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Preferential digestion of basement membrane collagen by an enzyme derived from a metastatic murine tumor

(collagenase/type IV collagen/basal lamina/cancer invasion/metastases)

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ABSTRACT **The specificity of human skin collagenase and of an enzyme from an invasive tumor were studied by using types I, II, III, IV, and V (AB) collagen as substrates. Human skin collagenase degraded types I, II, and III collagen, producing the characteristic ¾ and ¼ cleavage products, but failed to degrade type IV or V collagen. Collagenase prepared from the invasive tumor showed maximal activity after trypsin treatment. The tumor enzyme degraded type IV (basement membrane) collagen, producing fragments consistent with a single cleavage site but did not attack types I, II, III, and V collagen. Because type IV collagen prepared by pepsinization of placenta was also digested, it is likely that cleavage of type IV collagen by the tumor collagenase occurs within a largely helical domain. A type IV collagenase could play a significant role in tumor metastases and in normal tissues where basement membrane turnover takes place.**

Basement membranes are extracellular, partly collagenous matrices separating the parenchymal cells of various tissues from underlying connective tissue stroma. Basement membranes are believed to limit the penetration of macromolecules through blood vessel walls, to act as a scaffold for various epithelial cells, and to promote cell differentiation (1). The collagenous component of the basement membrane is termed type IV collagen and is distinct from other collagens, as judged by location (2) and chemical and immunological criteria (3–6). Current data suggest that the protein resembles a procollagen with a triple helical core of at least 300 nm and contiguous noncollagenous extensions (6).

Proteases that specifically attack native collagen have been described in mammalian tissues (7). These enzymes cleave the collagen molecule at a single site three quarters of the way from the NH_2 terminus of the molecule. Such enzymes degrade collagens type I, II, and III (8, 9). However, a granulocyte collagenase has been prepared that cleaves type I but not type III collagen (8). Recently Woolley *et al.* (10) and Timpl *et al.* (11) have reported that skin collagenase fails to degrade type IV collagen under conditions where types I, II, and III collagens are cleaved. This finding suggests that a separate enzyme might be involved in the turnover of basement membrane collagen.

Because collagenases are often identified in tissues that show histologic evidence of collagen breakdown, and because destruction of basement membranes are associated with tumor invasion (12, 13), we have identified a type IV collagenase in the medium used to culture a highly metastatic murine tumor. The activity of the tumor-derived enzyme was compared with a collagenase from human skin (14), with native types I, II, III, IV, and V collagen as substrates.

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MATERIALS AND METHODS

Preparation of Enzymes. Collagenase purified from cultures of human skin (14) was kindly supplied by John Jeffrey (Washington University School of Medicine).

The tumor enzyme was prepared from the medium in which explants of a highly metastatic murine tumor had been cultured. This tumor was originally derived from a pulmonary metastatis of the T241 sarcoma and produces local destruction of basement membranes during growth and metastasis (15, 16). The tumor elicits little or no inflammatory reaction up to the time of harvest. Eight days after injection of 10^6 cells, the tumor and remaining femoral muscle were dissected free of the femur and minced into 1-mm^3 fragments in Hanks' balanced salt solution containing penicillin (100 units/ml) and streptomycin (100 μg/ml). After the fragments were washed three times in Hanks' solution, they were placed in serum-free RPMI 1640 (GIBCO) medium in T75 flasks at 0.1 g per ml of medium. The media from the cultures were harvested on days 1, 3, 5, and 7 and replaced with fresh media. The media were assayed for enzyme activity without concentration. However, enzyme activity was enhanced by prior incubation with trypsin. In general, 0.2 ml of media used to culture tumor tissue was incubated with 0.05 ml of a 0.01% solution of trypsin (3× crystallized, Worthington) in 50 mM Tris·HCl/20 mM NaCl/5 mM $CaCl_2$, at pH 7.6. The mixture was incubated at 37°C for 5 min. Trypsin was inactivated by addition of 0.05 ml of a 0.05% soybean trypsin inhibitor solution (Sigma) in the same buffer.

Enzyme activity was precipitated from the media with ammonium sulfate at 25% and 50% saturation. The 0–25% and 25–50% precipitation pellets were redissolved in the same volume as the original media samples and dialyzed against the buffer described above at 4°C.

Preparation of Substrates. Type I collagen was prepared from guinea pig skin by the method of Nagai *et al.* (17). Type II cartilage collagen was purified from a rat chondrosarcoma (18), and type III collagen was purified from fetal calf skin by the method of Epstein (19). Type IV (basement membrane) collagen was obtained from a transplantable murine tumor different from the invasive tumor producing the collagenase (5, 6). This collagen was extracted with acetic acid and purified by precipitation and column chromatography as described (5, 6). After electrophoresis under reducing and denaturing conditions, two chains are observed in this material which migrate between the α chains and β components of other collagens.

Type IV collagen was also obtained from human placenta by pepsin treatment (20) and kindly supplied by Robert Glanville (Max Planck Institute for Biochemistry, Munich, West Germany). This material contains several collagenous components of different sizes. Type V collagen was prepared from rat skin by the method of Burgeson *et al.* (21).

Isotopic Labeling of Collagen. Tritium-labeled collagen was prepared by brief reductive alkylation with sodium [^{3}H]-borohydride by the method of Means and Feeny (22). No adverse effects of the labeling procedure occurred on the substrates as indicated by low trypsin sensitivity, unaltered migration pattern on gel electrophoresis, normal ability to form aggregates by the procedure described below, and quantitative precipitation of the labeled protein by antibody against type IV collagen. The maximum activity was 10^7 cpm/mg. Labeled collagen was stored in small aliquots at −70°C.

Collagenase Assay. Collagenase activity was determined by the release of radioactive peptides from labeled collagen by means of a method similar to that reported by Nagai *et al.* (17). Aggregates of native type I or IV collagen were prepared by first dissolving the protein in 0.5 M acetic acid followed by dialysis against 50 mM Tris·HCl/20 mM NaCl/5 mM $CaCl_2$, at pH 7.6. After a 4-hr incubation in the buffer at 37°C, insoluble material was collected by centrifugation at 2800 rpm for 15 min. The amount of collagen in the precipitate was estimated from the amount of labeled protein in the precipitate. Collagenase assays were performed in duplicate with 200 μl of enzyme solution and 5 μg of the labeled collagen precipitate suspended in 0.15 ml of buffer. Reactions were carried out at 37°C and terminated by centrifugation at 2800 rpm for 10 min at room temperature to sediment the protein in undigested aggregates. Aliquots of the supernatant solution were placed in Hydromix (Yorktown Research, S. Hackensack, NJ) and assayed for radioactivity in a Beckman liquid scintillation counter. The production of peptides from the substrate was also measured in each experiment by addition of trichloroacetic acid/tannic acid to the reaction mixture to precipitate undigested collagen according to the method of Peterkofsky and Diegelmann (23). The two methods of precipitating undigested collagen in these assays gave virtually identical results. Routine enzyme kinetics were linear with time and enzyme concentration. The range of replicate assays was always less than 10% of the mean.

Slab Gel Electrophoresis. In these studies collagenous protein was precipitated from the reaction mixture with 25% $(NH_4)_2SO_4$. The precipitate that formed was dialyzed against 5 mM acetic acid and lyophilized. Aliquots were electrophoresed on 5% polyacrylamide slab gels by the method of Laemmli (24) in the presence of 0.5 M urea or by the electrophoresis method of Nagai *et al.* (25), which is carried out at acid pH.

RESULTS

Preliminary studies demonstrated that an enzyme degrading type IV collagen was secreted by the explants of tumor tissue into the culture medium. Maximum enzyme activity was obtained from the third to the fifth day that explants of tumor tissue were cultured. Preferential digestion of type IV collagen (75%) relative to type I collagen (10%) was observed by the tumor enzyme preparation within the linear portion of the rate curve (Table 1 and Fig. 1). Under these conditions, purified human skin collagenase digested type I collagen maximally by 5 hr, but failed to digest type IV collagen even after 20 hr (Fig. 1).

The effect of trypsin activation, differential precipitation, and addition of various protease inhibitors on the tumor enzyme activity is shown in Table 1. Without trypsin activation, the media enzyme did not degrade type I collagen, but 36% of the type IV collagen was digested. After trypsin activation, up to 10% of the type I collagen was cleaved whereas 75% of the type IV collagen was solubilized. Most of the enzyme activity against type IV collagen was in the 25–50% ammonium sulfate precipitate, and we obtained an 8-fold concentration of activity relative to total protein in this fraction. Activity against both substrates was lost when the enzyme preparation was heated for 3 min at 100°C. The activity against type IV collagen was abolished by addition of EDTA and was inhibited by addition of fresh human serum to the reaction, but was not affected by the presence of soybean trypsin inhibitor. Trypsin treatment of the type I and type IV collagen substrates released less than 5% of the total radioactivity.

Table 1. Degradation of tritium-labeled type I guinea pig collagen, murine type IV collagen, and type IV human placental collagen

Enzyme	Collagen: Type I	Collagen: Type IV (murine tumor)	Collagen: Type IV (human placenta)
Murine tumor			
Nonactivated culture media	0	395 (36%)	
Activated culture media	408 (10%)	822 (75%)	1027 (64%)
0–25% $(NH_4)_2SO_4$		302 (28%)	
25–50% $(NH_4)_2SO_4$		850 (78%)	
With 10 mM EDTA		12 (1%)	
With 0.1% soybean trypsin inhibitor		842 (77%)	
With 5% human serum		520 (47%)	
Boiled		18 (1.5%)	32 (2%)
Bacterial collagenase (25 units)	4000 (100%)	859 (78%)	1401 (87%)
Purified human skin collagenase	2880 (72%)	10 (1%)	18 (1%)
Trypsin (0.01%)	144 (2.8%)	54 (4.9%)	68 (4.2%)

Media taken from the metastatic tumor cultures were activated by brief treatment with trypsin followed by soybean trypsin inhibitor as described in the text. Reaction mixtures containing enzyme and substrate were incubated at 37°C for 3 hr followed by precipitation of undegraded substrate with trichloroacetic acid/tannic acid at 4°C for 30 min and centrifugation at 2800 rpm for 15 min. Activity was measured by determining the radioactivity of unprecipitated labeled substrate fragments remaining in the supernatant fraction. The activities of purified human skin collagenase and murine tumor collagenase before and after partial concentration of activity by ammonium sulfate precipitation were compared. Each value given (cpm of radioactive peptides) is the mean of duplicate experiments after subtraction of background counts (less than 30 cpm). Values in parentheses are percent substrate degraded.

The components of the collagen substrates used in these studies were resolved by polyacrylamide gel electrophoresis (Fig. 2) both with and without various enzyme treatments. Ten minutes of exposure to bacterial collagenase destroyed all the components present in Fig. 2*A*. Exposure to trypsin, with an enzyme to substrate ratio of 1:2, produced partial degradation of collagen type III to ¾ and ¼ fragments (Fig. 2*B*), as previously reported (26). Alterations in the mobility of the components of type V collagen were also observed after this treatment. This treatment had little or no effect upon types I, II, and IV

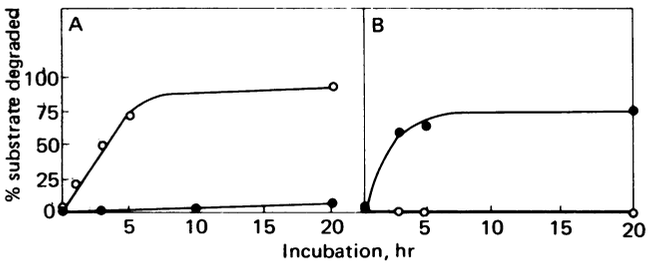

FIG. 1. Degradation of type I guinea pig collagen (○) and murine type IV collagen (●) by the tumor enzyme preparation or by purified human skin collagenase. The percent digestion was estimated by the release of tritium-labeled peptides from substrate in suspension (17), and activity was further verified by the method of Peterkofsky and Diegelmann (23). Activity is expressed as percent of bacterial collagenase-sensitive substrate (that is, the radioactivity degraded by 35 units of bacterial collagenase in the presence of *N*-ethylmaleimide was taken to be 100%). (*A*) Digestion of type I collagen and type IV collagen by trypsin-activated culture medium from the tumor; (*B*) digestion of the same substrates as in *A* by purified human skin collagenase. Each point shown is the mean of duplicate determinations. Trypsin released less than 5% of the total radioactivity in the substrates.

collagen. Human skin collagenase cleaved collagen types I, II, and III, producing the expected 3/4 and 1/4 fragments (Fig. 2*D*). This enzyme did not cleave type IV or V collagen. In contrast, the tumor enzyme selectively degraded type IV collagen (Fig. 2), showed little digestion of the type I, II, or III collagen molecules, and produced no significant change in type V collagen.

Individual preparations of tumor collagenase showed variable activity against type I collagen ranging from 0 to 16%. As previously reported (6), reduced type IV collagen substrate migrates as two bands estimated, by using type I collagen chains as molecular weight markers, to have molecular weights of 160,000 and 140,000. After digestion with the tumor-derived enzyme at 20°C for 8 hr, additional bands (identified by the arrows) were observed which were estimated to be 70% the size of the original bands (Fig. 2*C*). The amount of these lower molecular weight products increased with time of digestion and with increased amounts of enzyme.

The degradation products of type IV collagen were further studied by electrophoresis at acid pH, at which collagenous proteins separate solely on the basis of size (25). In this system, the α1(I) and α2 chains of type I collagen run as a single band, as do the β11 and β12 components. When the reduced and alkylated type IV collagen was electrophoresed, one major component, estimated to have a molecular weight of 155,000 was resolved (Fig. 3). After digestion with the tumor enzyme, the large digestion product had a molecular weight of 110,000, with a smaller component of molecular weight approximately 50,000 seen only when phenylmethylsulfonyl fluoride was included during the digestion.

In order to determine whether the tumor collagenase cleaved within the triple helical region of the type IV molecule, we incubated type IV from pepsin digests of human placenta with the enzyme (Table 1). As isolated, this protein contains four collagenous components (20). The tumor enzyme cleaved the 95,000 molecular weight component into two fragments by gel electrophoresis, whereas the skin enzyme was ineffective.

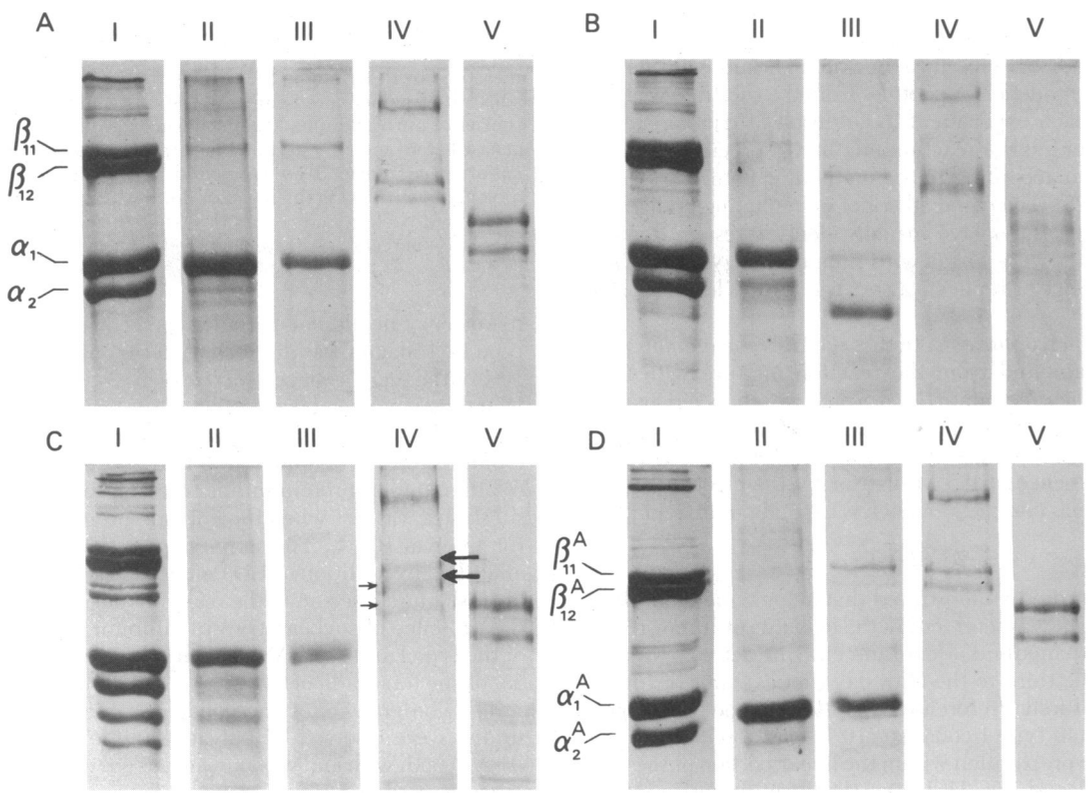

FIG. 2. Electrophoresis of types I, II, III, IV, and V collagen incubated with (*A*) no enzyme, (*B*) trypsin at a 1:2 enzyme to substrate ratio, (*C*) murine tumor collagenase, and (*D*) purified human skin collagenase. Incubations were performed at 27°C for 10 hr. The reactions were stopped by addition of EDTA (10 mM), and collagenous protein was precipitated with 25% ammonium sulfate. The human skin collagenase degraded collagen types I, II, and III to 3/4- and 1/4-length fragments, but failed to degrade type IV or type V collagen. In contrast, the tumor enzyme degraded type IV collagen (large arrows refer to the original bands, small arrows indicate the degradation products). Only a small proportion of the types I, II, and III collagen molecules were cleaved by the tumor enzyme whereas type V was not degraded. Trypsin degraded types III and V collagen.

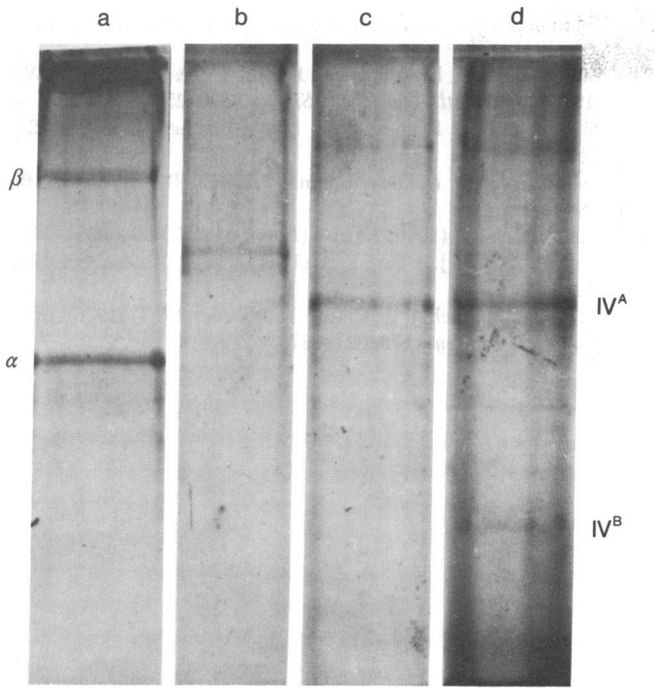

FIG. 3. Electrophoresis at acid pH of degradation products of murine type IV collagen produced by the tumor enzyme. Gel electrophoresis at acid pH (25) of: lane a, type I collagen; lane b, murine type IV collagen (reduced and alkylated under nondenaturing conditions), incubated without enzyme; lane c, native reduced and alkylated type IV collagen digested with the trypsin-activated tumor enzyme without phenylmethylsulfonyl fluoride added; and lane d, native reduced and alkylated murine type IV collagen digested with the trypsin-activated tumor enzyme carried out in the presence of phenylmethylsulfonyl fluoride (10 μmol). The larger degradation fragment (IV^A) from the 155,000 molecular weight component is estimated to have a molecular weight of 110,000; the smaller component (IV^B), a molecular weight of 50,000. Molecular weights were determined by comparison with cyanogen bromide fragments of α(I).

This 95,000 molecular weight component has been termed IVα by Glanville *et al.* (20).

DISCUSSION

Current evidence suggests that during the degradation of collagen specific collagenases carry out the initial breakdown. Those enzymes so far investigated on type I, II, and III collagen substrates have been found to cleave a single bond in each chain in the molecule, dividing it into two triple helical fragments (7). Although the bonds cleaved in types I, II, and III collagen are known, the factors that determine the specificity of the collagenase are not understood.

The studies reported here on the cleavage of type IV collagen from the tumor are consistent with cleavage at a single site in the molecule because two major fragments were observed on electrophoresis at acid pH. The smaller of these fragments is revealed when other proteases in the crude enzyme preparation are inhibited by phenylmethylsulfonyl fluoride. The sum of these fragments approaches that of the original chain. It seems likely that cleavage occurs within a largely helical segment of the molecule because aggregates of the protein are almost totally degraded at 37°C and a largely collagenous protein, the pepsinized placental type IV collagen, is degraded. The structure of type IV collagen is not well understood, and the exact localization of the cleavage site(s) probably awaits better definition of the type IV molecule.

The enzyme preparation used in these studies was obtained from primary explants of a metastatic tumor that causes the loss of basement membrane surrounding muscle bundles as well as vascular basement membrane in its path (16). Because few if any inflammatory cells are observed in the tumor or involved muscle at the time of tissue excision, it is unlikely that the enzyme studied here was derived from inflammatory cells. Similarly, explants of normal muscle did not produce detectable enzyme (not shown). We have previously shown that freshly isolated tumor cells caused the release of hydroxyproline when plated on preparations of pulmonary basement membrane (16). Additional proof that the tumor is the source of this enzyme is seen in culture, where isolated tumor cells passed for more than 20 generations also produced enzyme activity that degraded type IV collagen (not shown). Samples of tumor interstitial fluid also contained activity against type IV collagen.

The enzyme identified in these studies behaves like other mammalian collagenases with regard to susceptibility to inhibitors (Table 1). Its activity was abolished by EDTA, a chelating agent that inhibits collagenases but not cathepsins (7). Soybean trypsin inhibitor, a potent inhibitor of plasmin, did not alter enzyme activity. Activity was observed in the presence of phenylmethylsulfonyl fluoride, an inhibitor of serine proteases. Fresh human serum that inhibits other collagenases (7) reduced the enzyme activity. Also, like other mammalian collagenases, pretreatment with trypsin enhanced the collagenolytic activity presumably by activating a zymogen or destroying inhibitors (7, 14).

The substrate requirements of the collagenases are not understood although the amino acid sequences through the cleavage site of types I–III collagens are known (26). Previous studies have shown that a collagenase from granulocytes cleaves type I but not type III collagen (8), indicating that such enzymes can be collagen specific. Turnover of basement membrane probably requires an enzyme that does not attack other collagens. Such an enzyme need not be limited to tumor tissues because preliminary studies indicate that an enzyme degrading type IV collagen is produced by rat breast ducts and involuting breast tissue in culture. Because cleavage of type V collagen was not observed with either enzyme preparation, it is likely that still another collagenase is involved in its degradation.

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