Degradation of Native Type IV Procollagen by Human Neutrophil Elastase. Implications for Leukocyte-Mediated Degradation of Basement Membranes†

Daniel J. Pipoly and Edmond C. Crouch*

Department of Pathology and Respiratory and Critical Care Division, Department of Medicine, The Jewish Hospital of St. Louis at Washington University Medical Center, St. Louis, Missouri 63110

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ABSTRACT: Leukocyte-derived proteases may contribute to the destruction of basement membranes during inflammation. We have, therefore, examined the degradation of human type IV procollagen (PC) by purified human neutrophil elastase (HLE). Native [^{14}C]proline-labeled type IV PC was isolated from cultures of human HT-1080 cells and incubated with HLE for various times at 25 or 37 °C. Cleavage products were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and identified by CNBr peptide mapping. Incubation of type IV PC with HLE (<1:10 HLE:type IV weight ratio) resulted in cleavage of the proα1(IV) and proα2 chains (M_r 180 000 and 175 000) to discrete components of M_r >140 000. Peptide mapping indicated that the carboxy-terminal collagenase-resistant domains of both chains were rapidly and preferentially degraded. Longer incubations or incubations at higher enzyme:substrate ratios resulted in extensive and asymmetric internal cleavage with the generation of fragments similar in size distribution to the major pepsin-resistant fragments of type IV collagen. Our findings indicate that soluble, native human type IV PC is a substrate for HLE and is preferentially cleaved within the globular carboxy-terminal domains of the proα1 and proα2 chains. We suggest that even limited cleavage of type IV PC by HLE may disrupt intermolecular carboxy-terminal interactions believed to be important for basement membrane assembly and for maintaining basement membrane structure in vivo.

Leukocyte-mediated damage to basement membranes may represent an important mechanism of tissue injury (Fantone & Ward, 1985). Since type IV collagen is an essential structural component of basement membranes, we have examined the degradation of native type IV procollagen[1] (PC) by purified human neutrophil elastase.

Previous studies have described the degradation of soluble pepsin-extracted bovine lens capsule type IV collagen by neutrophil-derived neutral proteases (Uitto et al., 1980), and by elastase purified from human neutrophil granules (Mainardi et al., 1980a,b). In addition, neutrophils have been shown to release hydroxyproline from insoluble basement membranes isolated from glomerulus or bovine lens capsule [see, e.g., Davies et al. (1977, 1980) and Uitto et al. (1980)]. However, there are no published data on the degradation of the intact and secreted form of human type IV collagen[1] by HLE. Furthermore, the major sites of cleavage within the proα1(IV) and proα2(IV) chains and the relative susceptibility of the pepsin-sensitive carboxy-terminal domains have not been defined.

An examination of the degradation of an intact human type IV collagen substrate is important for several reasons: (1) pepsinized substrates have a decreased thermal stability [see, e.g., Uitto et al. (1980)] and may exhibit altered susceptibility to further proteolytic degradation; (2) differences in the primary structure of type IV collagens isolated from different species [see, e.g., Babel and Glanville (1984)] raises the possibility of interspecies variation in protease sensitivity; and (3) there is evidence that important intermolecular interactions are mediated by the pepsin-sensitive carboxy-terminal domains in vivo (Weber et al., 1984).

Our studies indicate that native type IV PC is a substrate for neutrophil elastase and suggest that degradation of the noncollagenous carboxy-terminal domains may contribute to the disruption of basement membrane at sites of inflammation in vivo.

Materials and Methods

Metabolic Labeling of HT-1080 Cells. HT-1080 cells were obtained from the American Type Culture (ATCC CCL 121). Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM Hepes, pH 7.5, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. For the preparation of type IV PC substrate, ten plates (Corning, 150 cm^2) of nearly confluent cells were preincubated for 30 min with serum-free DMEM supplemented with 50 μg/mL fresh ascorbic acid and 80 μg/mL β-aminopropionitrile fumarate (BAPN). Cultures were then labeled for 20 h in fresh preincubation medium containing 5 μCi/mL of L-[U-^{14}C]proline (New England Nuclear, >250 mCi/mmol).

Purification of Type IV Procollagen Substrates from Culture Medium. Human type IV PC was isolated from the culture medium of HT-1080 cells according to modifications of methods described for the isolation of type IV PC from cultures of human epithelial cells (Crouch & Bornstein, 1979). The culture medium and one wash of the dish with divalent cation-free phosphate-buffered saline (PBS) were pooled and clarified by centrifugation at 400g for 15 min. All subsequent procedures were performed at 4 °C except as indicated. Neutral protease inhibitors were added [0.2 mL phenylmethanesulfonyl fluoride (PMSF), 2 mM *N*-ethylmaleimide (NEM), 2.5 mM ethylenediaminetetraacetic acid (EDTA)], and the medium was further clarified by centrifugation for 30 min at 48000g. Proteins were concentrated by precipitation with 30% (w/v) ammonium sulfate without added carrier,

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* Address correspondence to this author at the Department of Pathology, The Jewish Hospital of St. Louis at Washington University Medical Center.

[1] We use the term "procollagen" (PC) to refer to the secreted form of a collagen.

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dialyzed vs. DEAE chromatography buffer (4 M urea, 50 mM Tris-HCl,[2] pH 7.5, containing 0.2 mM PMSF and 2.5 mM EDTA; pH adjusted at room temperature), and applied to a column of DE-52 cellulose (Whatman, 1.2 × 5 cm). Radioactive proteins eluting with the wash were concentrated by ultrafiltration on a YM-30 membrane (Amicon), dialyzed extensively vs. divalent cation-free PBS, counted by liquid scintillation spectrometry, and stored in aliquots at −20 °C. The specific activity of collagen in the radiolabeled substrate was approximately 1500–2000 ^{14}C dpm/μg.

For some experiments, type IV PC was further purified by CM-cellulose chromatography under nondenaturing conditions. Briefly, the unbound fractions from DEAE-cellulose were dialyzed vs. 2 M urea and 0.04 M sodium acetate, pH 4.8, and chromatographed on a column of CM-52 cellulose (Whatman, 1.2 × 5 cm) equilibrated with the same buffer at 4 °C. Type IV PC was eluted with 0.4 M NaCl, at a flow rate of 60 mL/h. The radioactive fractions were pooled, concentrated by ultrafiltration, dialyzed vs. PBS, and stored as above.

Human types I and III–V collagen were purified from limited pepsin digests of amnionic membranes or placenta by differential salt fractionation and ion-exchange chromatography under nondenaturing conditions (Kresina & Miller, 1979). Rat type I collagen was isolated from neutral salt extracts of lathyritic rat skin as described by Chandrakasan et al. (1976). Mouse type IV procollagen was prepared from EHS tumors as described by Kleinman et al. (1982). The amount of each collagen was estimated by hydroxyproline assay (Woessner, 1961).

Cleavage with HLE. Purified human leukocyte elastase (HLE, 875 units/mg, from purulent human sputum) free of cathepsin G activity was purchased from Elastin Products (Pacific, MO). The lyophilized protein was dissolved at 1.0 mg/mL in divalent cation-free phosphate-buffered saline, pH 7.4 (PBS), and stored at 4 °C. Elastolytic activity was assayed in the presence of 0.02% Brij 35 (Sigma) with [^3H]elastin as substrate (Banda et al., 1981). The enzyme migrated as three major bands on silver-stained SDS slab gels (M_r = 26 000–30 000). The elastinolytic activity was completely inhibited with 2 mM PMSF (Lively & Powers, 1978) or eglin *c* [see, e.g., Snider et al. (1985)].

Collagen substrates were incubated with HLE in PBS in the presence of 0.02% Brij for up to 24 h at 25 or 37 °C. The former temperature is below the expected melting temperature of the major helical domains of intact type IV PC and was used to ensure conformational stability of the collagen substrate [see, e.g., Uitto et al. (1980)]. Reactions were performed in 1.5-mL polypropylene microfuge tubes with a final reaction volume of 50 μL. Brij was required to prevent nonspecific binding to the assay tubes; preliminary experiments showed that the detergent had no effect on elastolytic activity or on the extent or pattern of type IV PC cleavage. For most experiments 1500 dpm of substrate (approximately 1 μg) was incubated with HLE at enzyme:substrate weight ratios of 1:1000, 1:100, 1:10, and 1:1. For incubations with unlabeled collagens we used 1–5 μg of substrate when proteins were to be examined by silver stain or 20–50 μg of substrate when proteins were to be stained with Coomassie blue. Incubated and nonincubated controls were included for all experiments. Reactions were terminated by addition of PMSF to a final concentration of 2 mM. Following the addition of 6× SDS–PAGE sample buffer, the samples were boiled for 2 min in the presence or absence of 50 mM dithiothreitol (DTT). Samples were temporarily stored at −20 °C prior to SDS–PAGE.

Identification of Cleavage Sites within the Collagenase-Resistant Terminal Domains of Type IV PC. The type IV substrate was dialyzed vs. 0.1 M acetic acid at 4 °C in the presence of pepstatin. Aliquots (32 000 ^{14}C dpm) were concentrated by lyophilization in conical centrifuge tubes, resolubilized in 60 μL of 150 mM NaCl, 10 mM $CaCl_2$, and 50 mM Tris-HCl, pH 7.5, and incubated with HLE at an enzyme:substrate weight ratio of 1:10 for 1 h at 25 °C (i.e., under conditions yielding limited terminal cleavage of the type IV chains). Following inactivation of HLE with 2 mM PMSF, the substrates were incubated with 10 μg of purified bacterial collagenase (Advance Biofactures, form III) for 2 h at 37 °C in a final reaction volume of 70 μL. Parallel aliquots of substrate were incubated in the absence of HLE and then digested with collagenase as above. Incubations were terminated by the addition of concentrated SDS–PAGE sample buffer and by boiling for 2 min. Samples were examined by SDS–PAGE as described above for HLE digests.

Oxidative Pretreatment of Type IV PC. Hydrogen peroxide (30% solution, Sigma) was diluted in PBS and added to the substrate solution at a final concentration of 0.05–50 mM. All solutions were prepared with freshly deionized and ultrafiltered water (Milli-Q, Millipore). Incubations were performed for up to 24 h at 37 °C and were terminated by the addition of a >5-fold excess of catalase (Sigma, bovine liver, 11 000 units/mg) for 15 min at 37 °C. Inactivation of hydrogen peroxide was confirmed by assay of hydrogen peroxide by the ferrithiocyanate method (Thurman et al., 1972). Incubated peroxide-free and catalase-treated controls were included with all experiments.

Myeloperoxidase (MPO) from purulent human sputum (31 units/mg) was obtained from Elastin Products (Pacific, MO), dissolved at 1 mg/mL in PBS, and stored at −80 °C. Peroxidase activity was determined spectrophotometrically by using *o*-dianisidine as hydrogen donor (Worthington Manual, 1972). Substrates were incubated with up to 20 mU of MPO in the presence of various concentrations of H_2O_2. Reactions were terminated by the addition of excess catalase. For some experiments, samples were then incubated in the presence or absence of HLE and processed for SDS–PAGE as described above.

SDS–PAGE and Fluorescence Autoradiography. Proteins were examined by SDS–PAGE on discontinuous 1.5-mm polyacrylamide slab gels containing 0.5 M freshly deionized urea (Crouch & Bornstein, 1978). A 3% stacking gel was used with a 6% or composite 6%/10% separating gel. For fluorescence autoradiography slab gels were fixed in 30% methanol–10% acetic acid, permeated with EnHance (New England Nuclear), dried, and exposed at −70 °C to sensitized Kodak XRP film. The relative recovery of radioactivity in proteins resolved by SDS–PAGE was determined by densitometry of appropriately exposed fluorograms (Laskey & Mills, 1975). For unlabeled substrates, gels were stained with Coomassie blue or by silver stain. The specific activity of radiolabeled type IV PC was estimated by comparing the density of stained radioactive proα chains with known amounts of mouse type IV PC. Molecular weights were determined relative to internal α1(I) monomers, dimers, trimers, and CNBr peptides.

[2] Abbreviations: Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HLE, human leukocyte elastase; MPO, myeloperoxidase; CM, carboxymethyl; DEAE, diethylaminoethyl; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

Cyanogen Bromide Peptide Mapping. CNBr peptide mapping was performed essentially as described by Barsh et al. (1981). Briefly, samples were reduced with 50 mM DTT and resolved by SDS–PAGE on a 0.75-mm, 3%/6% slab gel. Individual lanes were cut from the gel and incubated with CNBr in 70% formic acid. The slices were washed, equilibrated with SDS–PAGE sample buffer, horizontally positioned above a 1.5-mm, 5%/12.5% slab gel, and sealed in place with agarose. CNBr peptides were resolved by SDS–PAGE in the second dimension.

Proteolytic Cleavage of Proteins. Lyophilized proteins were incubated with 100 μg/mL pepsin (Worthington, 3352 units/mg) in 0.5 M acetic acid (50 μg/mL) for 24 h at 4 °C.

Immunochemistry. Monospecific antibodies to human placenta type IV collagen were prepared in rabbits, were affinity-purified, and were characterized as previously described (Crouch et al., 1986). Immunoprecipitations of dialyzed culture medium were also performed as described.

Results

Characterization of the Type IV Procollagen Substrate. Radiolabeled type IV PC was isolated from the culture medium of human HT-1080 cells (Alitalo et al., 1980). The substrate has been definitively identified as type IV PC on the basis of the following: (1) prior to reduction, the protein migrates near the interface of the stacking and separating gels, consistent with a trimeric molecule; (2) following reduction with DTT, the protein migrates on SDS–PAGE as two distinct, bacterial collagenase-sensitive polypeptide chains (M_r 180000 and 175000) which are present in a ratio of approximately 2:1; (3) the protein is cross-reactive with monospecific antibodies to human type IV collagen in indirect immunoprecipitation assays (Alitalo et al., 1980); (4) the protein is resistant to cleavage with interstitial collagenase (unpublished data); and (5) the protein shows a characteristic pattern of peptides following cleavage with CNBr (e.g., Figure 3) or staphylococcal V8 protease (Alitalo et al., 1980). Previous studies have also shown an appropriate pattern of posttranslational hydroxylation and glycosylation (Pihlajaniemi et al., 1981) and confirmed that the protein is assembled as heterotrimers of the proα1 and proα2 chains (Odermatt et al., 1984).

The purified type IV PC had a native triple-helical conformation as evidenced by its relative resistance to degradation by pepsin (100 μg/mL) for 24 h at 4 °C or for 2 h at 15 °C in 0.1 M acetic acid (Figure 1). Under these conditions, pepsin resulted in a slight increase in the electrophoretic mobility of the procollagen and its constituent proα chains consistent with loss of the carboxy-terminal globular domains (unpublished data; Timpl et al., 1981; Yurchencho & Furthmayr, 1984). There was also limited internal cleavage of the proα2 chain, consistent with the known sensitivity of human proα2(IV) to peptic degradation (Crouch et al., 1980). A comparable pattern of pepsin sensitivity has been described for native EHS type IV PC (Kleinman et al., 1982; Yurchencho & Furthmayr, 1984), and for type IV collagens isolated under nondenaturing conditions from several tissues [see, e.g., Kresina and Miller (1979)].

Examination of the purified radiolabeled substrate by SDS–PAGE and silver staining revealed that the major high molecular weight components comigrated with authentic type IV PC chains. The specific activity of type IV collagen in the radiolabeled substrate was approximately 1500–2000 ^{14}C dpm/μg and was sufficient to permit visualization of major cleavage products after 2–3 days of exposure.

Cleavage of Type IV PC by HLE. Incubation of type IV PC with HLE resulted in the generation of multiple discrete

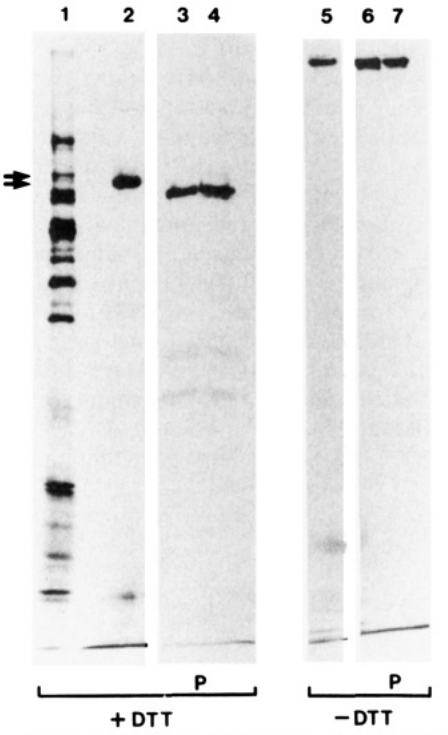

FIGURE 1: Characterization of type IV procollagen substrate. [^{14}C]Proline-labeled type IV PC was isolated from the culture medium of HT-1080 cells as described under Materials and Methods. Aliquots were dialyzed vs. acetic acid, lyophilized, and subjected to SDS–PAGE on 3%/6%/10% slab gels with or without prior reduction with dithiothreitol (DTT). Radiolabeled proteins were visualized by fluorography. Parallel aliquots were incubated with pepsin (P) prior to SDS–PAGE. Lane 1, rat lung fibroblast medium standards, reduced; lanes 2 and 5, type IV PC; lanes 3 and 6, type IV PC, treated with pepsin for 24 h at 4 °C; lanes 4 and 7, type IV PC treated with pepsin for 2 h at 15 °C. The positions of proα1 and proα2 chains are indicated at left. Note: Gels were overexposed to emphasize lower molecular weight contaminants and cleavage products.

cleavage products (Figure 2), which were identified by SDS–PAGE following incubation of gel slices with CNBr (Figure 3). The extent of cleavage increased with increasing incubation time, temperature, and/or enzyme concentration (Figure 2). There was no evidence of degradation when substrate was incubated for up to 24 h at 25 or 37 °C in the absence of enzyme or following addition of enzyme in the presence of 60 μM MeOSuc-$(Ala)_2$-Pro-Val-CH_2Cl, 2 mM PMSF, or an 8-fold molar excess of eglin *c*. Degradation did not require divalent cations and was not inhibited by 20 mM EDTA.

Incubation of substrate with 1 ng of HLE (E:S of 1:1000) at 25 °C resulted in minimal cleavage of the procollagen chains with a preferential decrease in the intensity of the proα2 chain (Figure 2A). Mapping studies, such as those described below, suggest that the two initial cleavage products of M_r 140000–155000 and 100000 were derived from the proα2 chain. More prolonged cleavage at higher enzyme:substrate (E:S) ratios resulted in complete degradation of the proα1 and proα2 chains with the stepwise generation of fragments similar in size distribution to the major pepsin-resistant fragments of human type IV collagen (M_r 165000, 155000, 140000, 100000, 70000, and 50000) [see, e.g., Crouch et al. (1980) and MacWright et al. (1983)]. Although the major high molecular weight fragments were further degraded at higher E:S ratios, small amounts of fragments of M_r 70000 and 50000, and a few lower molecular weight fragments migrating in the 10% separating gel, were still identifiable after a 24-h incubation at an E:S weight ratio of 1:1.

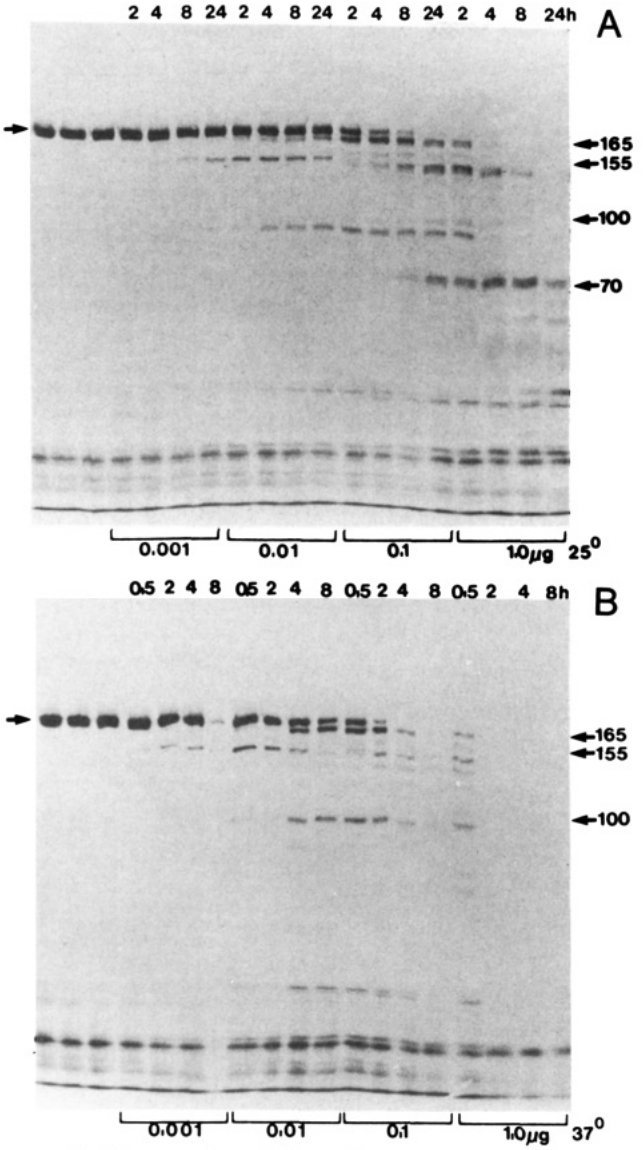

FIGURE 2: Cleavage of type IV procollagen with elastase. Aliquots of [^{14}C]proline type IV substrate were incubated with increasing amounts of human leukocyte elastase (HLE) for various times at 25 (A) or 37 °C (B). Following reduction with DTT, cleavage products were resolved by SDS–PAGE on a 3%/6%/10% slab gel and visualized by fluorography. Lane 1, substrate; lanes 2 and 3, substrate incubated for 24 h at indicated temperature. Incubation times (h) are indicated at top. The weight of HLE (μg) is indicated at bottom; 1 μg corresponds to an enzyme:substrate (E:S) weight ratio of approximately 1:1. The position of proα1(IV) is indicated at left. The relative molecular weights of the major fragments are indicated at right.

Incubation with HLE at 37 °C resulted in a similar pattern of high molecular weight cleavage products when digests were examined after comparatively short periods of incubation (compare panels A and B of Figure 2). However, the major fragments were unstable and were further degraded to low molecular weight peptides. In particular, the fragments of M_r 100000, 70000 and 50000, observed following incubation with 0.1 or 1.0 μg of HLE at 25 °C, were unstable and were rapidly degraded.

The major high molecular weight cleavage products were definitively identified by comparing the pattern of CNBr peptides derived from each type IV fragment under various conditions with peptides derived from intact proα1(IV) and proα2(IV) chains. Representative peptide maps of intact and HLE-degraded type IV PC are shown in Figure 3. Maps of the major cleavage products obtained following incubation with

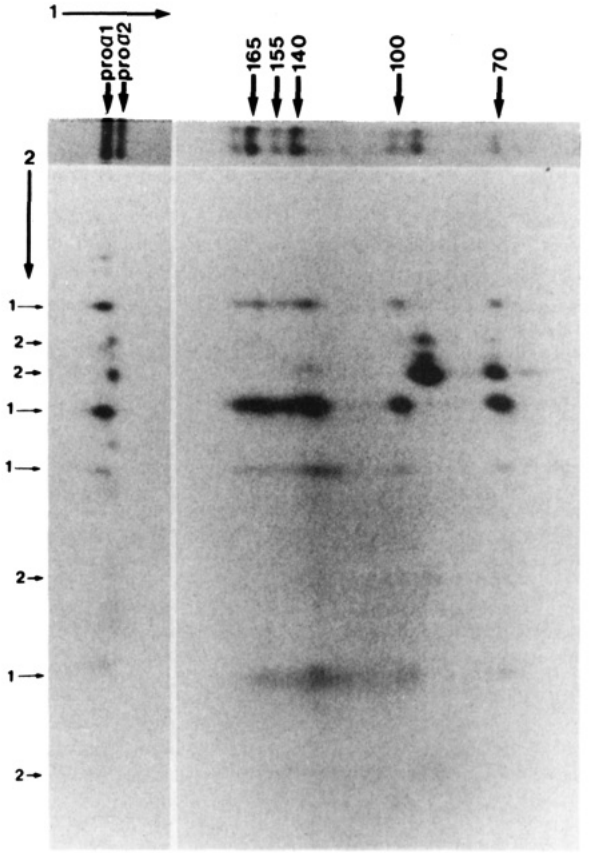

FIGURE 3: Representative CNBr peptide map of type IV procollagen cleavage products. Type IV PC (50000 cpm) was incubated with HLE at an estimated E:S weight ratio of 1:10 for 3 h at 25 °C. The cleavage products were resolved by SDS–PAGE on a 0.75 mm thick 3%/5%/10% slab gel following reduction with DTT (arrow 1). Individual lanes were cut from the gel and incubated with CNBr in 70% formic acid as described under Materials and Methods. The gel slices were washed with water, equilibrated with sample buffer, and horizontally positioned above a 5%/12.5% gel, and the peptides were resolved by SDS–PAGE in the second dimension (arrow 2). Left panel, undigested type IV PC substrate; right panel, type IV following digestion with HLE. The positions of proα1 and proα2 chains and major HLE-generated fragments are indicated at top. The positions of proα1 (1) and proα2-derived (2) CNBr peptides are indicated at left.

HLE at an E:S ratio of 1:10 for 3 h at 25 °C demonstrate the relative sensitivity of the proα1 and proα2 chains to elastase cleavage. Under these conditions, the major high molecular weight fragments (M_r 165000, 155000, and 140000) were derived from the proα1 chain, whereas the proα2 chain was largely degraded to fragments of M_r <100000 with only faint residual proα2-derived components of M_r 140000–155000. Lower molecular weight fragments derived from the proα1 chain (M_r 100000 and 70000) were also identified. The preponderance of relatively high molecular weight proα1-derived fragments indicated preferential cleavage near the amino and/or carboxy termini. CNBr maps of the proα1-derived fragments showed extensive homology with maps obtained for the amino-terminal pepsin-resistant domain of the α1(IV) chain (M_r 70000) (not shown). These observations suggested that initial cleavage of the triple-helical proα1(IV) chain occurs within or near the carboxy-terminal domain and that additional preferred cleavage sites are located within the carboxy-terminal half of the proα1 chain.

Cleavage of native type IV PC with purified bacterial collagenase results in the generation of peptides derived from the amino-terminal "7S" domains (M_r 30000–35000), which are cross-linked by disulfide bonds, and peptides derived from

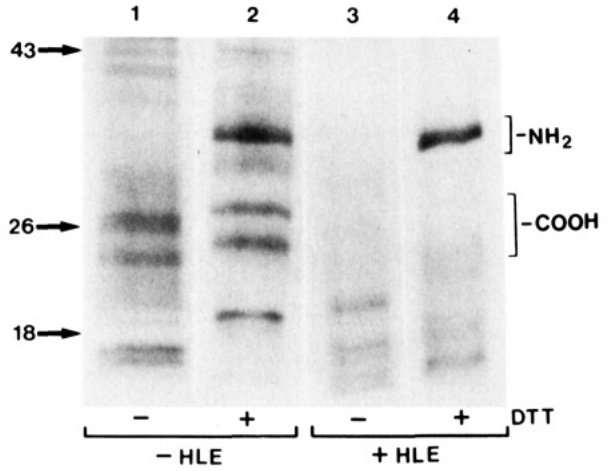

FIGURE 4: Degradation of type IV procollagen carboxy-terminal domains by elastase. Type IV PC was sequentially incubated with HLE and purified bacterial collagenase as described under Materials and Methods. Peptides were resolved by SDS–PAGE on a 5%/12.5% slab gel with or without prior reduction with DTT and visualized by fluorography. Lanes 1 and 2, collagenase-resistant peptides of the type IV substrate, incubated control; lanes 3 and 4, collagenase-resistant peptides following incubation of substrate with HLE at an E:S ratio of 1:10 for 1 h at 25 °C (compare with Figure 2A; 0.1 μg of HLE). The upper component in lane 2 (M_r ~35 000) migrated near the expected position of trimers without reduction and is derived from the collagenase-resistant amino-terminal domain (-NH_2); the lower molecular weight components in lanes 1 and 2 (M_r ≤30 000) lack interchain disulfide bonds consistent with the carboxy-terminal domains of the proα1 and proα2 chains (-COOH). The slower migration of these peptides in the presence of DTT presumably reflects unfolding of the chains secondary to reduction of intrachain disulfide bonds. Examination of this preparation of substrate prior to collagenase digestion revealed no discrete contaminants in this region of the gel. The positions of globular protein standards are indicated at left.

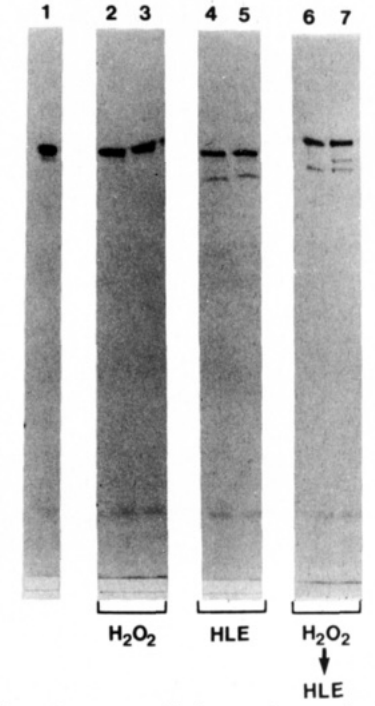

FIGURE 5: Effect of type IV oxidation on cleavage by elastase. Type IV PC was preincubated with H_2O_2 for 6 h at 37 °C. Following inactivation of H_2O_2 with catalase, the sustrate was incubated with 0.01 μg (E:S of 1:10) for 30 min at 37 °C. Samples were reduced with DTT, subjected to SDS–PAGE on a 3%/6%/10% slab gel, and visualized by fluorography. Lane 1, incubated control; lane 2, type IV PC incubated with 0.5 mM H_2O_2; lane 3, incubated with 5 mM H_2O_2; lanes 4 and 5, incubated with HLE; lanes 6 and 7, preincubated with 0.5 or 5 mM H_2O_2, respectively, prior to cleavage with HLE.

the carboxy-terminal "NC1" domains (M_r ≤30 000), which lack interchain disulfide bonds (Fessler & Fessler, 1982; Timpl et al., 1981; Weber et al., 1984). We, therefore, examined the size distribution of collagenase-resistant peptides following limited digestion with HLE in order to further localize the site(s) of initial elastase cleavage. For these experiments, type IV PC was incubated with HLE under conditions previously shown to yield limited terminal cleavage of the proα1 chains. The central collagenous domains were then degraded with purified bacterial collagenase, and the collagenase-resistant peptides were resolved by SDS–PAGE on a 5%/12.5% slab gel (Figure 4). As expected, incubation of the native substrate with collagenase resulted in the disappearance of intact proα1 and proα2 chains and the generation of disulfide- and non-disulfide-bonded peptides corresponding to the amino- and carboxy-terminal domains, respectively (Figure 4, lanes 1 and 2). Incubation of the HLE-degraded substrate with collagenase demonstrated essentially complete recovery of disulfide-bonded components comigrating with intact amino-terminal domains and the disappearance of the lower molecular weight proα1- and proα2-derived carboxy-terminal peptides (Figure 4, lanes 1 and 2). These data indicate that there are elastase-sensitive sites within the nonhelical carboxy-terminal domains of the proα1 and proα2 chains. Furthermore, the high recovery of disulfide-bonded components following HLE cleavage confirmed that the amino-terminal domains are comparatively resistant to degradation by HLE.

There was no cleavage of the pepsin-resistant domains of human types I or V collagen following incubation with HLE (1:10 weight ratio) for 24 h at 25 °C. Identical incubations with pepsinized human type III collagen showed partial cleavage to fragments similar in size to the major fragments released by vertebrate collagenase, as previously described (Gadek et al., 1980; Mainardi et al., 1980b). Incubation of salt-extracted lathyritic rat type I collagen resulted in a slight increase in mobility on SDS–PAGE consistent with cleavage of the amino- and/or carboxy-terminal telopeptides (Starkey et al., 1977).

Treatment of Type IV Procollagen with Oxidants. It has been suggested that leukocyte-derived oxidants contribute to the degradation of matrix macromolecules, including collagen, in inflammatory reactions in vivo (Riley et al., 1985). There is also in vitro evidence to suggest that oxidants may directly degrade collagens and/or increase their susceptibility to proteolytic degradation (Curran et al., 1984; Kerr et al., 1985; Riley & Kerr, 1985). In addition, leukocyte-derived oxidants, including H_2O_2, have been identified at sites of neutrophil contact with glomerular basement membranes under conditions leading to basement membrane degradation in vitro (Vissers & Winterbourn, 1984; Vissers et al., 1980). We, therefore, examined the effects of H_2O_2 and neutrophil-derived myeloperoxidase (MPO) on type IV PC and its sensitivity to subsequent degradation by HLE.

Type IV PC was not degraded during prolonged incubation (up to 24 h) with H_2O_2 (0.005–5 mM) at 37 °C (Figure 5, lanes 2 and 3). Specifically, there was no release of TCA-soluble radioactivity and no detectable decrease in the intensity of proα bands following SDS–PAGE and fluorography. In addition, substrates incubated for 4 h at concentrations >0.5–5 mM were resistant to CNBr cleavage, consistent with oxidation of methionyl residues. Incubation of type IV PC with MPO (20 milliunits or 130 μM) for up to 8 h at 37 °C resulted in no detectable degradation in the presence of chloride and in the presence or absence of H_2O_2. Interestingly, incubation of type IV PC with MPO in the presence of 0.05–0.5 mM

H_2O_2 resulted in the generation of nonreducible dimers and higher aggregates of proα chains, consistent with oxidative cross-linking. At higher H_2O_2 concentrations we observed no evidence of cross-linking, suggesting oxidative inactivation of MPO. In fact, there was >90% reduction in peroxidase activity following incubation of purified MPO with >0.5 mM H_2O_2 for 5 min at 37 °C. In order to determine whether peroxide-mediated oxidation might alter the susceptibility of type IV collagen to cleavage by HLE, substrate was preincubated with up to 5 mM H_2O_2 for 8 h at 37 °C. This oxidative pretreatment did not significantly alter the extent or pattern of cleavage (e.g., Figure 5, lanes 4–7).

Discussion

Native soluble human type IV PC is a substrate for human neutrophil elastase in vitro. Our results indicate that type IV PC is initially cleaved within the globular carboxy-terminal domains of the proα1 and proα2 chains and subsequently undergoes stepwise internal cleavage of the proα1 and proα2 chains.

The amino acid sequences recognized by HLE within the central helical domain appear to be quite different from that observed for murine sarcoma collagenase and monocyte type IV collagenase (Fessler et al., 1984; Garbisa et al., 1986). The sarcoma collagenase shows selective symmetric cleavage within both chains near a "kink" region within the amino-terminal portion of the molecule. By contrast, internal degradation of type IV PC heterotrimers with HLE proceeds by asymmetric cleavage of the proα1 and proα2 chains with the generation of multiple overlapping α1- and α2-derived fragments. Since the major cleavage products are similar in size to the major pepsin-resistant fragments of human type IV collagen [see, e.g., Crouch et al. (1980) and MacWright et al. (1983)], internal cleavage probably occurs near interruptions within the -Gly-X-Y- sequence which have previously been shown to be sites of preferred peptic cleavage [see, e.g., Schuppan et al. (1980)]. Thus, the complex pattern of cleavage products generated by HLE in part reflects differences in the distribution of interrupted and protease-sensitive sequences within the proα1 and proα2 chains (Schwarz et al., 1986). Such interruptions may also provide sites for cleavage by neutral proteases, such as the mast cell chymase (Sage et al., 1979; Crouch et al., 1980).

Type IV molecules spontaneously associate in vitro to form extended aggregates stabilized by end-to-end interactions involving the amino- and carboxy-terminal domains (Bachinger et al., 1982; Duncan et al., 1983; Timpl et al., 1981; Yurchencho & Furthmayr, 1984). There is increasing evidence that these interactions contribute to the structural organization and mechanical properties of basement membranes in vivo [see, e.g., Dixit et al. (1981) and Weber et al. (1984)]. Timpl et al. (1981) have proposed that type IV molecules form an extended network mediated by homologous end-to-end interactions, i.e., by antiparallel association of four amino-terminal domains and the dimeric association of carboxy-terminal domains. These interactions are subsequently stabilized by intermolecular covalent cross-links. Yurchenco and Furthmayr (1984) have proposed an alternative model in which type IV dimers associated by their carboxy-terminal domains interact to form ordered polygonal arrays. In this model, the carboxy-terminal domains are allowed to participate in lateral interactions between collagen molecules, whereas the amino-terminal domains are free to mediate interactions between layers of the polygonal lattice. Significantly, both models ascribe important roles for the noncollagenous carboxy-terminal domains. Thus, cleavage of the carboxy-terminal domains of the proα1(IV) and proα2(IV) chains by HLE may be particularly effective in disrupting, or preventing, the assembly of basement membrane structures. Degradation of these domains may be more damaging than limited asymmetric internal cleavage, which might leave the overall structural organization of the basement membrane intact.

It is important to be cautious in extrapolating from in vitro studies of protease sensitivity to events at sites of inflammation and leukocyte migration and activation in vivo. For example, protease–sensitive sites may be masked (or exposed) by self-aggregation or heterologous intermolecular interactions, as well as by alterations in substrate conformation. In addition, high concentrations of tissue or plasma protease inhibitors (e.g., α1-proteinase inhibitor, α2-macroglobulin) may limit degradation by leukocyte-derived proteinases except within protected extracellular compartments in vivo (Campbell, 1986). Discharge of leukocyte granules into such microenvironments could also provide a mechanism for achieving high concentrations of active HLE or other leukocyte-derived protease (e.g., cathepsin G) at localized sites of basement membrane degradation.

Oxidation of type IV PC with H_2O_2 did not alter the extent or pattern of degradation by neutrophil elastase. Furthermore, there was no evidence for the direct oxidative degradation of type IV PC, despite prolonged incubation with H_2O_2 at concentrations as high as 50 mM in the presence or absence of MPO. Previous studies have described the degradation of fibronectin and other proteins by H_2O_2 (Fligiel et al., 1984). On the other hand, Weiss et al. (1984) observed that degradation of endothelial cell-derived matrix by stimulated neutrophils was primarily dependent on HLE and was not influenced by inhibitors of various oxygen metabolites. Although it will be important to examine the possible effects of hydroxyl radical or superoxide anion, the present data suggest that oxidation does not play an important role in leukocyte-mediated degradation of type IV collagen.

In summary, we have shown that native, soluble type IV procollagen is a substrate for HLE and that HLE preferentially degrades type IV within the carboxy-terminal domains. We suggest that these domains may be preferentially lost or degraded at sites of inflammation in vivo, resulting in the disruption of intermolecular interactions important for normal basement membrane structure and function.

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