Cleavage of native type I collagen by human neutrophil elastase

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The ability of purified human neutrophil elastase (EC 3.4.21.37) to cleave native type I collagen has been investigated. Soluble human, bovine or rat type I collagen was incubated with neutrophil elastase for 16 h at 25 °C before catalysis was stopped with 3,4-dichloroisocoumarin. Analysis by SDS/PAGE of the collagen digests revealed 3/4-length fragments similar in size to those produced by interstitial collagenase. The collagenolytic activity was dose dependent and was not due to a contaminating metalloproteinase or cysteine proteinase, as it was not inhibited by 1,10-phenanthroline, EDTA or L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane. The identity of the cleavage products was confirmed using a new antibody that recognizes the unwound α2(I)-chain. This detected the 3/4-length fragment of type I collagen following neutrophil elastase cleavage. In addition to cleaving soluble collagen, neutrophil elastase also cleaved reconstituted, radiolabelled type I collagen fibrils, at a rate of 16 μg/min per nmol. These results indicate that neutrophil elastase can cleave native type I collagen in the helix, an activity that might contribute to its roles in connective-tissue pathology.

INTRODUCTION

Type I collagen is the most abundant protein in the body. It constitutes more than 95% of total collagen in bone, cornea, dentin and tendons and more than 85% of that of dermis, gingiva and heart valves [1]. Owing to its covalently cross-linked fibrillar structure, it provides mechanical support and maintains structural integrity and tensile strength. The breakdown of type I collagen is important in physiological processes such as wound healing and bone resorption and pathological processes such as tumour metastasis, tendonitis and rheumatoid arthritis, and often collagenolysis is associated with inflammation and neutrophil recruitment [2].

The mechanism of degradation of collagen has been the subject of considerable interest, since in the native state it is resistant to attack by most proteolytic enzymes. It has been shown that extracellular breakdown of fibrillar collagen helices at neutral pH is accomplished by the action of at least three specific collagenases: interstitial collagenase [matrix metalloproteinase (MMP)-1; EC 3.4.24.7], neutrophil collagenase (MMP-8; EC 3.4.24.34) and collagenase-3 (MMP-13; EC 3.4.24.–) [3–6]. They all initially cleave at a specific Gly-Leu/Ile bond to generate characteristic 1/4 and 3/4 fragments that are then degraded further by the collagenase itself [7] as well as by gelatinolytic enzymes, such as gelatinases A (EC 3.4.24.24) [8] and B (EC 3.4.24.35) [9], neutrophil elastase (NE) [10] and plasmin (EC 3.4.21.7) [11] at neutral pH and cathepsins B (EC 3.4.22.1) [12] and L (3.4.22.15) [13] at acidic pH. Under normal conditions (except in bone), the intracellular route for the degradation of type I collagen mediated by lysosomal cysteine proteinases prevails, but in periods of accelerated breakdown, such as in inflammation, morphogenesis and metastasis, the extracellular route becomes important [2].

Neutrophils provide host defence mechanisms against bacterial and fungal infection, but they can also cause pathological tissue destruction mediated by the release of a complex assortment of agents that can be divided into two groups, which correspond to their localization to either of two sites in the cell: the plasma membrane or the intracellular granules. The plasma membrane is the site of the enzyme, NADPH oxidase, that participates directly in the generation of at least three oxygen metabolites: superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$) [14]. The intracellular granules, on the other hand, contain proteolytic enzymes. The granules can further be divided into primary and secondary; the primary (azurophil) granules contain large quantities of the serine proteinases, NE, cathepsin G (EC 3.4.21.20) and myeloblastin (EC 3.4.21.76). The secondary (specific) granules contain neutrophil collagenase and urokinase-type plasminogen activator (EC 3.4.21.73). A third small vesicular organelle (tertiary granule) contains gelatinase B [15]. Upon neutrophil activation, degranulation occurs and proteolytic enzymes are released.

NE is implicated in several pathological conditions, including obstructive pulmonary diseases, such as emphysema and bronchiectasis [16,17], and rheumatoid arthritis [18]. Owing to its broad specificity, it can degrade many extracellular proteins, including elastin, proteoglycan, denatured collagens, fibronectin and laminin. It can also cleave native collagen type III in the helix [19] and has been shown to cleave the telopeptides of collagen types I, II [20] and III [19].

Starkey [20] has investigated the cleavage of type I collagen by NE and clearly demonstrated telopeptide cleavage, but produced no direct evidence for helical cleavage. We have now re-investigated this question, and to this end have made a new anti-peptide antibody, which we show recognizes denatured forms of type I collagen and which we have used in this study to identify collagen fragments following proteolytic cleavage.

MATERIALS AND METHODS

Materials

Recombinant human MMP-1 (interstitial collagenase) was kindly given by Professor T. E. Cawston (University of Newcastle,

Abbreviations used: 3,4-DCI, 3,4-dichloroisocoumarin; AP, alkaline phosphatase; CM, carboxymethyl; E-64, L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane; MMP, matrix metalloproteinase; NE, neutrophil elastase; SBTI, soya-bean trypsin inhibitor; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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UK.) Native human and bovine type I and type III collagens, soya-bean trypsin inhibitor 3,4-dichloroisocoumarin *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (EC 3.2.21.4), DNase I from bovine pancreas (EC 3.1.21.1), CNBr-activated Sepharose 4B, carboxymethyl (CM)-Sepharose CL-6B, complete and incomplete Freund's adjuvant, *Clostridium histolyticum* collagenase (EC 3.4.24.3), L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane (E-64), EDTA, and disodium *p*-nitrophenyl phosphate were all purchased from Sigma (Poole, Dorset, U.K.). Aprotinin was purchased from Bayer (Newbury, Berks., U.K.). Purified human NE was obtained from Calbiochem (Nottingham, U.K.) or prepared as described below. Ac-Ala-Ala-Ala-pNa was purchased from Bachem Feinchaemikalien AG (Bubendorf, Switzerland). Alkaline phosphatase (AP)-conjugated goat anti-rabbit Ig was purchased from Southern Biotechnology (Birmingham, AL, U.S.A.). Coomassie Brilliant Blue R-250, nitrocellulose membranes, and AP-substrate solution kit were all purchased from Bio-Rad Laboratories (Hemel Hempstead, Herts., U.K.). 24-well plates were purchased from Costar (High Wycombe, Bucks., U.K.). All other reagents were of analytical grade.

Preparation of type I collagen

Native rat type I collagen was isolated from tail tendons as described previously [21].

NE purification and activity assay

NE was purified from an empyema according to the methods of Baugh and Travis [22], and Heck et al. [23]. In brief, 250 ml of pus from one patient was mixed with 250 ml of 4 % (v/v) Triton X-100 and stirred for 3 h at 4 °C, adjusted to 1 M NaCl/1 % (v/v) Triton X-100 and to a final volume of 1 litre, and then centrifuged at 50000 ***g*** for 30 min. The supernatant was dialysed against DNase buffer (0.05 M Tris and 35 mM $MgCl_2$, pH 7.5) and incubated with DNase (0.4 μM) at 37 °C for 5 h. It was centrifuged at 50000 ***g*** for 20 min at 4 °C, and the supernatant was collected. A total of 50 ml was loaded on to a 10 ml column of CNBr-activated Sepharose coupled to aprotinin, at a rate of 15 ml/h. The column was eluted with 0.05 M sodium acetate/1 M NaCl, pH 5.0, and 6 ml fractions were collected. Both NE and cathepsin G were eluted in a single peak, which was dialysed against binding buffer (0.05 M sodium acetate and 0.1 M NaCl, pH 5.5). A total of 60 ml of the dialysed pool was applied to a CM-Sepharose column (150 mm × 16 mm) at a rate of 40 ml/h. NE was eluted with 0.05 M sodium acetate/0.45 M NaCl, pH 5.5, followed by 0.05 M sodium acetate/1.0 M NaCl, pH 5.5, to obtain cathepsin G. The elastase concentration was determined using $A^{1\%}_{1cm,280}$ 9.85 [24]. The enzyme was stored at 4 °C in 30 mM sodium acetate/70 mM acetic acid, pH 5.0. Enzyme activity was assayed according to the method of Buttle et al. [25], using Ac-Ala-Ala-Ala-pNA as substrate. One unit of activity corresponded to the release of 1 nmol of 4-nitroaniline/min under these conditions. The specific activity of our NE preparation was 22 units/mg. In some experiments, a commercial preparation of purified NE was employed with essentially identical results.

Preparation of an anti-peptide antibody for the detection of denatured type I collagen

Criteria for selection of a suitable peptide sequence were the same as described previously [26]. The sequence from residues 133–154 of the α2(I)-chain, namely -Gly-Pro-Ala-Gly-Pro-Pro(OH)-Gly-Lys-Ala-Gly-Glu-Asp-Gly-His-Pro(OH)-Gly-Lys-Pro(OH)-Gly-Arg-, was predicted to be hydrophilic. This sequence, AH2W1, is located close to the N-terminus of the helix. It was synthesized with a C-terminal polylysine tree to act as a carrier [27] by Dr. Arthur Moir (Protein Synthesis Unit, The Krebs Institute, University of Sheffield, U.K.). New Zealand white rabbits were immunized five times at fortnightly intervals by intraperitoneal injection of 250 μg of the peptide emulsified with complete (first immunization) or incomplete (subsequent immunizations) Freund's adjuvant. Serum samples from the rabbits were then tested in an ELISA for reactivity with both immunizing peptide and denatured human type I collagen.

NE cleavage of soluble collagen

Triple-helical collagen was dissolved in 50 mM Tris/HCl/0.5 M NaCl/20 mM $CaCl_2$, pH 7.6, containing the indicated concentrations of collagen and enzyme at 25 °C unless otherwise stated. 3,4-DCI was reconstituted in ethanol and was used to stop the enzyme reactions at a final concentration of 100 μM. Under these conditions, the enzyme activity was abolished, and no cleavage of type I collagen nor type III collagen was detected by SDS–PAGE (results not shown). TPCK-treated trypsin was dissolved in the same buffer and used in control reactions at 25 or 37 °C. These reactions were stopped with SBTI (0.5 mg/ml). For comparative purposes, MMP-1 was used to cleave type I collagen. MMP-1 was activated by incubation with 1 mM *p*-aminophenylmercuric acetate for 1 h at 37 °C. Bovine type I collagen dissolved in 0.1 M Tris/HCl, pH 7.6, containing 10 mM $CaCl_2$ (2.5 mg/ml), was incubated with active MMP-1 for 40 h at 25 °C, then the enzyme was inhibited by 20 mM EDTA. The final molar ratio of collagenase to collagen was 1:50.

SDS–PAGE and immunoblotting

SDS–PAGE was performed according to Laemmli [28]. Collagen fragments were analysed on either 7.5 % or 10 % polyacrylamide gels under reducing or non-reducing conditions. The gels were stained with 0.2 % (w/v) Coomassie Brilliant Blue in 50 % (v/v) methanol/10 % (v/v) acetic acid and destained with 20 % (v/v) methanol/5 % (v/v) acetic acid. In some cases, the gels were scanned for densitometric analysis using the IS-1000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). The electrophoresed samples were transferred to nitrocellulose membranes and blocked for 1 h at ambient temperature with PBS/3 % (w/v) BSA (PBS/BSA) before immunoblotting. Blocked membranes were incubated for 1 h at ambient temperature with the rabbit anti-peptide antibody AH2W1 or control serum (see below) diluted 1:500 in PBS/3 % BSA. After three washes with PBS/0.1 % (v/v) Tween (PBS/Tween), the membranes were incubated for 30 min at ambient temperature with the AP-conjugated goat anti-rabbit second antibody, diluted 1:1000 with PBS/BSA. The membranes were washed three times with PBS/Tween and once with distilled water. AP-substrate solution was prepared from a commercial kit employing 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium. It was added to, and incubated with, the membranes at ambient temperature until optimal colour had developed. The reaction was then stopped by rinsing with distilled water.

Assay for fibrillar collagen degradation

This assay was carried out according to Cawston and Barrett [21] with some modifications. Briefly, aliquots (300 μl) of rat type I [^{3}H]collagen (a gift from Professor T. E. Cawston) in PBS (0.5 mg/ml) were allowed to polymerize at 37 °C overnight in 24-

well plates. NE (0.01 unit/ml; 0.5 μg/ml) was added to the fibrillar collagen in 400 μl of 50 mM Tris/HCl/0.5 M NaCl/20 mM $CaCl_2$, pH 7.6, and the mixtures were left at 37 °C overnight. Supernatants were analysed for the presence of soluble radiolabelled material by diluting 200 μl of the supernatant in 5 ml of scintillant and counting the radioactivity using a Beckman LS500C β-counter. The total counts per well were determined by digesting the residual, uncleaved collagen with *C. histolyticum* collagenase (1 mg/ml). The degree of contamination with non-fibrillar collagen was established by incubating three wells per plate with trypsin (1 mg/ml).

RESULTS

Cleavage of soluble type I collagen by NE

Human type I collagen (0.8 mg/ml) was incubated with 0.01 unit/ml (0.5 μg/ml) purified NE for 16 h, and the reaction was stopped by adding 3,4-DCI (100 μM). An incubation temperature of 25 °C was used to ensure that the collagen remained triple helical [29]. The collagen fragments were separated by SDS/PAGE. The results, shown as a representative experiment (Figure 1), demonstrate that NE cleaved human and bovine type I collagen, generating fragments similar in size to those of the

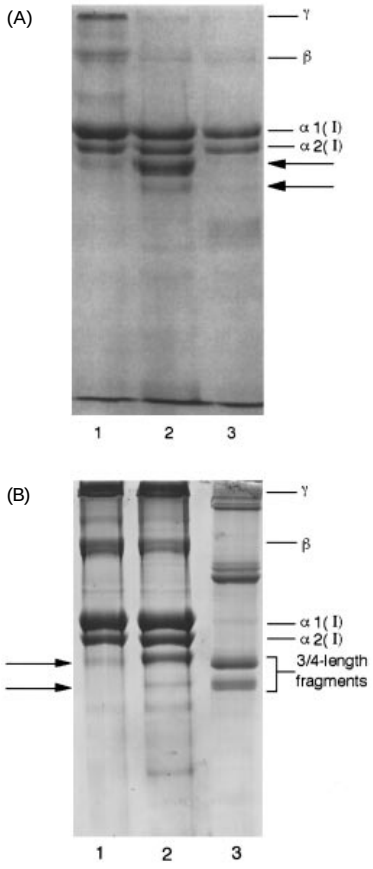

Figure 1 Degradation of human and bovine type I collagen by NE

Samples of human (**A**) or bovine (**B**) type I collagen were incubated with NE (0.01 unit/ml) or without enzyme for 16 h at 25 °C, and the reaction mixtures were electrophoresed on 7.5% polyacrylamide in the presence of SDS. (**A**) Lane 1, uncleaved type I collagen; lane 2, NE-cleaved type I collagen; lane 3, type I collagen cleaved initially by NE as above and then by 1 mg/ml trypsin for 16 h at 30 °C. (**B**) Lane 1, uncleaved type I collagen; lane 2, NE-cleaved type I collagen; lane 3, type I collagen cleaved by 100 nM MMP-1 for 40 h at 25 °C. In both panels, arrows indicate the major fragments generated by NE.

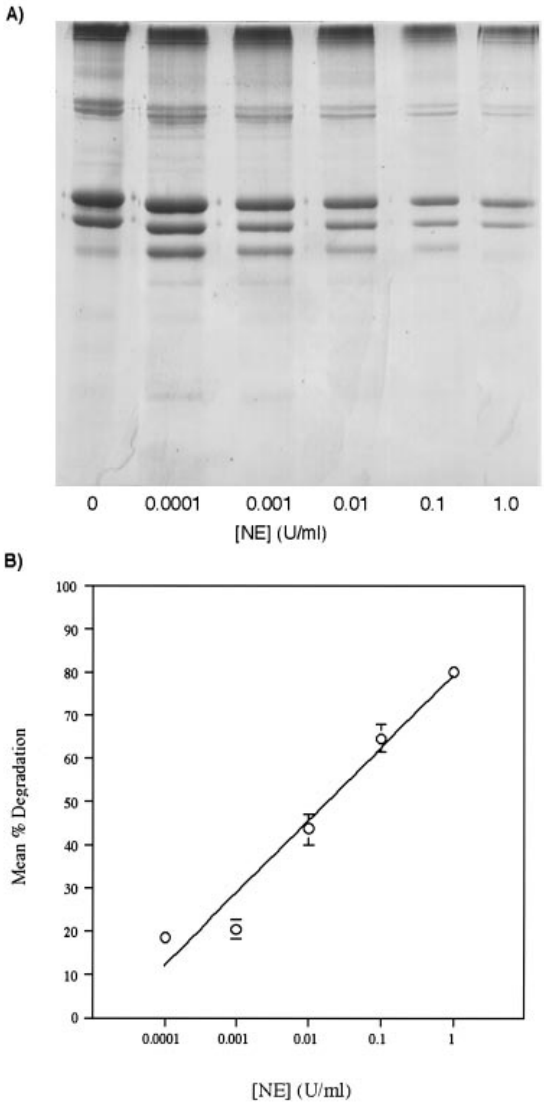

Figure 2 Dose-dependent collagenolytic activity of NE against soluble type I collagen

Human type I collagen was incubated with various concentrations of NE for 16 h at 25 °C. The reactions were stopped by adding 3,4-DCI (100 μM), and the reaction mixtures were electrophoresed on 6% polyacrylamide in the presence of SDS (**A**). Loss of total collagen at each NE concentration was determined by densitometric analysis (**B**). Abbreviation: U, unit.

3/4-length fragments produced by MMP-1 (see Figure 1B). No 1/4-length fragments could be detected, even on 12% gels (results not shown), presumably because they had undergone further degradation by NE. The amount of γ and β components was reduced, consistent with the previous finding that NE can cleave in the telopeptide region of type I collagen, thus removing the cross-links and generating more α-chains [20] and compensating for the loss of α chains through helical cleavage. In control experiments, collagen (0.8 mg/ml) incubated with trypsin (1 mg/ml) at either 25 or 37 °C was not detectably cleaved (results not shown).

In order to confirm that NE cleavage of type I collagen resulted in denaturation of the substrate, NE and type I collagen were incubated, the enzyme reaction was stopped with 3,4-DCI, as above, and the mixture was left for 40 min. DCI is labile in

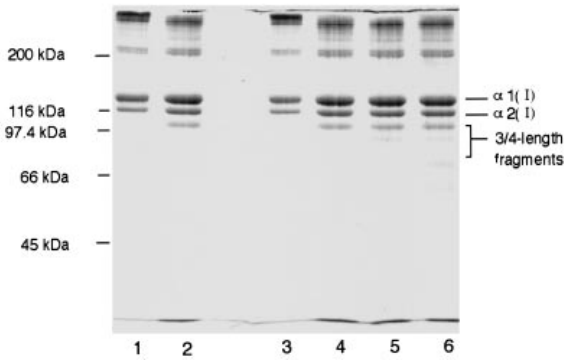

Figure 3 NE cleavage of type I collagen is not due to the presence of a contaminating proteinase

NE was pre-incubated with proteinase inhibitors, then used to digest collagen as described in the Materials and methods section. The reaction mixtures were then electrophoresed on 7.5% polyacrylamide in the presence of SDS. Lane 1, collagen without enzyme; lane 2, NE-cleaved collagen; lane 3, NE plus 1 mM 2,3-DCI; lane 4, NE plus 1 mM 1,10-phenanthroline; lane 5, NE plus 20 mM EDTA; lane 6, NE plus 1 mM E-64.

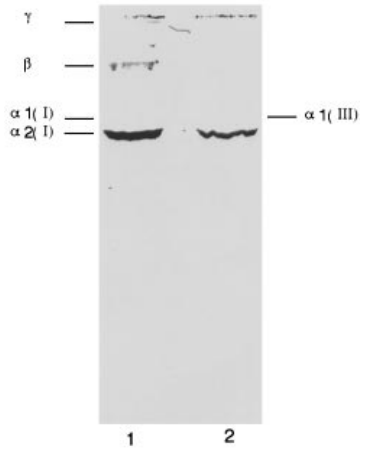

Figure 4 Specificity of antibody AH2W1

Human type I and type III collagens were heat denatured for 20 min at 80 °C, then electrophoresed on 7.5% polyacrylamide in the presence of SDS, transferred to a nitrocellulose membrane and immunoblotted with AH2W1 antibody. Lane 1, type I collagen; lane 2, type III collagen.

aqueous solution with a $t_{0.5}$ of 18 min [30]. Therefore, after 40 min the effective concentration of DCI would have fallen to approx. 20 μM. Trypsin (0.5 mg/ml) was then added and incubated with the cleaved collagen for 16 h at 30 °C, before this reaction was stopped with SBTI. The reaction mixture was analysed by SDS/PAGE. The result (Figure 1A, lane 3) clearly shows that trypsin was capable of degrading the elastase-generated 3/4 fragments without affecting the intact α-chains.

The dose dependence of type I collagen cleavage by NE was examined by densitometric scanning of SDS/PAGE gels. Human type I collagen was incubated with increasing concentrations of NE (0.0001 to 0.1 unit/ml). The cleaved collagen preparations were separated by SDS/PAGE, and loss of total collagen ($\alpha+\beta+\gamma$ chains) was measured. The representative gel shown in Figure 2(A) demonstrates extensive loss of α-chains at high enzyme concentrations. This gel and those from two identical

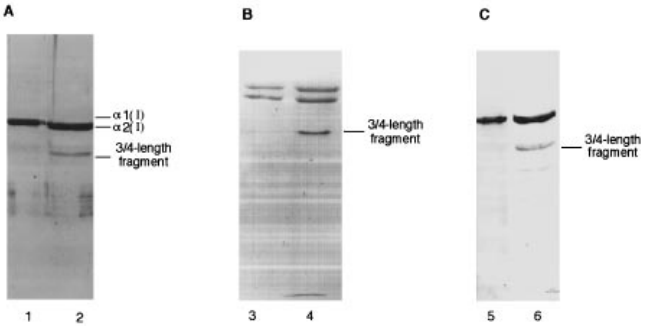

Figure 5 Identifying NE-cleavage products of human, rat and bovine type I collagen

Western immunoblots of collagen digested with NE using AH2W1 antibody were carried out as described in the Materials and methods section. Blots (**A**), (**B**) and (**C**) are human, rat and bovine collagen respectively. Lanes 1, 3 and 5, collagen control; lanes 2, 4 and 6, NE-cleaved collagen. All samples were heat denatured before electrophoresis.

experiments were scanned for semi-quantitative analysis. The results (Figure 2B) demonstrate a clear dose-dependent effect, which approximates to a straight line when plotted on a log-linear scale.

Effect of proteinase inhibitors on NE cleavage of type I collagen

Proteinase inhibitors were used to investigate whether the collagenolytic activity of our NE preparation was due to a contaminating MMP or cysteine proteinase. The serine proteinase inhibitor, 3,4-DCI at a concentration of 1 mM, fully inhibited the degradation of type I collagen by NE (Figure 3, lane 3).

Neutrophil collagenase (MMP-8) is known to cleave type I collagen efficiently, and we therefore considered it to be particularly important to rule out any effect of this enzyme or any MMP. To this end, the NE preparation was pre-incubated with 1 mM 1,10-phenanthroline or 20 mM EDTA. Neither of these inhibitors had any detectable effect on the cleavage of type I collagen by NE (Figure 3, lanes 4 and 5). Similarly, the cysteine proteinase inhibitor, E-64 (1 mM), had no effect (Figure 3, lane 6).

Characterization of the anti-peptide antibody AH2W1

The reactivities of the rabbit antiserum AH2W1 with human types I and III collagen were investigated by Western blotting. Collagen was dissolved in SDS/PAGE sample buffer at 2 mg/ml, heat-denatured and loaded at 10 μg/well. The antibody specifically detected the α2(I)-chain of human type I collagen but not the α1(I)-chain; the α1(III)-chain of human type III collagen was also not detected (Figure 4). The preparation of human skin type III collagen was clearly contaminated with type I collagen, since an α2(I) chain was detected using AH2W1 (Figure 4, lane 2).

The immunological detection of NE-cleavage products of human, bovine and rat type I collagen

NE is known to cleave human type III collagen specifically in the triple helix near the collagenase cleavage site, thereby generating 1/4- and 3/4-length fragments [19]. Since many preparations of human type I collagen also contain type III collagen, we have made use of the AH2W1 antibody to probe for fragments of type I collagen generated by NE. The results of human, rat and bovine NE-digested type I collagen are shown in Figure 5. The

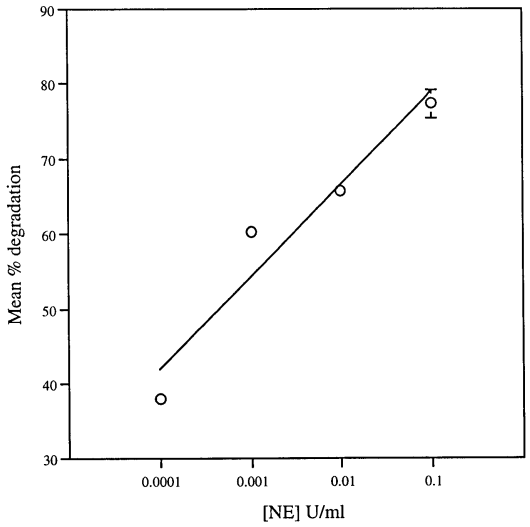

Figure 6 Dose-dependent collagenolytic activity of NE against fibrillar type I collagen

24-well plates were coated with radiolabelled collagen as in the Materials and methods section. NE was added to different wells, in triplicate, at various concentrations and incubated at 37 °C for 16 h. Supernatants were analysed for the presence of soluble radiolabelled material, and the results expressed as percentages of release of total collagen. The mean value of radiolabelled collagen released by trypsin was subtracted from all experimental values. Abbreviation: U, unit.

Table 1 Quantification of fibrillar type I collagen degradation

Aliquots (300 μl) of rat radiolabelled type I collagen (0.5 mg/ml) in PBS were allowed to polymerize at 37 °C overnight into 24-well plates. NE (0.01 unit/ml; 0.5 μg/ml), clostridial collagenase (1 mg/ml), and trypsin (1 mg/ml) were added to the fibrillar collagen in 400 μl of 50 mM Tris/HCl/0.5 M NaCl/20 mM $CaCl_2$, pH 7.6, and the mixtures were left at 37 °C for 16 h. Supernatants were analysed for the presence of soluble radiolabelled material. Their results are normalized to 100% release by clostridial collagenase. Values are means ± S.D. of triplicate wells.

Treatment	Inhibitor	Radiolabelled-collagen release per well (%)
Clostridial collagenase	—	100
Control (buffer)	—	8 ± 0.05
Trypsin	—	21 ± 0.02
NE	—	88.2 ± 0.81
NE	1,10-Phenanthroline	86.7 ± 1.3

AH2W1 antibody specifically detected the uncleaved, denatured α2(I) chain. In the NE-cleaved collagen preparations there was a second band (approx. 78 kDa), equivalent to that of a 3/4-length fragment. This band was generated using type I collagen from all three species. In our rat collagen preparation, antibody AH2W1 cross-reacted with an epitope in the uncleaved, denatured α1(I) chain. This epitope was apparently degraded by NE, since no additional fragments were seen in the NE-cleaved rat collagen. The result with this antibody confirms that the 3/4-fragment was derived from type I rather than type III collagen.

Collagen fibril degradation by NE

We considered it important to determine whether NE was capable of cleaving polymerized collagen fibrils. In three separate experiments, fibrillar collagen was incubated with increasing concentrations of NE (0.0001 to 0.1 unit/ml). There was a dose-dependent release of degraded collagen (Figure 6). Over 16 h, 67% of the fibrillar collagen (after subtraction of the trypsin control value) was degraded by 0.01 unit/ml NE (Table 1), which is equivalent to a rate of 16 μg/min per nmol. NE pre-incubated with 1,10-phenanthroline (100 μM) gave the same result as NE alone (Table 1). This further confirms that the collagenolytic activity seen is due to NE and not to a contaminating metalloproteinase.

DISCUSSION

Our results clearly demonstrate that NE is capable of cleaving within the helical portion of native type I collagen under conditions that preclude any helix denaturation [29]. To our knowledge this is the first demonstration of this potentially important activity. The collagenolytic action of the NE preparation was not due to a contaminating neutrophil collagenase, as confirmed by the lack of any inhibition by 1,10-phenanthroline or EDTA. The fragments were clearly generated from type I collagen and not type III collagen, as shown by immunoblotting with the α2(I)-chain-specific antibody AH2W1, as well as by the use of rat-tail type I collagen as a substrate in some experiments. Unlike skin or placenta, acid-extracted collagen from rat-tail tendons does not contain detectable amounts of type III collagen.

Previous studies have provided contradictory evidence regarding the ability of NE to cleave type I collagen in the helix. Starkey [20] described the presence of transient collagen fragments following NE cleavage and suggested that these were due to helical cleavage of type I collagen. However no attempt was made to characterize these fragments, which could have resulted from cleavage of contaminating type III collagen molecules in the rabbit-skin type I collagen preparation that was used. Other authors have failed to observe cleavage of the type I collagen helix by NE [19,31,32]. In agreement with Starkey [20], our data suggest that the type I collagen helical fragments are transient, since they often appeared as faint bands in SDS/polyacrylamide gels or immunoblots. This is presumably due to subsequent gelatinolytic activity of NE and suggests that the NE-generated fragments are unstable, even at 25 °C. An alternative explanation is that NE may cleave intact helical collagen at multiple sites in the α2(I) chain. However, we could only detect one fragment with the antibody AH2W1.

Loss of α-chains due to cleavage was not always observed, because they were continuously replaced by telopeptide cleavage of the β and γ components, as shown by densitometric scanning of the bands. For this reason, it can sometimes be difficult to observe the helical cleavage of type I collagen in Coomassie Brilliant Blue-stained gels. However, the use of our antibody, which does not cross-react with type III collagen, clearly demonstrates the production of a fragment following NE cleavage within the helical region.

No 1/4-length fragments could be detected in our experiments. Collagenase-generated 3/4 and 1/4 pieces have been shown to be less thermally stable than the uncleaved molecule, having the T_m reduced by 4 °C and 7 °C respectively [33]. Danielsen suggested that any modifications to these fragments would affect their thermal-stability characteristics [34]. We therefore presume that the NE-generated 1/4 fragment is more susceptible to denaturation than the 3/4 fragment, allowing NE to act as a gelatinase to cleave further the 1/4 fragment. Modification of the type III collagen 1/4 fragment by NE has been demonstrated previously [19]. In the absence of any detectable 1/4 fragment, we were unable to identify the exact NE-cleavage site. However, epitope AH2W1 is located close to the N-terminus of the α2(I)-

chain and is present in the NE-generated fragment of molecular mass of approx. 78 kDa. We can therefore infer that the NE cleavage site is located at approximately the position of the collagenase cleavage site.

NE (0.01 unit/ml) cleaved rat type I collagen fibrils at 37 °C at a rate of 16 μg/min per nmol. Knäuper et al. [6] used essentially the same rat collagen-fibril assay as that described here to study hydrolysis by collagenases. Fully activated interstitial collagenase (MMP-1) cleaved the fibrils at a rate of 120.5 μg/min per nmol, whereas partially active and fully activated neutrophil collagenase (MMP-8) cleaved at 106.7 and 338.5 μg/min per nmol respectively. NE is therefore 10–20 times slower than these collagenases at cleaving native type I collagen. However, this does not preclude a pathological role for NE in chronic inflammatory conditions, when concentrations of the enzyme may be very high [35].

We have found that cleavage of the type I collagen triple helix by NE was slower than cleavage of the type III collagen helix, and that type II collagen was not detectably cleaved in its helix (W. Kafienah, D. J. Buttle and A. P. Hollander, unpublished work). This hierarchy of susceptibility to helical cleavage by NE (III > I > II) may reflect the relative degree of laxity of the triple helix in the region of the collagenase cleavage site [36,37], which is relatively rich in imino acid residues. Native type III collagen can be cleaved in this region by trypsin [38], an enzyme that has no effect on the native helix of collagen types I and II.

The neutrophil has been implicated as a mediator of tissue-destructive events in inflammatory diseases ranging from rheumatoid arthritis and myocardial reperfusion injury to respiratory distress syndromes, blistering skin disorders and ulcerative colitis [17], and in wound healing [2]. In addition to free-radical-mediated destruction of tissues, neutrophil primary granules contain at least three proteinases that have the potential to degrade the extracellular matrix: the serine proteinases NE, cathepsin G and myeloblastin. Acting together, these enzymes have the capacity to destroy all the extracellular-matrix proteins, including elastin, proteoglycans, fibronectin, laminin and various collagens. Our results show that NE is capable of extensively degrading reconstituted fibrillar collagen. Since soluble, monomeric collagen exists at negligible levels in the stroma, fibrillar collagen is a more relevant biological substrate [1]. Extrapolating *in vitro* studies of protease sensitivity to events at sites of inflammation and leukocyte migration and activation *in vivo*, however, needs further examination. Clearly, high concentrations of tissue or plasma proteinase inhibitors (e.g. α1-proteinase inhibitor, α2-macroglobulin) may limit degradation by leukocyte-derived proteinases *in vivo*. However, discharge of neutrophil granules into a protected extracellular microenvironment [17,39] could result in high concentrations of active NE at these sites [32].

In conclusion, the ability of NE to attack the helical region of type I collagen reveals a potential mechanism for the degradation of the extracellular matrix of most connective tissues in inflammatory conditions.

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