Localization of Collagenase at the Basal Plasma Membrane of a Human Pancreatic Carcinoma Cell Line

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ABSTRACT

We have recently presented biochemical evidence for collagen and gelatin degrading activities associated with plasma membranes of various human cancer cell lines. In this report we describe the localization of interstitial collagenase at the basal plasma membrane of human pancreatic cancer cell line RWP-I, using immunofluorescence and ultrastructural immunogold labeling techniques. Collagenase was expressed on the extracellular face of the plasma membrane. Furthermore, the immunogold labeling was concentrated on the long, finger-like microvillous projections typically seen on the basal cell surface, while the short, brush-like projections characteristic of the apical cell surface were unlabeled. When the cytoplasmic face of the membrane was made accessible, the number of reactive sites increased markedly, indicating a high concentration of enzyme at the inner surface of the plasma membrane.

When plasma membrane fractions of RWP-I cells were prepared by differential centrifugation, high salt washes virtually failed to extract collagenase activity from the membrane, while detergent extraction with n-octyl glucoside, a detergent used in the purification of integral membrane proteins, yielded soluble collagenase activity. When detergent extracted membrane fractions were passed over an anticollagenase immunosorbent column, collagenase was specifically bound, as demonstrated by the TC6 and TC8 degradation of type I collagen by the bound material. Gelatinolytic activity did not bind to the column. Furthermore, immunoprecipitation of 125I-labeled detergent extracts of tumor membranes yielded a single Mr 55,000 band consistent with the zymogen form of the connective tissue collagenase. These morphological and biochemical findings suggest that collagenase is a tightly associated component of the basal plasma membrane, where it occupies a strategic location for directional proteolysis during cell migration and invasion.

INTRODUCTION

Dynamic interactions between cells and their extracellular milieu play a crucial role in neoplastic processes of tumor invasion and metastasis, as well as in normal phenomena such as tissue remodeling during embryogenesis and inflammatory responses. Basement membrane, extracellular matrix, and connective tissue fibrillar proteins present a natural barrier to the migration of cancer cells. Much of our insight into the proteolytic degradation of the extracellular components during this process comes from in vitro studies of malignant cells, which serve as a readily available model for cell migration in general. A spectrum of proteinases, such as interstitial collagenase (1, 2), type IV collagen degrading enzymes (3), gelatinase, serine proteinases (4), cathepsin B (5), and plasminogen activator (6) have been implicated in cancer invasion (7, 8).

Interstitial type collagen is the major structural protein of all tissues. Tumor cells can penetrate dense stroma only if a proteolytic function associated with the plasma membrane.

Collagenase, synthesized by normal connective tissue cells in culture, has been purified and its action on collagen types I, II, III (12, 13) and X (14) has been characterized. The enzyme has been shown to be a prototype of a secretory protein. It is synthesized as preprocollagenase, processed to a proenzyme within microsomal membranes and immediately secreted without intracellular storage (15).

In contrast, we described a metalloproteinase activity in the total cell extract of highly metastatic mouse melanoma cells that digests collagen type I and type IV and gelatin (16). Furthermore, the collagenolytic activity of human small cell carcinoma cells was highly enriched in the plasma membrane fraction (17). These observations suggest the existence of cell surface bound collagenase in human cancer cells and propose a proteolytic function associated with the plasma membrane. Their sanctuary status in the plasma membrane places them optimally for the effective destruction of substratum in a controlled, directional way. This also allows the local concentration of the enzyme to be maintained and may prevent it from binding to proteinase inhibitors present in the extracellular milieu (18).

In this study we have used an ultrastructural and immunological approach to identify interstitial collagenase in RWP-I cells. Tumor collagenase was predominantly localized at basal type microvilli of plasma membrane where cells contact the substratum, suggesting that directional collagenolysis occurs at the cell-stroma interface.

MATERIALS AND METHODS

Cell Line

The human RWP-I pancreatic cancer cell line, kindly provided by Dr. D. L. Dexter (Department of Medicine, Roger Williams General Hospital, Providence, RI), has previously been characterized (19). For this study only cell cultures were used. Aliquots of these cells were injected into athymic mice and gave rise to large s.c. tumors. RPMI 1640 (GIBCO, Grand Island, NY) and 5–10% fetal calf serum (Flow Laboratories, Walkersville, MD) in 95% air and 5% CO2 at 37°C was used.

Antisera

Human procolllagenase from the culture medium of rheumatoid synovial cells was affinity purified by exploiting cross-reactivity with anti-rabbit collagenase (20). The anti-rabbit collagenase antibody was directed against purified rabbit synovial fibroblast collagenase and has been described previously (20). Its monospecificity was established by double immunodiffusion, immunoprecipitation of 125I-labeled collagenase, and immunoblotting analysis. Cross-reactivity with human rheumatoid synovial procollagenase has been shown (20). The human antigen extracted on an anti-rabbit collagenase affinity column ran as a doublet with molecular weights of 52,000 and 55,000 on SDS-PAGE.

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[2] The abbreviations used are SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; anti-pColl, sheep anti-(human rheumatoid synovial procollagenase) IgG; RAS, unconjugated rabbit anti-(sheep IgG) IgG; RWP-I, human pancreatic cancer cell line; MMP-3, matrix metalloproteinase-3, synonymous withstromelysin; APMA, p-aminophenylmercuric acetate; PBS, phosphate-buffered saline.
The eluted antigen showed activity in a standard collagenase assay after activation by 4-aminophenyl mercuric acetate. Antiserum was produced by immunizing a sheep. The IgG fraction (referred to as anti-pColl) was isolated at a final concentration of 18 mg/ml. Anti-pColl recognizes interstitial type human collagenase and procollagenase, but not gelatinase and MMP-3/stromelysin as confirmed by immunoblot analysis. The working dilution was 1:60 (300 μg/ml).

**Immunofluorescence Microscopy**

Immunolocalization of collagenase at the light microscopic level was carried out at room temperature. RWP-I cells were grown on coverslips to semiconfluence. The adherent cells were washed in PBS and fixed in 2% formaldehyde for 15 min. Others were washed in 150 mM NaCl, 50 mM Tris, 5 mM CaCl2, pH 7.5. No difference in staining was noted between these two groups. Half of the coverslips were permeabilized in 0.25% Triton X-100/PBS for 5 min and the other half were kept in PBS only. The specimens were then incubated with primary antisera (diluted 1:60 in PBS) for 60 min. After thorough washes in PBS, fluorescein isothiocyanate-conjugated rabbit anti-sheep IgG (H + L) [IgG (Cappel, West Chester, PA) was applied (diluted 1:150 in PBS) for 30 min. Preadsorbed anti-pColl as well as normal sheep serum were used as controls. The preparations were mounted with Aquamount, viewed with a Zeiss Axiosmat microscope, and photographed with a Kodak TMAX film.

**Immunogold Labeling of Collagenase Sites at Ultrastructural Level**

Colloidal gold particles (10 nm) coupled to rabbit anti-sheep IgG (H + L) IgG at 40 μg/ml protein concentration was purchased from EY Laboratories, Inc., San Mateo, CA. The specificity of the reagent was shown by incubating a nitrocel lulose strip impregnated with 1 μg sheep IgG with 5 μl gold probe (20 μg/ml); bovine serum albumin (diluted 1:60 in PBS) for 60 min. Preadsorbed anti-pColl as well as normal sheep serum were used as controls. The preparations were mounted with Aquamount, viewed with a Zeiss Axiosmat microscope, and photographed with Kodak TMAX film.

**Distribution Analysis of Gold Grains on Plasma Membranes**

The distribution of gold grains on the surface of 10 cells was assessed by image analysis in order to determine whether collagenase sites on the plasma membrane are concentrated on cell projections. The cells were selected at random and photographed at ×4000 which did not allow an evaluation of the presence or location of gold particles. All cells included a nuclear profile. All gold particles on the circumference of each cell were counted and grouped into sites on cell projections versus sites on the cell body. Small aggregates of gold grains were scored as one site and grains had to be directly attached to the membrane to be counted. Cell processes which were not in continuity with the cell body were considered part of the cell if the profiles were no further from the cell than the length of attached processes in the same plane of section. To normalize the number of grains in each categories, the parameters were measured differentially with an electron image analysis device (Zeiss ZIDAC). Scanning electron micrographs of similar cells were taken to assess the 3-dimensional structure of the projections. For this, cells were grown on coverslips, fixed in glutaraldehyde, critically point dried, and examined with an AMR scanning electron microscope.

**Preparation of Plasma Membrane Associated Collagenase**

Postnuclear cell membranes were isolated from RWP-I cells using nitrogen cavitation followed by differential centrifugation as previously described in Ref. 16 and then subjected to sequential extraction. Extrinsic membrane proteins were extracted by 2 mM KCl treatment (16). The remaining intrinsic membrane proteins were solubilized by treatment of total Polytron homogenate (50,000 × g pellet with 2.5% sodium deoxycholate on ice) followed by a second 100,000 × g centrifugation. The supernatant was divided into an upper half (lipid phase) and a lower half (protein phase). The pellet was treated with 1% n-octyl glucoside (Sigma Chemical Co., St. Louis, MO) overnight at 4°C and subjected to a last 100,000 × g centrifugation. The extracted proteins from each of the four supernatants were dialyzed against 25 mM cacodylate/5 mM CaCl2/0.05% Brij 35 (Sigma), pH 7.2. Samples undergoing immunoprecipitation were concentrated by the addition of solid ammonium sulfate to 60% saturation and dialyzed against the above buffer. Aliquots of pellets and supernatants were activated with trypsin, then inactivated with soybean trypsin inhibitor and assayed for 3H-collagenolysis using 2 μg of labeled substrate at 27°C for 18 h as described in Ref. 17. RWP-I conditioned medium was collected after 48 h from serum-free cell cultures containing 1 × 10^7 cells/flask and spun at 770 × g for 10 min followed by 50,000 × g for 1 h. The proteins in the supernatant were precipitated with solid ammonium sulfate to 60% saturation, then resuspended, and dialyzed against the above buffer. Aliquots of pellets and supernatants were activated with trypsin, then inactivated with soybean trypsin inhibitor, and assayed for collagen degradation, using 2 μg of 3H-labeled collagen at 27°C for 18 h as described in Ref. 17. The protein concentration of each extract and of conditioned medium was determined by the fluoroscein method (21).

**Immunoblotting**

Samples (100 μl) of RWP-I whole cell homogenate and sequential membrane extracts in various PBS dilutions were applied to nitrocel lulose filters in a slot blot apparatus (Bio-Rad Bio-Dot SF) and the membrane was washed several times with PBS; 100 μl nonimmune rabbit IgG and PBS served as control. The nitrocellulose filters were soaked in reconstituted 5% (w/v) nonfat dried milk for 40 min. They were incubated with anti-pColl diluted 1:125 in PBS overnight at 4°C. After extensive washing, the filters were reacted with donkey anti-sheep IgG IgG (2.9 μg/ml) (referred to as RAS) (Chemicon, El Segundo, CA) for 18 h at 4°C (see controls). A final wash in 6 changes of PBS followed. Pellets of stained specimens appeared pink while unstained specimens appeared tan. The pellets were fixed in 3% glutaraldehyde/0.2 M sodium cacodylate buffer and processed for electron microscopy. Ultrathin sections were cut, stained with lead citrate and uranyl acetate, and examined under a Zeiss EM 10 microscopy.

**Immunoprecipitation**

Secreted procollagenase from human rheumatoid synovial fibroblast cultures was purified to a final concentration of 750 μg/ml using an anti-(rabbit collagenase) immunoadsorbant column as described in Ref. 15. Anti-pColl (90 μg) was incubated with 53 μg antigen in 70 nL and diluted in PBS to a final concentration of 0.044 unit (1 unit degrades 1 μg of collagen/min) of total collagenase activity were applied to the antigen.

**Samples**

Samples (100 μl) of RWP-I whole cell homogenate and sequential membrane extracts in various PBS dilutions were applied to nitrocel lulose filters in a slot blot apparatus (Bio-Rad Bio-Dot SF) and the membrane was washed several times with PBS; 100 μl nonimmune rabbit IgG and PBS served as control. The nitrocellulose filters were soaked in reconstituted 5% (w/v) nonfat dried milk for 40 min. They were incubated with anti-pColl diluted 1:125 in PBS overnight at 4°C. After extensive washing, the filters were reacted with donkey anti-sheep IgG IgG-alkaline phosphatase conjugate (Sigma) 1:1000 for 1 h at 37°C and the blot was developed in 0.1 M Tris-HCl, pH 9.5/0.1 M NaCl/5 mM MgCl2 containing nitro blue tetrazolium (Sigma; 0.33 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma; 0.165 mg/ml). The reaction was terminated by rinsing it in water.

**Immunonoautoradiography of Collagenase**

RWP-I membrane extracts (260 μl) containing 0.044 unit (1 unit degrades 1 μg of collagen/min) of total collagenase activity were applied
to a column of Affi-Gel 10 (Bio-Rad, Richmond, VA) coupled with anti-pColl IgG (1.5 ml), which was equilibrated with 50 mM Tris-HCl/0.15 M NaCl/10 mM Ca\(^{2+}/0.05\%\) Brij 35/0.02% NaN\(_3\), pH 7.5. Specifically bound procollagenase was eluted with the above buffer containing 6 M urea and fractions of 200 μl were collected. The fractions were then applied to spin columns to remove urea as described in Ref. 22. Each fraction was assayed for the degradation of \(^{14}\)C-acetylated type I collagen and \(^{14}\)C-acetylated gelatin introducing 3200 cpm per tube (22) in the presence of 1 mM APMA and 0.04 ng of MMP-3/stromelysin for 24 h at 24°C and 37°C, respectively. MMP-3 is required for the maximal activation of procollagenase (23, 24).

For the analysis of type I collagen degradation, 30 μl of the peak collagenase fraction were incubated with 15 μg type I collagen in the presence of 1 mM APMA/0.04 ng MMP-3 in a total volume of 50 μl at 28°C for 48 h as described above and then analyzed on SDS-PAGE (25).

**Immunoprecipitation of Collagenase**

One hundred μl of concentrated n-octyl glucoside membrane extracts (104 μg) were radioiodinated by using Iodo-Gen (1,3,4,6-tetrachloro-3a,6a-diphenylglycouril) (26) and free \(^{125}\)I ions were removed by spin columns (22). The sample was then subjected to immunoprecipitation by using 5 μl of anti-pColl IgG in a total volume of 400 μl according to the method of Nagase et al. (15). Antigen-antibody complexes were dissociated by boiling in SDS-PAGE sample buffer and the antigen was analyzed by SDS-PAGE and subsequent autoradiography.

**RESULTS**

**Immunolocalization of Membrane Bound Tumor Collagenase.** To characterize the extent and overall distribution of collagenase, RWP-I monolayer cultures were studied with an immunofluorescence technique by using anti-pColl. A discrete punctate fluorescence was detected on approximately 50% of the cells, indicating a moderate level of expression of collagenase on the extracellular surface of the plasma membrane (Fig. 1b). In contrast, when cells were permeabilized with Triton X-100 prior to incubation with the immunoprobe, the staining intensity increased markedly (Fig. 1c). The staining pattern was predominantly diffuse with some focal densities. This increase in staining intensity was due to intracytoplasmic sites of enzyme as well as sites on the cell surface. Preabsorption of the antibody with the affinity purified immunogen (Fig. 1a) as well as replacement of anti-pColl by normal sheep serum (data not shown) completely abolished staining of permeabilized and nonpermeabilized cells.

**Polarized Localization of Collagenase in RWP-I Cells.** At the electron microscopic level, collagenase immunoreactivity was detected at the cell surface in a nonrandom distribution. Although there was considerable variation between individual cells, approximately 90% of all cell cross-sections were labeled with gold grains. The short plump microvilli on the apical surface were virtually devoid of gold labeling (Fig. 2b), while the long, thin finger-like processes, which are typical for the cell base, showed a moderate degree of collagenase reactivity (Figs. 2a and 3a). The number of reactive sites increased greatly when both the extracellular and the cytoplasmic face of the plasma membrane became accessible to the immunoprobe (Fig. 3). The detached fragments of plasma membrane were heavily labeled with gold grains in an irregular fashion. These fragments also showed a distinct morphology. They usually formed long convoluted strings with many small uniform ruffles decorating the main thread (Fig. 3b and control Fig. 4). Labeling was associated with these structures. To show that these peculiar string configurations were plasma membrane rather than rough endoplasmic reticulum or nuclear membrane, we have the following evidence. (a) Some cells showed very early stages of plasma membrane detachment, with loops peeling off (Fig. 4a). Their morphology was identical to the detached membrane fractions (Fig. 3b); (b) previously, we demonstrated that the 100,000 x g pellet of RWP-1 cell homogenates contains a membrane band which is highly enriched in collagenase activity and exhibits the same ruffled profile as seen in this study (see Ref. 27, Fig. 2, lower right). Other cell organelles such as nuclei, mitochondria, and various vesicular structures showed only occasional sparse labeling. No dense core granules which might contain collagenase were noted.

Three types of controls demonstrated the specificity of the immunolabeling at the electron microscopic level. Preabsorption of anti-pColl with excess of purified human synovial procollagenase (Fig. 4a, b) and substitution of anti-pColl with normal sheep serum (data not shown) completely abolished immunogold labeling. Preincubation of the anti-pColl treated specimens with unconjugated secondary antibody (RAS) blocked the subsequent immunogold labeling virtually completely (Fig. 4c). Immunolabeling of intact cells was abolished as well (data not shown).

**Quantitative Distribution Analysis of Collagenase Sites on Intact Plasma Membrane.** A comparison of the number of particles per unit area of membrane on cell body versus projections gave a measure of the differential distribution on the
COLLAGENASE IN HUMAN PANCREATIC CARCINOMA

(a) Basal plasma membrane projections, which are long and finger-like, are decorated with anti-pColl immunogold complexes at the extracellular face. (b) Apical projections, which are short and brush-like, are unlabeled; × 24,000.

Fig. 3. (a) Membrane on cell projections (small arrow) and on the cell body (big arrow) are immunolabeled with anti-pColl. (b) Detached plasma membrane fragments are heavily labeled with anti-pColl immunogold complexes, indicating a much larger number of enzyme sites at the cytoplasmic face. Note the irregular distribution and the formation of ruffles (arrow) as shown in Fig. 4; × 24,000.

intact cell surface (see Fig. 3a). We assume that a single gold particle or an aggregate of gold particles attached to the membrane represents one collagenase site. Each pair of numbers in Table 1 was generated from the same cross-section of a cell. The total perimeter of cell body versus projecting membrane profiles on each cell circumference was measured and the data normalized. Table 1 shows that the plasma membrane of projections exhibited twice as many collagenase sites than smooth areas.

Extraction of Collagenolytic Activity from Detergent Treated Plasma Membranes. Trypsin activated RWP-I whole cell homogenate, postnuclear preparations, and plasma membrane...
enriched fractions degraded 10, 40, and 1560 ng of type I \(^{[3]H}\) collagen/mg protein/18-h incubation, respectively. Treatment with buffered 150 mM NaCl, pH 7.5, followed by 2 M KCl for 30 min resulted in no significant loss of membrane collagenolytic activity. In contrast, treatment with \(n\)-octyl glucoside resulted in extraction of 96% of the final collagenolytic activity (1115 ng substrate degraded/mg protein/18 h). RWP-I conditioned medium also contained secreted collagenolytic activity; 48-h conditioned medium degraded 41 ng of \(^{[3]H}\)collagen/mg protein/18-h incubation following trypsin activation. Ammonium sulfate precipitated (0–60% saturated) conditioned medium digested 244 ng of \(^{[3]H}\)collagen/mg protein/18 h following trypsin activation.

Immunoblotting of Detergent Extracted Plasma Membrane Fractions. The distribution of immunoreactive collagenase was followed through each step of the membrane extraction sequence. Aliquots of whole cell homogenate contained highly concentrated collagenase-like material which could only be extracted by the final \(n\)-octyl glucoside step (Fig. 5). However, high salt extraction alone, which removes loosely associated proteins, or butyl alcohol extraction, which removes certain intrinsic membrane proteins yielded very little immunoreactive material. These results suggest that collagenase is a tightly associated membrane protein.

Selective Binding of Membrane Collagenase to an Immunoaffinity Column. Detergent extracted RWP-I membrane fractions, which selectively bound to an anti-pColl affinity column, were examined for collagenolytic and gelatinolytic activity (Fig. 6). The degradation of \(^{3}H\)-type I collagen and \(^{[3]H}\)gelatin after APMA and stromelysin activation at 27°C and 37°C, respectively, across the column showed that collagenase activity was eluted from the affinity column with 6 M urea, while gelatinase
did not bind to the column and was recovered in the flow through fraction. The elution profile of collagenase seems to be composed of two individual peaks, probably due to antibody populations of lower and higher affinity. When peak fractions of the eluate were incubated with type I collagen at 27°C and then analyzed by SDS-PAGE, the TC* and the TCb products were formed. No other degradation products were found (Fig. 6, inset). These products are considered specific for collagenase. Conversely, the peak of the gelatinase fraction produced multiple smaller fragments after incubation with gelatin at 37°C (data not shown).

Immunoprecipitation of Collagenase. To identify the species and determine the molecular weight of the membrane bound collagenase, the detergent extract was radioiodinated and precipitated with anti-pColl. As shown in Fig. 7, a single species with a molecular weight of about 55,000 is recognized (Fig. 7, Lane 2). The membrane bound collagenase required activation to produce collagenolysis, indicating that the enzyme is present in a latent form (data not shown). The M, 55,000 species is consistent in molecular weight with the procollagenase of connective tissue (15, 28). Other metalloproteinases, which have partial homology with collagenase, are not recognized by our antibody.

**DISCUSSION**

In the present study we have demonstrated tumor collagenase at the plasma membrane. The enzyme seems to be primarily...
present in the zymogen form and is activated with organonemercials and MMP-3 in vitro. In vivo, other matrix proteinases, such as the plasminogen activator/plasmin system could function as activators as well as regulators of membrane associated collagenase (29). Also, based on sequence homologies at the site of activation between all members of the matrix metalloproteinase family known so far, autoactivation and mutal activation have recently been shown to be important mechanisms for these enzymes (23, 24, 30). The collagenase molecules are concentrated at the cytoplasmic face of the membrane with some degree of expression at the extracellular surface. To our knowledge, this is the first ultrastructural immunolocalization of a metalloproteinase. Our studies were possible because tumor collagenase cross-reacts with an immunoprobe raised against rheumatoid synovial fibroblast collagenase. Immunoprecipitation indicates that the tumor collagenase is a M, 55,000 molecule consistent with the molecular weight of the secreted connective tissue procollagenase. Furthermore, the immunological cross-reactivity as well as the pattern of substrate degradation suggest a high degree of conservation in the primary structures between the two molecules, and even possible identity. The fact that tumor collagenase was extractable from plasma membrane with detergent, but not with high salt washes, is evidence that the enzyme is tightly associated with the membrane and not just loosely adsorbed. When the activity of membrane associated collagenase is compared with secreted collagenase in conditioned medium, it appears that the cell membrane represents a major portion of enzymatic activity in RWP-I cells. However, differential half-life, activation status, and local inhibitor concentration ultimately will determine the activity in vivo.

Our morphological data can reflect a number of possible associations of enzyme and plasma membrane; e.g., (a) the enzyme may be directly anchored within the lipid bilayer of the membrane. Phosphatidyl inositol has been described as a covalently linked lipid anchor for a number of membrane associated enzymes (31). (b) The enzyme may undergo transmembraneous secretion but be partially or completely captured by a high affinity membrane bound collagenase receptor. This type of pathway has been shown to apply to the urokinase-type plasminogen activator in normal and malignant cells (see Ref. 32 for a brief review). The failure to extract collagenase activity from plasma membrane preparations with high salt solutions makes this possibility unlikely but does not entirely exclude it. (c) Collagenase may be transiently accumulated at the inner surface of the plasma membrane and subsequently be released as soluble enzyme via an exocytosis-like secretory pathway. The ultrastructural image we observe could then be due to enzyme in transit through the membrane. (d) The expression of collagenase on the cell surface may be a transient phenomenon that precedes the shedding of portions of the plasma membrane as vesicles. Such a pathway has been described for cathepsin B-like cysteine proteinase (33). Also, electron microscopic studies on invasive carcinoma in vivo suggested destruction of connective tissue by membrane vesicles in the immediate vicinity of the invading epithelial cells (34, 35). Although we favor the possibility of a genuine collagenolytic activity of plasma membrane at points of contact between cell and substratum, our data do not definitively distinguish among the above alternatives. A combination of mechanisms is also conceivable.

The pathway of collagenase in normal mesenchymal cells has been well described and appears to be a simple secretory one, but it is not known whether there is concentration at the cell surface. In pulse chase experiments of human skin and rabbit synovial fibroblast cultures, collagenase appears rapidly in the culture medium within 35 min after synthesis (15, 28). Immunofluorescent collagenase sites are found abundantly within the connective tissue matrix, whereas the cytoplasm is relatively free of enzyme (36–38). These findings are consistent with the secretion and diffusion of soluble enzyme into the matrix.

The notion of proteolytic activity at points of contact has recently become more important. Urokinase-type plasminogen activator has been localized to points of local contact in normal and sarcoma cells (39). Fibrinolytic-type plasminogen activator is confined to sites of contact involving the activation of an integral membrane proteinase by pp60weets which is present at the inner surface of the plasma membrane (40). Our findings of selective distribution of collagenase at basal type microvillous projections suggests directional proteolysis via contact sites at the cell-stroma interface. Electron microscopic studies of reconstituted basement membranes have demonstrated that tumor cell invasion of extracellular matrix proceeds by the formation of specialized pseudopodia that form adhesion contacts and maximize local hydrolysis by membrane bound proteinases at the interfacing surface area (39). Various human breast cancer cell lines required direct contact for the degradation of endothelial basement membrane (41). Clearing of substrate with release of degradation products of basement membrane type IV collagen, laminin and fibronectin occurs only along the path of tumor cells and beneath cellular processes, suggesting that it is due to cell surface proteinases rather than secreted enzymes (42–44).

Collagenase localization studies in tumor cells have been limited until now. Only light microscopic studies using immunofluorescence have been performed. In head and neck tumors (45, 46) and in melanoma (38), the intercellular stroma exhibits bright immunofluorescence when stained for collagenase. In permeabilized cells, there is a sharp border between cytoplasm and matrix, thus outlining the silhouette of the tumor cells, while more than 90% of the tumor cells themselves are negative. This is consistent with the existence of collagenase domains on the cell surface, particularly in view of the limited resolution of immunofluorescence. Our high resolution ultrastructural study lends further support to this concept.

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