and an unoccupied channel (*).
DISCUSSION

The reconstituted EHS basement membrane matrix provides a unique three-dimensional model for the study of tumor cell invasion. Although the exact arrangement and association of the basement membrane-specific macromolecules present in the reconstituted matrix (or in authentic basement membranes) is not completely known, these components appear to associate to form the matrix in a specific pattern with a constant stoichiometry (5, 6). It has been demonstrated previously that soluble laminin and type IV collagen will precipitate when mixed together and that heparan sulfate proteoglycan will increase the amount of laminin that becomes incorporated into the precipitate (12). Other experiments have established the specific interaction between laminin and type IV collagen (5, 13) and between laminin and proteoglycan (14). It has been suggested that these three components interact to form the basic subunit of the basement membrane complex, the “matrisome” (5, 6). Kleinman et al. (5) have shown that the presence of the heparan sulfate proteoglycan was essential for the formation of the reconstituted matrix and that glycosaminoglycans such as heparin or heparan sulfate failed as effective substitutes.

The HT1080 cells demonstrated rapid adhesion and spreading on the surface of the reconstituted basement membrane matrix. This was expected since the HT1080 cells have been shown to attach to laminin-coated surfaces (15). In addition, Timpl et al. (4) reported recently that HT1080 cells will also attach to type IV collagen in the absence of laminin. More importantly, they demonstrated that the cells attached to plastic surfaces coated with the triple-helical portion of type IV collagen but not to surfaces coated with the 7S domain or NC1 globular end of the collagen molecule.

In the past, HT1080 cells have been cultured on various types of extracellular matrices, including subendothelial matrix (3, 16) and aortic smooth muscle cell matrix (17). The unique behavior of the HT1080 cells on the EHS-derived matrix has not been observed on these other types of matrices and seems to be related to the unique molecular composition and/or physical nature of the EHS matrix.

After HT1080 cell attachment to the EHS-derived matrix, rapid cell migration on the matrix was noted. The cell population appeared to be heterogeneous with respect to this property, since not all cells showed rapid cell locomotion. Most cells left distinct trails that were readily visible by phase-contrast microscopy. These trails represented grooves in the matrix that were made during the process of migration and may in fact have been a result of tumor-cell-mediated hydrolysis of the matrix. These depressions seemed not only to record the locomotive activity of a cell’s migratory behavior but probably also indicate the degradative activity of the cell on the surface of the three-dimensional matrix.

Once formed by the tumor cells, the channels were used by neighboring cells for their migration. This was confirmed in other experiments in which the tumor cells were allowed to form the etched tracks in the matrix and were then removed by
Fig. 7. Ultrastructure of the invasion of the matrix by tumor cell pseudopodia, from the same sample as in Fig. 6 but viewed at high magnification. In A, filopodial projections of the fibrosarcoma cell are seen to penetrate deeply into the surrounding matrix. Note distinct areas of matrix dissolution at sites along the surface of these membrane projections (arrows) (×16,000). B, cross-section of a tumor cell with small membrane pseudopods that have invaded into the matrix. The tip of one such projection is visible on the left (arrow). A larger diameter filopodium is seen on the right, with an extensively lysed zone of matrix surrounding the membrane projection. Note the presence of membrane vesicles at the surface of the matrix (arrowheads) and the presence of a membrane invagination on the surface of the pseudopodium (small arrow) (×33,500).
detergent lysis. Subsequently, when fresh tumor cells were seeded onto the matrix, most of these cells were found to migrate preferentially on these preformed tracks. The reason for this preferential movement in a preformed track is not understood, but it may reflect a conditioning of the matrix by the original migrating tumor cells. Alternatively, the availability of a roughly semi-circular groove may provide the tumor cell with a greater surface area of matrix, thus increasing cell adhesion contacts and traction efficiency. In an apparently related phenomenon, Haemmerli and Strauli (18) reported that leukemia cells that had invaded the rabbit mesentery were frequently observed to migrate back and forth on short tracks in the extracellular matrix. Furthermore, neighboring tumor cells accumulated in these tracks. It seems likely that the “networking” by HT1080 cells may require the formation of intimate cell-cell contacts; in this regard, we have seen evidence of extensive zonula adherens-like junctions between HT1080 cells invading the EHS-derived matrix (not shown).

Deep tumor cell invasion into the matrix resembles the surface “networking” occurring in a three-dimensional space. Unlike the tracking on the surface of the gel, which was evident within several hours, invasion downward required several days and was apparently initiated by a minority of the cell population. It appeared that the cells preferred to remain on the top of the matrix and would only initiate movement into the gel when a high cell density was reached. This may indicate that tumor cells must first partially condition the matrix by loosening or digesting matrix components, or alternatively that the lack of available substrata may promote tumor cell infiltration. In fact, when colonies of HT1080 cells on plastic were overlaid with matrix, migration upward into the matrix occurred initially at areas of high cell density. However, if tumor cells were suspended in the EHS extract prior to gelation, tumor cell migration and tunneling through the matrix was noted within the first few hours (not shown).

The behavior of normal skin fibroblasts on the EHS-derived matrix was drastically different from that observed for the tumor fibrosarcoma cells. First, the fibroblast cells, although able to attach to the matrix, did not immediately spread on the matrix and required several days before significant spreading or migration had occurred. In contrast, within 1 h after the HT1080 cells were seeded on the matrix, most cells had spread, and many had initiated cell migration. Second, there was little evidence of the fibroblasts forming the tracking and networking observed with the tumor cells. Third, the fibroblasts frequently caused pulling and distortion of the matrix gel, whereas the fibrosarcoma cells tended to cause clearing of the matrix. The distinct differences in behavior between the two cell types are presumably related to the malignant phenotype of the HT1080 cells. However, further comparisons with other neoplastic and normal cell types should be performed. Recently Hadley et al. (19) reported that Sertoli cells seeded on or within the EHS matrix formed differentiated polarized cords of cells. Although their invasive behavior was not specifically examined, the cells did not appear to extensively migrate on or invade the matrix and remained as tightly associated cell aggregates.

Invading tumor cells showed a leading frontal edge that contained filopodia and lamellipodia. Active extension of these specialized areas of the surface membrane appeared to represent the major mechanism by which the cell gradually penetrated into the matrix. Deeply infiltrating membrane projections were seen in intimate contact with the matrix, while near the base of the microvilli distinct zones of matrix clearing were visible. This suggests that cellular projections such as filopodia or lamellipodia that penetrate into the matrix may do so by the local enzymatic hydrolysis of matrix macromolecules. However, mechanisms other than proteolysis may be important, such as mechanical penetration, internalization of matrix, and non-hydrolytic solubilization of intact macromolecules. The invasive process apparently required the destruction and dissolution of the matrix, since no compression or distortion of the matrix was observed. [In contrast, Volk et al. (20) and Sas et al. (21) have reported that tumor cells can reorganize substrate-bound matrix proteins into cables.] Whatever the mechanism, it seems likely that as matrix clearance proceeds, the tumor cell moves into the void, and this cyclic process is continuously repeated (1, 3, 27). While matrix clearing is evident at the site of pseudopod invasion, it is also possible that some matrix clearing occurs at other cell surfaces free of membrane projections. This is suggested by the almost carved and relatively smooth surface of matrix surrounding the invading tumor cells (Fig. 6).

Various studies have suggested that cell pseudopodial projections may function in invasion of basal lamina and various connective tissue matrices by both normal and neoplastic cell types (22–28). Normally invasive cells such as polymorphonuclear leukocytes extrude pseudopodia as the cell penetrates amnion basal laminae (22). Osteoclasts dissolve and invade bone matrix at a specialized ruffled edge that consists of highly motile collections of membrane pseudopodial projections. Trophoblasts that invade the endometrium and underlying uterine connective tissue form extensive pseudopodial projections. During angiogenesis, capillary endothelial cells initiate new vessel formation by penetrating their basal lamina with microvillar projections, and the capillary sprouts continue their infiltration of the surrounding connective tissue stroma by a leading edge of cellular pseudopodial projections (23). B16 melanoma cells invade extracellular matrices with an extensive array of pseudopodial projections (3, 16). The initial penetration of basal lamina and underlying connective tissue by tumor cells has been shown to occur in the area of the cytoplasmic projections of those cells that are in direct contact with these substrates (26). For the efficient dissolution of the extracellular matrix, direct physical contact of the tumor cell surface with the matrix substratum seems to be required (16, 17).

Tumor cells have been shown to secrete soluble proteinases or to possess cell-surface-bound proteinases (1, 2, 16, 17), and it is reasonable to conclude that the more plasma membrane surface area available, the greater will be the cells' ability to hydrolyze and penetrate extracellular matrices. In addition, there is some evidence that certain proteinases, such as plasminogen activator, may be present on cellular extensions (29). Chen et al. (30) showed recently that virus-transformed fibroblasts degrade substratum-attached fibronectin at areas of cell contact. Pseudopodia containing a focal concentration and polarized arrangement of degradative enzymes would not only be useful in the penetration of extracellular matrices or even host tissue barriers, but in addition, these surface appendages would also provide the needed anchoring units for pulling the invading cell forward.

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