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Cartilage Degradation by Stimulated Human Neutrophils: Elastase Is Mainly Responsible for Cartilage Damage

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Although neutrophilic granulocytes are assumed to contribute to cartilage degradation during rheumatic diseases, there is still a discussion whether reactive oxygen species (ROS) or proteolytic enzymes that are both released by the neutrophils are most relevant to cartilage degradation. To gain further insight into these processes, an *in vitro* approach to study the interaction between the products of stimulated neutrophilic granulocytes and cartilage was used: Neutrophils from the blood of healthy volunteers were treated with different stimulators (e.g., Ca^{2+} ionophores) in order to induce degranulation. Supernatants of neutrophils were afterward incubated with thin slices of pig articular cartilage. Some experiments were also performed in the presence of selected enzyme inhibitors. Supernatants of cartilage were subsequently assayed by one- and two-dimensional high-resolution proton NMR spectroscopy, and the content of soluble carbohydrates in the supernatant was additionally determined by biochemical methods. The selective inhibition of elastase decreased most significantly the extent of cartilage degradation, whereas all other inhibitors had much smaller effects. These results were additionally confirmed by measuring the effect of isolated elastase on articular cartilage in the absence and presence of different inhibitors. It is concluded that elastase released [EC 3.4.21.37] by neutrophils is the most relevant enzyme for cartilage degradation.

Key Words: cartilage degradation; polymorphonuclear leukocytes; NMR spectroscopy; elastase; arthritis; joint degeneration.

INTRODUCTION

Degenerative joint diseases like rheumatoid arthritis and osteoarthritis are a major cause of disability and early retirement in industrialized countries and, therefore, of great socioeconomic significance. Unfortunately, mechanisms of cartilage degradation are not yet completely understood (*1*).

Since polymorphonuclear leukocytes (PMNs) occur in large numbers in the synovial (joint) fluids of patients suffering from rheumatic diseases (*2*), these cells are nowadays assumed to possess considerable relevance to cartilage degradation. For instance, it was shown that the pannus tissue that is formed in earlier stages of rheumatic diseases

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is rich in PMNs (*3*). Additionally, products of neutrophils were shown to be able to destroy the collagen moiety (*4*) as well as the polysaccharides of cartilage (*5*).

During joint inflammation, PMNs invade the joint space and release upon stimulation different cartilage-damaging products. Proteolytic enzymes (*6*) and a variety of reactive oxygen species (ROS) (*7*) are both under discussion as mediators of cartilage destruction. Unfortunately, the large number of potential cartilage-damaging agents as well as the huge variety of possible targets within the cartilage layer (proteins, polysaccharides, and cartilage cells) complicate extremely the introduction of an appropriate model. An additional problem is the potential interaction between the ROS and the released enzymes (*8*).

Although experiments on the effects of enzymes like elastase, trypsin, and collagenase (*9*) or saccharidases (*10*), as well as ROS like HOCl (*11*) or hydroxyl radicals (*12*) on cartilage were performed, there are only a very few recent investigations dealing with the use of stimulated neutrophils (*13*). This is surprising since only these experiments would warrant an approach close to the *in vivo* conditions.

There are also some additional difficulties in the determination of the extent of cartilage degradation and the identification of the final products of the involved reactions. Cartilage consists of different proteins (mainly collagen as well as the link and the core proteins of the proteoglycans) and polysaccharides (mainly chondroitin-4- and chondroitin-6-sulfate as well as keratan sulfate and hyaluronan), the so-called glycosaminoglycans (GAG) (*14*). Since these individual components form a complex, highly ordered network, native cartilage polymers are insoluble. Under pathological conditions, however, degradation of the cartilage matrix—accompanied by the formation of soluble components with a lower molecular weight—occurs. Therefore, the determination of soluble proteins and polysaccharides in the cartilage supernatants was often used as a measure of cartilage degradation (*13,15*).

Unfortunately, all these assays provide only information on the concentration of cartilage degradation products but do not clarify the structure of these products (*13*). Especially small degradation products like acetate or formate, which are formed when the polysaccharides of cartilage react with hypochlorite (*11*) or hydroxyl radicals (*12*), respectively, are difficult to detect. Here, sophisticated methods like high-resolution NMR spectroscopy are more appropriate analytical tools (*16,17*).

In this investigation high-resolution NMR spectroscopy in combination with biochemical assays is used for the investigation of the extent of cartilage degradation induced by products of stimulated neutrophils. Selective enzyme inhibitors are used to suppress selectively the effects of enzymes released by the cells. From the obtained results it is concluded that primarily elastase is responsible for cartilage degradation since its selective inhibition diminishes the extent of cartilage degradation most significantly.

MATERIALS AND METHODS

Chemicals

Chemicals for buffer preparation (KH_2PO_4, Na_2HPO_4, and glucose), dimethyl sulfoxide (DMSO), D_2O (isotopic purity 99.96%), 3-(trimethylsilyl)-1-propionate (TSP), Alcian blue, guanidinium hydrochloride, and sodium formate were obtained in

the highest commercially available purity from Fluka Feinchemikalien GmbH (Neu-Ulm, Germany).

All chemicals for the stimulation of neutrophils (*N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and A23187) were obtained from Sigma (Deisenhofen, Germany). Both compounds were dissolved in DMSO to give 10^{-2} M stock solutions. The following enzyme inhibitors were used: methoxy-succinyl-Ala-Ala-Pro-Ala-chloromethyl ketone (Sigma), phenylmethylsulfonyl fluoride (Fluka), and diisopropylfluorophosphate (Fluka). All compounds were dissolved in DMSO to give 10^{-2} M stock solutions. All stock solutions were stored in the dark at 4°C.

For comparative purposes, purified elastase [EC 3.4.21.37] was purchased from Calbiochem (Bad Soden, Germany) and used without further purification.

Cartilage Preparation

Pig articular cartilage was obtained from the knee joints of juvenile pigs (about 12 months old and without traces of disease) within a few hours after slaughter. After removal of the bone, the cartilage was cut into small pieces and immediately used as the substrate for the agents released by PMNs (*13*).

Cell Preparation and Oxidative Activity

Polymorphonuclear leukocytes were isolated from heparinized (10 IU/ml) blood from healthy volunteers (*18*). The preparation included a dextran-enhanced sedimentation, Ficoll-Hypaque density centrifugation, lysis of remaining red blood cells with distilled water, and washing of cells with Hanks' balanced salt solution. PMNs were counted in a CASY cell counter (Schärfe System GmbH, Reutlingen, Germany) and were used at a concentration of 3×10^6 cells/ml (*13*).

Oxidative activity of PMNs was checked in all cases as a measure of cell viability by luminol-amplified chemiluminescence (*18*). These experiments were performed on a microplate luminometer MicroLumat LB 96 P (EG&G Berthold, Wildbad, Germany). PMNs (1×10^5 cells) were preincubated for 5 min with luminol (5×10^{-5} M, final concentration) at 37°C. All chemicals were added immediately before starting the measurement.

Incubation of Cartilage Specimens with Cell Supernatants

PMNs (3×10^6 cells/ml) were preincubated at 37°C for 5 min. Subsequently, cells were stimulated (15 min) with fMLP (10^{-6} M) in the presence of A 23187 (10^{-5} M). The indicated concentrations were found to yield a maximum of enzyme release. After 10 min of centrifugation (2500 rpm, 4°C), 0.5 ml supernatant of the neutrophil suspension was added immediately to freshly prepared cartilage slices (100 mg) and incubated at 37°C for different times. After incubation, samples were spun down to remove debris and insoluble material. The resulting, clear supernatants were used for subsequent analysis.

NMR Measurements

Proton-NMR measurements were performed on a Bruker DRX-600. Typically, 0.40 ml cartilage supernatant was placed in a 5-mm-diameter NMR tube and 50 μl of D_2O was added to provide a field frequency lock. The intense water signal was

suppressed by the application of presaturation on the water resonance frequency. Usually, 128 free induction decays were acquired with a total delay of 8 s to allow full spin-lattice (T_1) relaxation of the protons (90° flip angle: 7 μs).

All spectra were recorded with a spectral width of about 13 ppm and 32K data points. No window functions were used prior to Fourier transformation. No zero filling was used. Chemical shifts were referenced to 2.5×10^{-4} M internal sodium 3-(trimethylsilyl)-1-propionate that also served as concentration standard (*19*). Although TSP is known to bind to proteins (*19*), we did not find major differences, equally if TSP or for means of comparison formate was used as concentration standard. Resonances were identified by their known chemical shifts and subsequent enhancement after addition of a small amount of the corresponding pure compounds. Homonuclear ^{1}H-^{1}H-correlation experiments (COSY) were performed using the standard pulse sequence provided by Bruker (*20*).

Alcian Blue Determination of Acidic Carbohydrates

The determination of acidic carbohydrates in cartilage supernatants was performed with the dye Alcian blue (*21*). A master curve was obtained using chondroitin sulfate from bovine trachea. Ten microliters of the corresponding cartilage supernatants was diluted with 90 μl water and mixed with 2 ml Alcian blue reagent (*13,21*). After complete precipitation of the polysaccharide–dye complex, the precipitate was redissolved in 4 M guanidinium hydrochloride. The intense blue color of the resulting solutions was determined spectrophotometrically at 601 nm (*13*).

RESULTS AND DISCUSSION

Establishing Working Conditions

There are two different ways that neutrophils damage native cartilage (*22*). The first is the release of proteolytic enzymes. Especially elastase is assumed to contribute to cartilage destruction, because of its comparably low molecular weight (34 kDa) and its high concentration in neutrophilic granules. It is assumed that elastase penetrates into the cartilage and smoothes the way for enzymes (e.g., collagenase) with higher molecular weights (*23,24*).

The second possibility is the release of reactive oxygen species. Here, the effects of $O_2^{\cdot -}$ (*25*), H_2O_2 (*26*), HOCl (*11,17*), and $HO^{\cdot}$ (*12*) were found in model experiments using cartilage slices or isolated components of cartilage. However, the value of these investigations is limited because concentrations of reactive oxygen species were used far above the physiologically relevant level. Since many ROS react with potential targets according to the principle of competition (*27*), a high ROS concentration may favor reactions that would never occur under *in vivo* conditions.

To overcome these problems and as an approach to mimic the *in vivo* conditions we have performed experiments with pig articular cartilage slices that were incubated with supernatants of stimulated neutrophils. In our opinion this experimental procedure is the most direct way to assess the potential synergistic effects of all agents released by the neutrophils (*13*).

In the presence of products released from the neutrophils, the insoluble polymers of cartilage are converted into soluble degradation products with lower molecular

weights. The determination of the concentration of proteins and carbohydrates in the supernatant of cartilage can, therefore, be used as a reliable, but simple, indicator of cartilage degradation.

Our first aim was to investigate to what extent differently stimulated neutrophilic granulocytes contribute to cartilage degradation and to what extent the concentration of carbohydrates in the cartilage supernatant changes with incubation time. We have used exclusively the Alcian blue determination of acidic carbohydrates since the release of carbohydrates from the cartilage seems to be a more reliable marker of cartilage destruction, whereas protein release is less specific (*13*).

Figure 1 shows the carbohydrate concentrations in supernatants of pig articular cartilage samples treated with supernatants of unstimulated (white bars) and A 23187-stimulated neutrophils (black bars) dependent on the incubation time. A 23187 was used because this compound is known to induce a marked degranulation from neutrophilic granulocytes. In the case of the unstimulated neutrophils only a very slight increase of the carbohydrate content is observed even at longer incubation times. This is a clear indication that unstimulated neutrophils release only low amounts of enzymes.

In contrast, when cartilage slices are treated with the supernatant of A 23187-stimulated neutrophils, a dramatic increase of the glycosaminoglycan concentration in the supernatant of the cartilage is detectable. Under these conditions, the carbohydrate concentration is after 4 h about five times higher than that of the unstimulated neutrophils. This is in agreement with the assumption that enzymes released from the neutrophils retain their activities for a longer time (*13*). These differences, however, make an exact time control for the incubation experiments with cartilage and neutrophils absolutely necessary. Although the applied Alcian blue assay works well for acidic carbohydrates and provides fast and reliable results, nearly all colorimetric

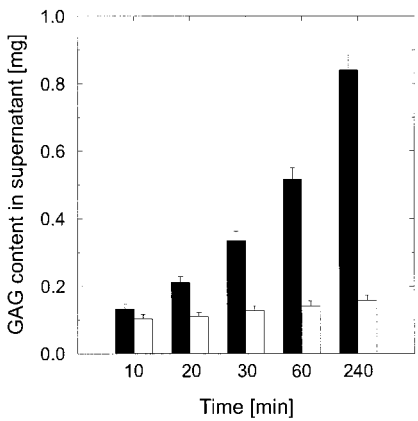


FIG. 1. Time-dependent release of glycosaminoglycans from cartilage slices (100 mg) incubated with unstimulated (white bars) and A 23187-stimulated (black bars) neutrophilic granulocytes (3×10^6 cells/ml) into the cartilage supernatant. The glycosaminoglycan (GAG) concentration was determined by the Alcian blue assay. Ten microliters of each solution was used for assaying the amount of GAG released into the supernatant. Means and standard deviations of three independent measurements are shown.

assays have the considerable disadvantage that the results are strongly influenced by the chemical structure of the analyte and especially by its molecular weight (*13*). This is still more pronounced for protein determinations (especially for the Bradford assay) and, therefore, we have not used protein determinations for the analysis of cartilage supernatants (*28,29*). Unfortunately, the Alcian blue determination of acidic polysaccharides is also influenced by the molecular weight of the glycosaminoglycans released from the cartilage (*13*). Therefore, the application of additional, more sophisticated methods to investigate the cartilage supernatants is advisable.

We have shown in a number of studies (*11–13,16,30*) that high-resolution NMR spectroscopy is a suitable method for the determination of degradation products of cartilage. This especially holds if those degradation products are relatively small. In Fig. 2 the ^{1}H NMR spectra of the supernatants of pig articular cartilage after incubation with supernatants of unstimulated (left) and A 23187-stimulated neutrophils (right) are shown. Spectra were recorded after 30 (a,e), 60 (b,f), 120 (c,g), and 240 min (d,h) of incubation and are both scaled in exactly the same way in reference to the TSP standard at 0.00 ppm (not shown).

The different signal to noise ratios of the spectra on the left and the right are caused by a methodological problem: In the presence of stimulated neutrophils, proteins and polysaccharides are released into the cartilage supernatant resulting in solutions of high viscosity. This confers enhanced linewidths and a lower quality of water suppression

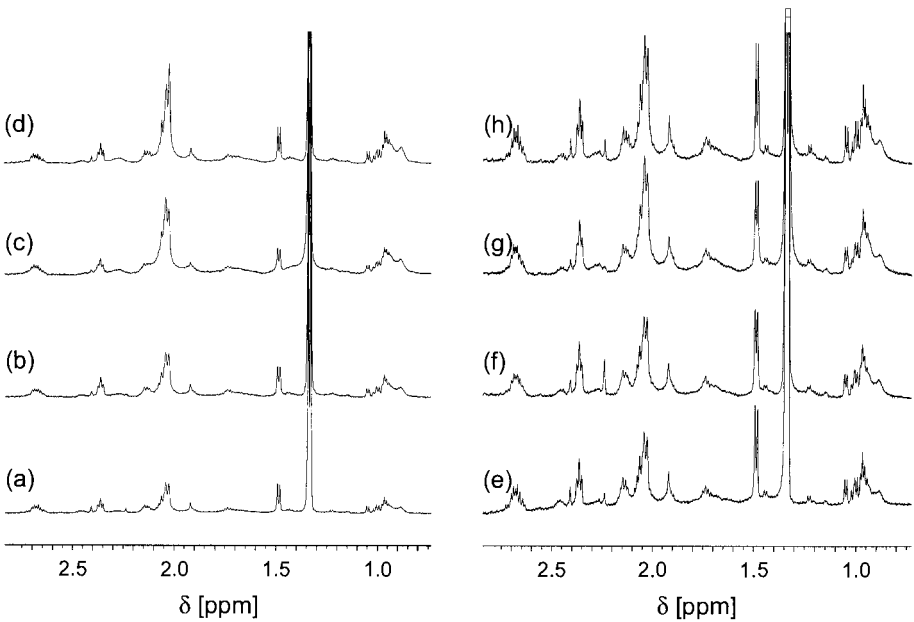


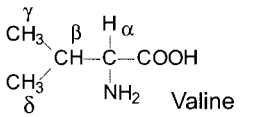
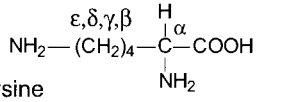
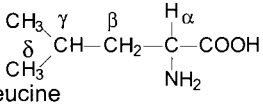
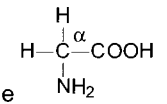
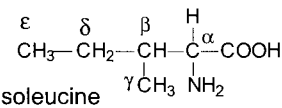
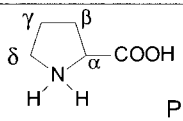
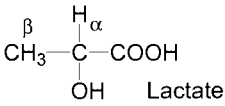
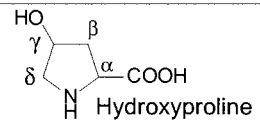
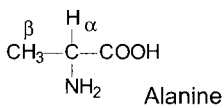
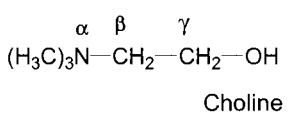
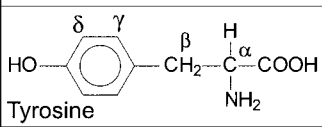
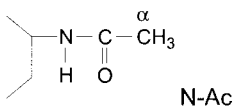
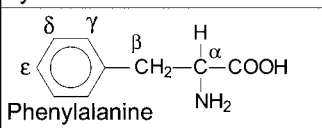
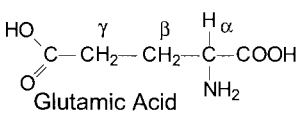
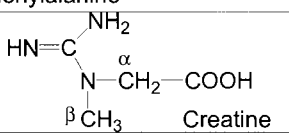
FIG. 2. ^{1}H NMR spectra of the supernatants of articular cartilage incubated with the supernatants of neutrophilic granulocytes (3×10^6 cells/ml). Spectra on the left were obtained with unstimulated cells, spectra on the right with A23187-stimulated cells. Spectra were recorded dependent on incubation time (a,e, 30 min; b,f, 60 min; c,g, 120 min; d,h, 240 min).

resulting in decreased signal to noise ratios. This affects all resonances to a comparable extent.

Spectra on the left and on the right differ considerably. Signal intensities (and, therefore, the metabolite concentrations) are higher in comparison to the TSP standard at 0.00 ppm if stimulated neutrophils are used. This is in good agreement with the data obtained by carbohydrate determination (cf. Fig. 1). A survey of important metabolites detectable in cartilage supernatants and the corresponding proton NMR chemical shifts are given in Table 1. Unfortunately, it was necessary in all cases to truncate the spectra and to show only a relatively small range of chemical shift: Since the applied buffer contains relatively high amounts (10^{-2} M) of glucose for the

TABLE 1

Chemical Structures and ^{1}H Chemical Shifts of Compounds Detectable in the NMR Spectra of the Supernatants of Articular Cartilage

 Valine	α = 3.61 β = 2.27 γ = 1.03 δ = 0.98	 Lysine	α = 3.74 β = 1.89 γ = 1.44 δ = 1.70 ε = 3.01
 Leucine	α = 3.75 β = 1.71 γ = 1.71 δ = 0.95 ε = 0.94	 Glycine	α = 3.56
 Isoleucine	α = 3.67 β = 1.97 γ = 1.00 δ = 1.46/1.25 ε = 0.92	 Proline	α = 4.13 β = 2.35/2.08 γ = 2.01 δ = 3.43/3.34
 Lactate	α = 4.12 β = 1.32	 Hydroxyproline	α = 4.35 β = 2.43/2.17 γ = 3.50 δ = 3.37
 Alanine	α = 3.79 β = 1.47	 Choline	α = 3.21 β = 3.52 γ = 4.06
 Acetate	α = 1.91	 Tyrosine	α = 3.89 β = 3.30/3.02 γ = 7.19 δ = 6.89
 N-Ac	α ~ 2.04	 Phenylalanine	α = 3.95 β = 3.27 γ = 7.31 δ = 7.41 ε = 7.37
 Glutamic Acid	α = 3.77 β = 2.06 γ = 2.34	 Creatine	α = 3.93 β = 3.03