Cartilage degradation by stimulated human neutrophils: reactive oxygen species decrease markedly the activity of proteolytic enzymes

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Background: Although neutrophilic granulocytes clearly contribute to cartilage degradation in rheumatic diseases, it is unclear if reactive oxygen species (ROS) or proteolytic enzymes are the most important components in cartilage degradation and how they interact.

Results: Neutrophils were stimulated by chemicals conferring a different degree of ROS formation and enzyme release. Supernatants of neutrophils were incubated with thin slices of pig articular cartilage. Supernatants of cartilage were assayed by NMR spectroscopy, MALDI-TOF mass spectrometry and relevant biochemical methods.

Stimulation conditions of neutrophils correlated well with the extent of cartilage degradation. Due to the release of different enzymes, cartilage degradation could be best monitored by NMR since mainly low-mass degradation products were formed. Astonishingly, the suppression of the formation of ROS resulted in decreased cartilage degradation.

Conclusion: ROS formed by neutrophils are not directly involved in cartilage degradation but influence the activity of proteolytic enzymes, which are the main effectors of cartilage degradation.

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Introduction

Degenerative joint diseases like rheumatoid arthritis or osteoarthritis are a major cause of disability and early retirement in industrialized countries and are, thus, of great socioeconomic significance. Unfortunately, detailed mechanisms of cartilage degradation processes are not yet completely understood [1].

Since polymorphonuclear leukocytes (PMNs) occur in vast numbers in the synovial fluids of patients suffering from rheumatic diseases [2], these cells are assumed to have the highest impact on cartilage degradation. For instance, neutrophils and neutrophil-derived products (especially proteases like elastase) contribute to the formation of the pannus tissue in rheumatoid arthritis [3] and are also able to destroy the collagen moiety [4] as well as the polysaccharides of cartilage [5].

In the course of joint inflammation, PMNs invade the joint space and release different cartilage-damaging products. In this context, proteolytic enzymes [6] as well as different reactive oxygen species (ROS) [7] are still under discussion as the main cartilage-destroying agents. A survey of the events leading to cartilage degradation by neutrophils under the formation of low-mass compounds is given in Figure 1. Unfortunately, the large number of cartilage-damaging agents as well as the huge number of possible targets within the cartilage layer (proteins, polysaccharides, and cells) complicate extremely the experimental behavior and the introduction of an appropriate model is difficult. An additional serious problem is the possible interaction between the ROS and the released enzymes [8].

Although experiments on the influence of cartilage-damaging proteases like elastase, collagenase [9] or saccharidases [10], as well as ROS like HOCl [11] or hydroxyl radicals [12] on cartilage were performed, there is still a lack of experiments using stimulated neutrophils. This is astonishing since only this would warrant an approach close to the in vivo conditions.

On the other hand, there are some additional difficulties in the determination of the extent of cartilage degradation and the final products of the reactions. Cartilage consists of a variety 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) [13]. Since these individual components form a complex, highly-ordered network, native cartilage polymers are in-

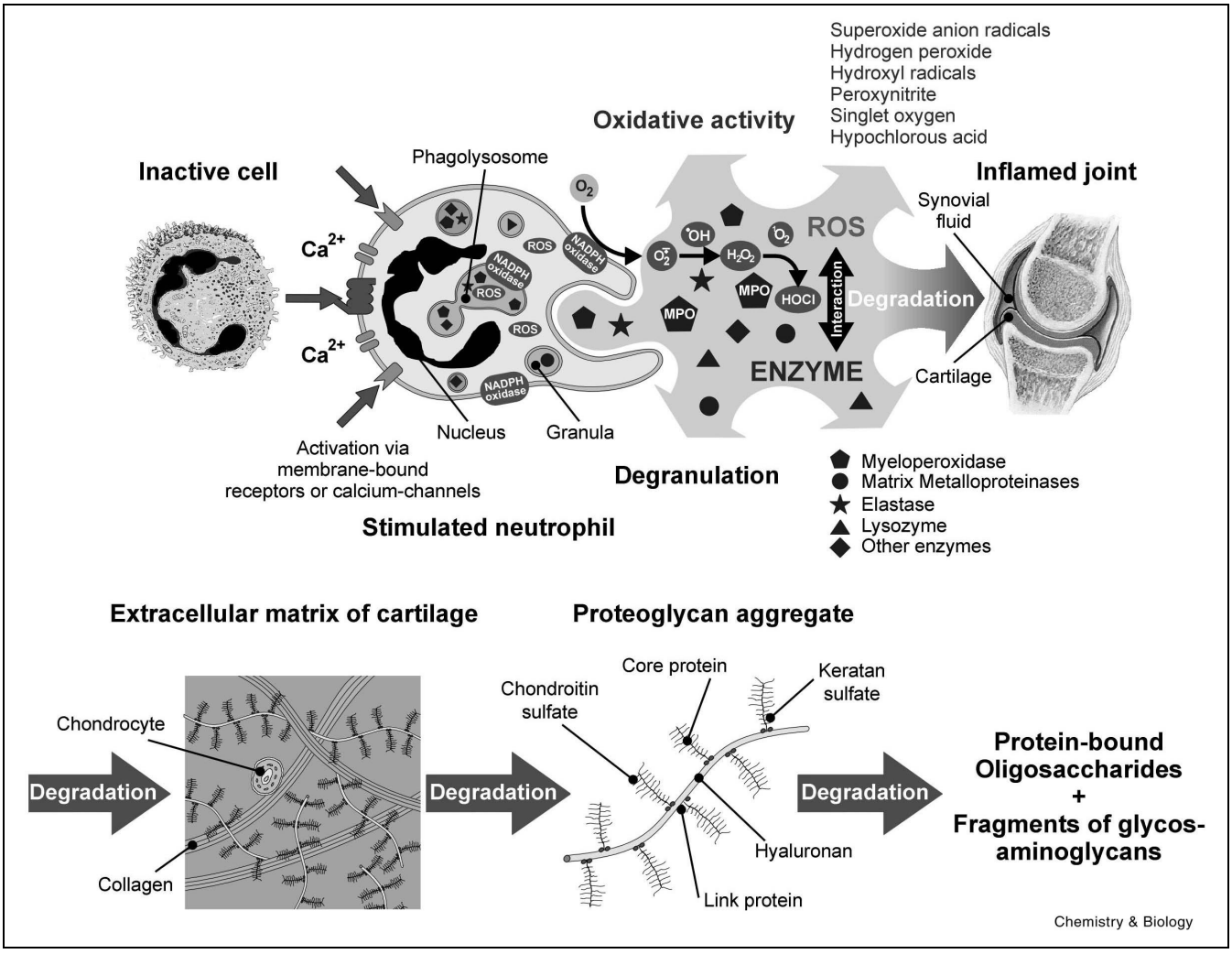

Figure 1. Proposed mechanism of cartilage degradation during rheumatic diseases: Neutrophils invade from the blood flow into the joint space. Upon stimulation they release different ROS and proteolytic enzymes. These damage-conferring products lead to the degradation of the high-mass components of articular cartilage under the formation of low-mass components.

soluble in water. Under pathological conditions, however, degradation of cartilage, accompanied by the formation of soluble components with a lower molecular weight, occurs. Therefore, the determination of soluble proteins as well as soluble polysaccharides in the cartilage supernatant has been often used as a measure of cartilage degradation [14].

Unfortunately, all these assays provide only an information on the total amount of soluble cartilage degradation products but do not analyze the chemical structure of such products. Especially, low-mass degradation products like acetate or formate, which are formed when the polysaccharides of cartilage react with hypochlorite or hydroxyl radicals, respectively, are not detectable. Here, sophisticated methods like high-resolution NMR spectroscopy are more appropriate analytical tools [15,16].

In the present investigation we will show that modern analytical techniques like NMR spectroscopy and mass spectrometry provide important information on the extent of cartilage degradation caused by products of stimulated neutrophils. Relatively long incubation times were chosen to emphasize the effects of proteolytic enzymes. It can be concluded from the experiments that mainly the enzymes released by PMNs are responsible for cartilage degradation. On the other hand, strong interactions between the enzymatic activity and the amount of released ROS were observed, whereby effects of enzymes towards cartilage were most pronounced in the absence of ROS. This fact

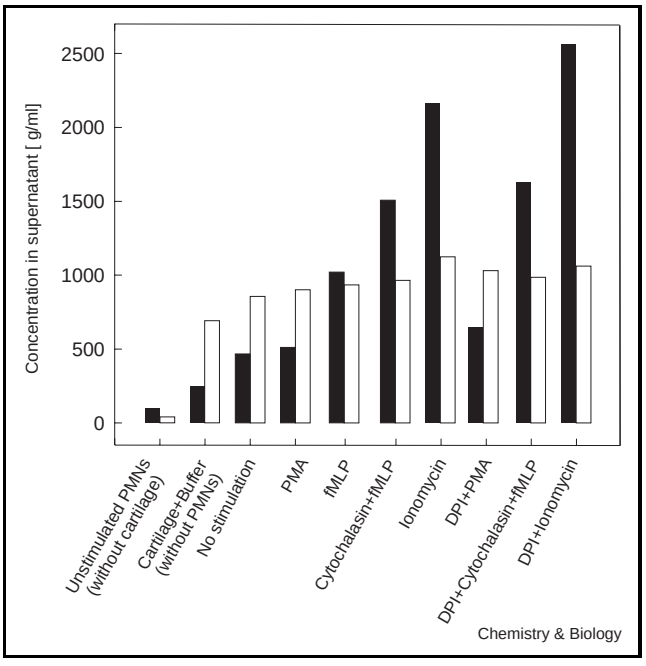

Figure 2. Carbohydrate (black bars) and protein (white bars) concentrations of the supernatants of articular cartilage (100 mg) incubated with the supernatants of neutrophilic granulocytes (4×10^6 cells/ml) which were stimulated by different reagents. 30 µl of each supernatant was used. The individual concentrations were calculated from the corresponding master curve obtained with chondroitin sulfate or bovine serum albumin (standard deviations are estimated to be about 5% in all cases, $n=3$). Abbreviations: PMNs, polymorphonuclear leukocytes; PMA, phorbol-myristate-acetate; fMLP, formyl-methionyl-leucyl-phenylalanine; DPI, diphenyliodonium chloride.

resembles closely the conditions described for patients with chronic granulomatous disease [8].

Results

It is most likely that the enzymes and ROS released by neutrophilic granulocytes damage the cartilage tissue under the formation of soluble degradation products. Thus, concentrations of proteins and carbohydrates in the supernatant should be reliable indicators of cartilage degradation. Figure 2 shows the carbohydrate (black bars) and protein concentrations (white bars) in supernatants of pig articular cartilage samples treated with supernatants of differently stimulated neutrophils.

In the absence of the cartilage (first bars), the supernatants of neutrophils do neither show significant protein nor carbohydrate concentrations. On the other hand, cartilage incubated with pure buffer shows a considerably enhanced amount of soluble carbohydrates and proteins. This is caused by the presence of denaturated and, thus, soluble collagen in the native cartilage samples. Because of this comparably high amount of soluble protein even in the absence of neutrophils, the protein concentration does not vary significantly between the different samples (standard deviations are estimated to be about 5% in all cases). Therefore, the carbohydrate determination seems to be a more suitable method to assess the degree of cartilage degradation.

In comparison to the cartilage samples incubated with sole buffer, concentrations of carbohydrates are only slightly enhanced in the presence of unstimulated as well as PMA-stimulated neutrophils. On the other hand, when neutrophils were stimulated with fMLP and especially cytochalasin B/fMLP or ionomycin, a significant concentration enhancement of carbohydrates can be observed. This indicates that primarily the link proteins of cartilage proteoglycans (cf. Figure 1) are digested by proteolytic enzymes resulting in a considerable enhancement of soluble carbohydrate fragments [9]. Since cartilage consists mainly of collagen (about 75% of its dry weight) [13], the activity of collagenolytic enzymes should be accompanied by markedly enhanced protein concentrations in cartilage supernatants. Because this increase of protein concentration was not observed in our experiments, most proteolytic enzymes of neutrophils (e.g. elastase) are clearly not able to digest the cartilage collagen, but do only damage the protein moiety of proteoglycans [9].

Astonishingly, in the presence of diphenyliodonium chloride (an inhibitor of the cytochrome *b* moiety of the NADPH oxidase and, thus, for the generation of superoxide anion radicals and all other physiologically relevant ROS [8]) the effects of supernatants of neutrophils on cartilage are stronger expressed. This primarily holds for the carbohydrate content after ionomycin stimulation. Although this effect clearly points out that ROS are not the most important species for cartilage degradation, the enhancement after DPI treatment is not yet completely understood. We assume that the activity of proteolytic enzymes is decreased in the presence of superoxide anion radicals and other ROS, conferring a lower degree of cartilage degradation.

To obtain an independent tool for the determination of the extent of cartilage degradation, all cartilage supernatants were also examined by ^{1}H NMR spectroscopy. Figure 3 shows the ^{1}H NMR spectra of PMN supernatants (a), cartilage in the presence of the supernatant of unstimulated neutrophils (b), and cartilage incubated with pure buffer (c). Since the applied buffer contains relatively high amounts (10 mM) of glucose, all spectra are clearly dominated by the glucose resonances [17,18]. Fortunately, all glucose resonances occur in a relatively small range of chemical shift ($\delta\sim3.2$–3.9 ppm) and do not overlap with resonances of interest.

Glucose resonances are also detectable in spectra (b) and

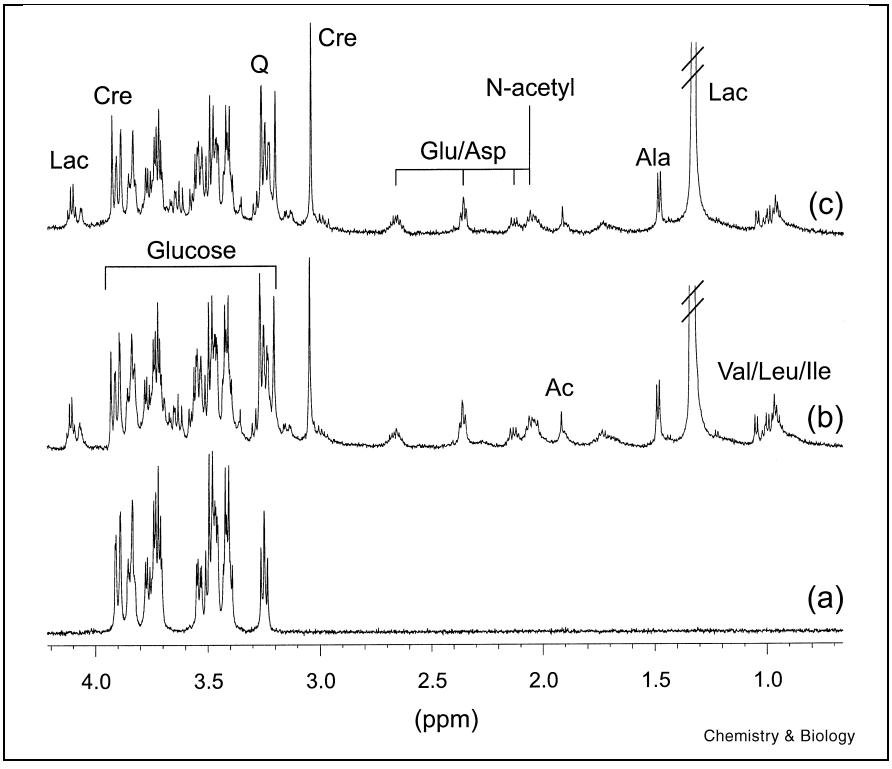

Figure 3. Comparison of the ^{1}H NMR spectra of Hank's buffer which was used for neutrophil preparation **(a)**, articular cartilage incubated with unstimulated neutrophils **(b)**, and articular cartilage upon buffer incubation **(c)**. Abbreviations used in peak assignment: Cre, creatine; Lac, lactate; Q, quaternary ammonia groups; Ac, acetate; *N*-acetyl, methyl protons of *N*-acetyl groups of polysaccharides of cartilage. Resonances of amino acids are indicated by the three-letter code.

(c), but are accompanied by additional resonances, whereby the lactate resonance (δ= 1.31 ppm for the methyl group) is the most intense one, reflecting the metabolic activity of the cartilage cells (this resonance has been cut off for clarity). Further resonances correspond to amino acids like alanine, valine, leucine, isoleucine, glutamic acid and aspartic acid as well as creatine (3.04 and 3.90 ppm) [17,18].

The extent of cartilage degradation is very low under these conditions, because only small amounts of low molecular *N*-acetylated carbohydrates ($\delta\sim$ 2.0 ppm) [19] and degradation products of cartilage proteins ($\delta\sim$ 0.9 ppm according to the methyl groups of different amino acids like leucine, isoleucine, valine) [9,18,20] are detectable in spectra (b) and (c). Both resonances are reliable indicators of cartilage degradation: As shown in Figure 4, intensities of carbohydrate ($\delta\sim$ 2.05 ppm) and protein resonances ($\delta\sim$ 0.9 ppm) are more and more enhanced moving from cartilage incubated with supernatants of unstimulated neutrophils (a) to neutrophils stimulated with PMA (b), fMLP (c), cytochalasin B/fMLP (d) and finally ionomycin (e). The last two stimulators cause the most expressed degradation of proteoglycans as well as of the collagen moiety of cartilage. The enhancement of the *N*-acetyl resonance can be most easily detected in the NMR spectra [19]. It is evident that this resonance consists of three different peaks attributable to chondroitin-4-, chondroitin-6-, and keratan sulfate, which are the most abundant constituents of the polysaccharides of the extracellular matrix of cartilage [21]. Due to its low abundance in articular cartilage, hyaluronan is not expected to contribute to this resonance to a large extent [11]. The high molecular weight of native hyaluronan in cartilage and the resulting considerable line-width are further reasons that prevent its detection by NMR, whereas the lower molecular weight of chondroitin sulfate (about 50 kDa in comparison to 800 kDa) favors its detection [22].

Although the quantitative analysis of the obtained ^{1}H NMR spectra provides not very accurate data due to the considerable line-width of the resonances of interest, spectra were integrated for a quantitative analysis. The ratio of the integral intensities of the *N*-acetyl region (2.2–1.8 ppm) and the collagen region (1.1–0.8 ppm) is 0.76 (without stimulation, a), 0.75 (PMA, b), 0.73 (fMLP, c), 1.16 (cytochalasin B/fMLP, d) and 1.26 (ionomycin, e). The observed changes indicate that the content of NMR-detectable *N*-acetyl groups rises much stronger than the collagen/protein content in cartilage supernatants. This is an additional indication that the polysaccharides of cartilage are more sensitive against the action of the released enzymes compared to the collagen moiety.

Despite of its much lower sensitivity, an enhanced carbohydrate concentration is also detectable by ^{13}C NMR spectroscopy. In Figure 5 the ^{13}C NMR spectra of pure buffer,

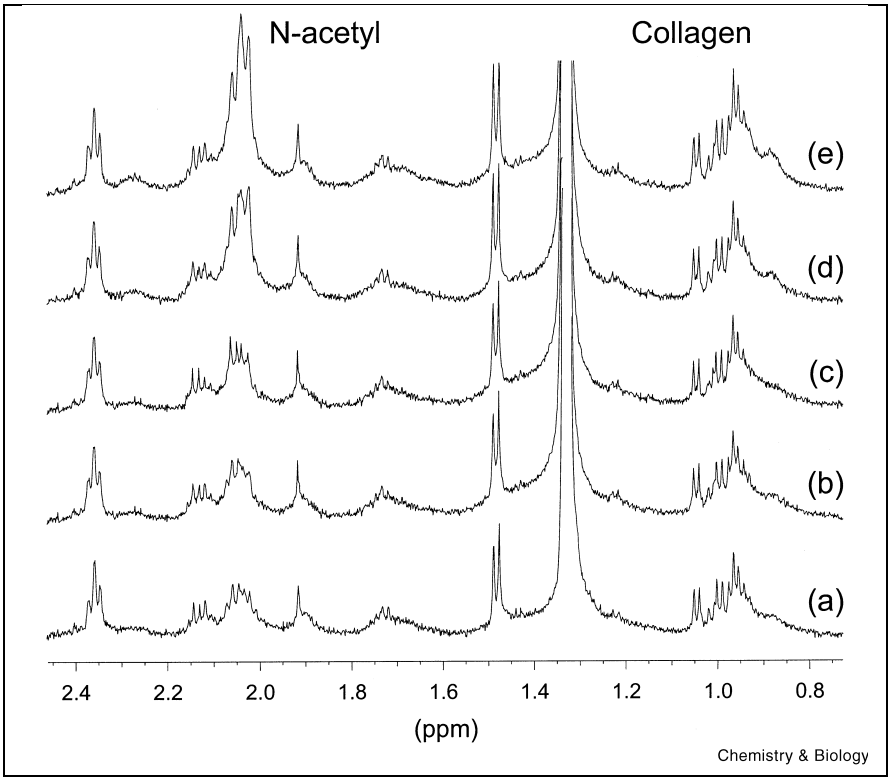

Figure 4. ^{1}H NMR spectra of supernatants of pig articular cartilage incubated for 240 min with supernatants of PMNs. The following stimulators were used: **(a)** without stimulator, **(b)** PMA, **(c)** fMLP, **(d)** cytochalasin B/fMLP and **(e)** ionomycin. *N*-acetyl refers to mobile *N*-acetyl groups which are solubilized upon treatment of cartilage with stimulated neutrophils whereas collagen indicates collagen fragments.

containing primarily glucose (a) and the supernatant of cartilage incubated with cytochalasin B/fMLP-stimulated neutrophils are shown as representative examples (b). The number of resonances in the typical carbohydrate region (between 110 and 60 ppm) clearly increases when stimulated neutrophils are used (b). Additional resonances of the carbonyl (~180 ppm) and the *N*-acetyl groups (~23 ppm) of polysaccharides of cartilage occur. These resonances are primarily caused by the release of chondroitin sulfate [23] from the cartilage tissue and, thus, support the data derived from ^{1}H NMR spectroscopy. Especially the detection of only two resonances for the C-1 of glucuronic acid and *N*-acetylgalactosamine indicates that mainly chondroitin sulfate is released into the supernatant.

The most astonishing results, however, were obtained when cartilage was exposed to the supernatant of stimulated PMNs in the presence of DPI. In order to show the formation of ROS by PMNs in dependence on stimulation conditions, the oxygen burst of neutrophilic granulocytes was also monitored by luminol chemiluminescence [24]. In Figure 6 the influence of different stimulators on the oxidative activity of PMNs is shown. It is evident that fMLP stimulation leads only to a minor chemiluminescence response (a), whereas the addition of PMA (d) yields a considerable light emission [24]. In comparison to PMA the cytochalasin B/fMLP stimulation (b) as well as the ionomycin stimulation (c) induced a less expressed oxygen burst. These differences are in agreement with the different mechanisms of signal transduction processes leading to ROS production in neutrophils [24]. Nevertheless, chemiluminescence intensities and, thus, the oxidative burst of neutrophils, are decreased to less than 5% of the initial value, if chemiluminescence measurements are carried out in the presence of DPI (data not shown).

A marked influence of DPI is also detectable by ^{1}H NMR spectroscopy. Figure 7 shows the ^{1}H NMR spectra of cartilage supernatants incubated with unstimulated cells (a) or ionomycin-stimulated cells (c). The spectra (b) and (d) were recorded under the same experimental conditions, i.e. with unstimulated cells or ionomycin, respectively, but in the presence of DPI. It is obviously visible from spectra (c) and (d) that the addition of DPI results in enhanced concentrations of NMR-detectable carbohydrates (~2.05 ppm) and collagen fragments (~0.9 ppm). This effect was observed with all stimulators in this study (data not shown). On the other hand, DPI alone (in the absence of a further stimulator) does not markedly change the spectra (cf. Figure 7a,b). We have also studied the influence of DPI and all other stimulators on cartilage in the absence of neutrophilic granulocytes. However, DPI as well as fMLP, PMA or ionomycin did not show any changes in comparison to pure buffer.

In the presence of ionomycin-stimulated cells, the effects

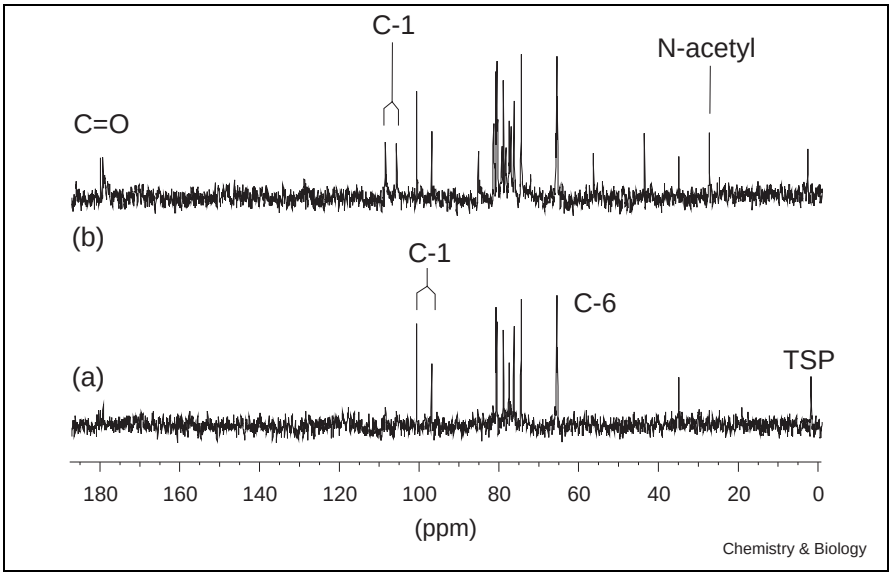

Figure 5. Comparison of two representative ^{13}C NMR spectra of cartilage incubated with pure buffer **(a)** and in the presence of ionomycin-stimulated neutrophils **(b)**. The assignment of the glucose resonances was performed according to [18] and of the chondroitin sulfate resonances according to [23]. TSP denotes 3-(trimethylsilyl)-1-propionate, which served as internal reference ($\delta(Si(CH_3)_3) = 1.70$ ppm).

of degradation are increased by about 30% in the presence of DPI (relative integral intensities of *N*-acetyl groups and collagen protons: 1.61/1.31) in comparison to the absence of DPI (1.23/0.98; cf. Figure 4e). This effect indicates that ROS diminish the activity of different proteolytic enzymes. Therefore, the suppression of ROS formation by DPI enhances considerably the enzymatic activity of the corresponding proteases, resulting in more intense NMR resonances.

Finally, established methods of the protein and carbohydrate biochemistry were used to confirm the NMR results. A very fast developing tool in this context is the MALDI-TOF (matrix-assisted laser desorption and ionization time-of-flight) mass spectrometry [25]. Although MALDI-TOF mass spectrometry was originally developed for protein analysis, it has been shown recently that also strongly acidic oligosaccharides formed by the enzymatic degradation of cartilage can be detected even without previous derivatization [26].

Unfortunately, we were unable to detect any carbohydrates in cartilage supernatants by MALDI-TOF mass spectrometry. This is most likely caused by the high negative charge density and the still rather high molecular weight of polysaccharides [26]. However, protein spectra of cartilage supernatants could be recorded. In contrast to the intriguing work of Zaia et al. [27] we were not able to detect peaks according to a molecular mass of more than about 16 kDa. However, in comparison to these authors our supernatants of cartilage were not purified. Since MALDI-TOF spectra of proteins cannot be analyzed quantitatively [28], only a typical set of positive ion mass spectra is given in Figure 8. In (a) the effect of unstimulated cells as negative control is shown, whereas in (b) and (c) the neutrophils were stimulated with cytochalasin B/fMLP or ionomycin, respectively. Spectrum (d) shows the effect of a 1 mg/ml papain solution as positive control.

Obviously, more vigorous stimulation conditions primarily enhance the formation of low-mass compounds: Whereas the experiment with unstimulated neutrophils (a) yields an intense peak according to a molecular weight of about 15.5 kDa, this peak is markedly diminished when cytochalasin

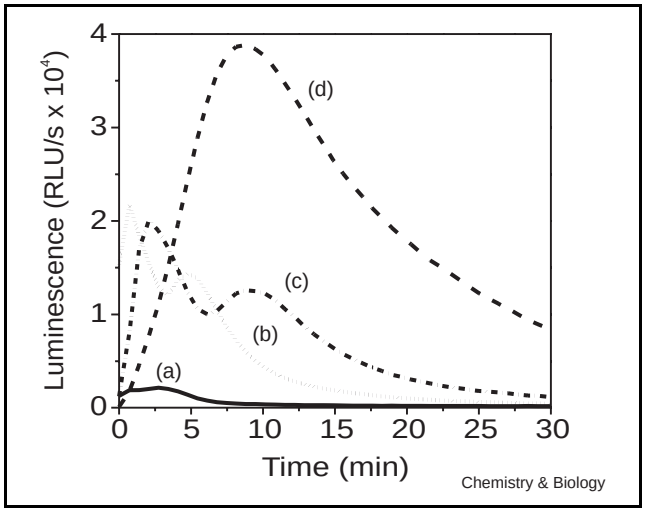

Figure 6. Luminol-chemiluminescence measurements showing the amount of ROS released by stimulated neutrophils in dependence on stimulation conditions. **(a)** fMLP; **(b)** cytochalasin B/fMLP; **(c)** ionomycin; **(d)** PMA. The presence of DPI nearly completely abolishes chemiluminescence (data not shown).

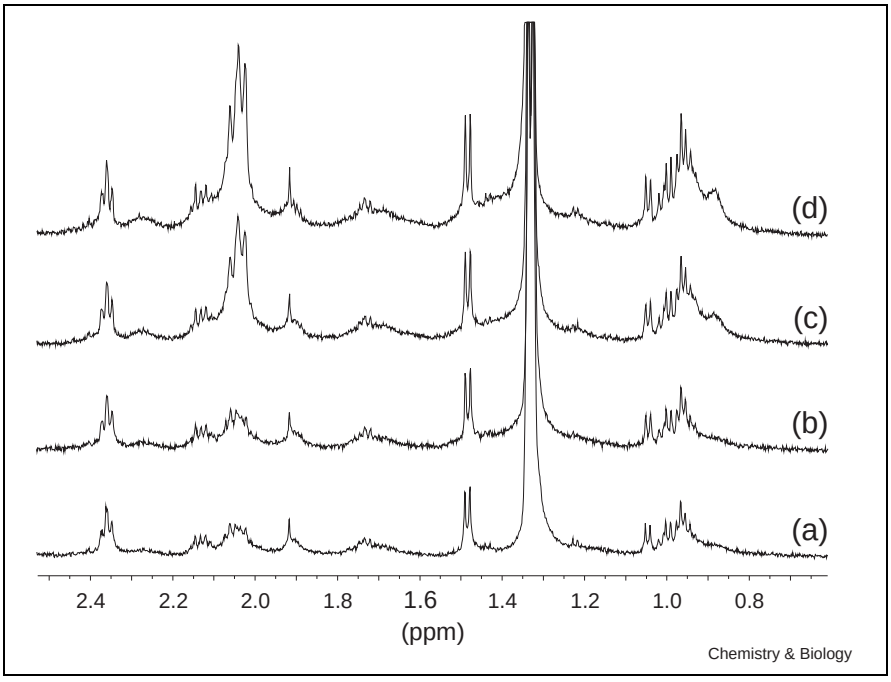

Figure 7. Comparison of different ^{1}H NMR spectra of supernatants of articular cartilage incubated with unstimulated cells **(a,b)** and ionomycin-stimulated neutrophils **(c,d)**. **(a)** and **(c)** were recorded in the absence, and **(b)** and **(d)** in the presence of DPI, respectively.

B/fMLP- or ionomycin-stimulated PMNs are used. The reduction of this peak is accompanied by the appearance of broader peaks at a molecular weight between 6.5 and 8 kDa. The by far most expressed effect is observed under the influence of papain. This enzyme cleaves proteins nearly randomly and, thus, produces very small fragmentation products, whereby the intense peak at about 15.5 kDa vanishes completely. Besides these changes the papain treatment also results in the formation of small peptides and even single amino acids, which are most easily detectable by NMR [9]. On the other hand, these products are hardly detectable by MALDI-TOF since in this mass region the matrix gives also a number of signals, which strongly interfere with the peaks of interest.

Analogous results are obtained by the conventional SDS–

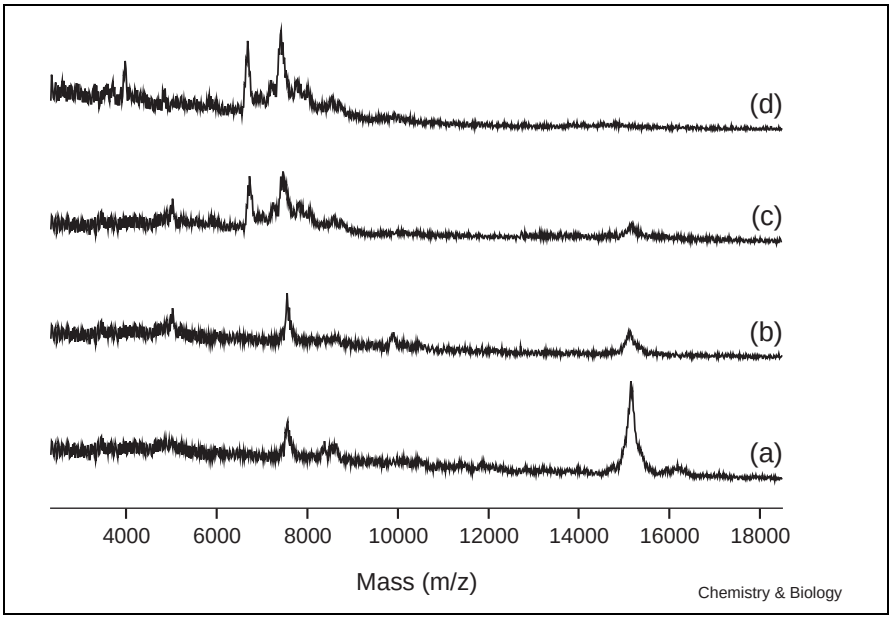

Figure 8. Positive ion MALDI-TOF mass spectra of supernatants of articular cartilage incubated with supernatants of neutrophils in the presence of different stimulators. **(a)** Unstimulated neutrophils, **(b)** cytochalasin B/fMLP, **(c)** ionomycin. In **(d)** the cartilage was digested with 1 mg/ml papain as positive control to obtain a maximum extent of cartilage degradation.

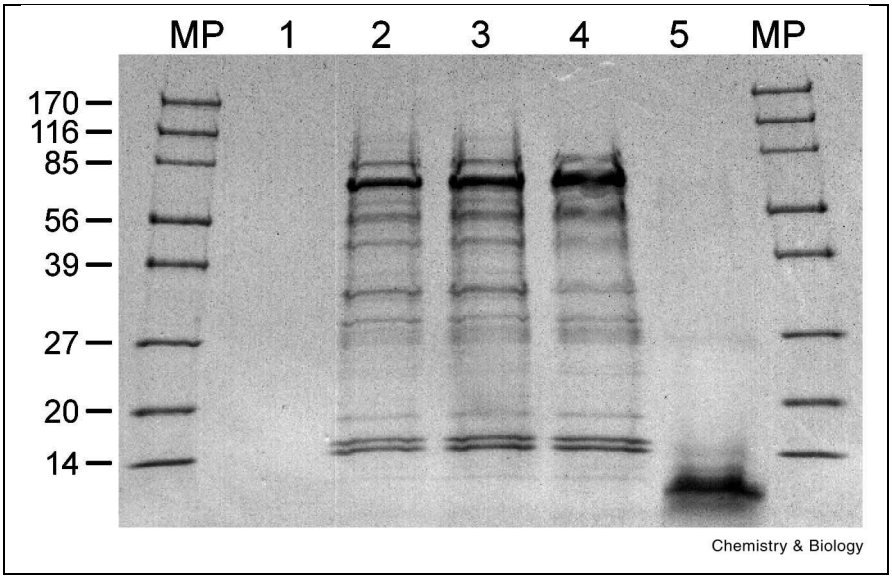

Figure 9. Electrophoresis of supernatants of articular cartilage obtained upon incubation with stimulated neutrophils. The different lanes refer to: **(1)** pure supernatant of neutrophils; **(2)** cartilage+buffer; **(3)** cartilage+unstimulated cells; **(4)** cartilage+ionomycin-stimulated cells; **(5)** cartilage+1 mg/ml papain solution. MP indicates the marker proteins.

PAGE. Figure 9 shows a gel of different supernatants of articular cartilage incubated with the supernatants of neutrophils. Bands are not detected for the supernatant of neutrophils alone (lane 1) but a number of bands emerges upon treatment of cartilage with pure buffer (lane 2), unstimulated neutrophils (lane 3), and ionomycin-stimulated PMNs (lane 4). Although those bands cannot be assigned to defined proteins [29], it is evident that only small differences between the different stimulation conditions occur. However, more vigorous stimulation conditions do not lead to the appearance of additional bands, but to a diminution of the intensity of larger proteins. This effect is most expressed in the case of papain (lane 5). Here, only rather small proteins are detectable as a broad, but intense band at the front of the gel. All larger proteins (cf. lanes 2–4) are completely removed by the action of papain under the formation of very small proteins which are not detectable anymore by the conventional SDS–PAGE. The papain itself is visible as a weak band at 24 kDa.

Discussion

Although the pathological mechanisms of degenerative joint diseases merit huge interest because of the increasing number of patients suffering from these diseases in the industrialized countries, mechanisms of cartilage degradation are still widely unknown [1]. Due to the massive accumulation of neutrophils in pathologically changed synovial fluids, these cells are assumed to play a considerable role in rheumatic diseases [30].

There are two different ways how neutrophils are able to damage the native cartilage tissue and its components. The first way is the release of different proteolytic enzymes. In this context, especially elastase is assumed to contribute considerably to cartilage destruction, because of its comparably low molecular weight (34 kDa) and its relatively high concentration in neutrophilic granules. It is assumed that elastase penetrates into the cartilage and, thus, smooths the way for enzymes like collagenase which possess a higher molecular weight [31,32].

The second way for cartilage damaging is the release of different ROS. Here, notable effects of species like $O_2^{\bullet -}$ [33], H_2O_2 [34], HOCl [11] and $HO^{\bullet}$ [12] were clearly detected in model experiments using cartilage slices or isolated components of cartilage. However, the value of these investigations is limited in the sense that in most cases concentrations of ROS far above the physiologically relevant level were used. Since many ROS react with their targets according to the principle of competition, a high concentration of ROS may favor reactions which would never occur at lower concentrations.

To overcome these problems and as an approach to simulate the in vivo conditions we have performed experiments with pig articular cartilage slices, which were incubated with supernatants of stimulated neutrophils. In our opinion this experimental procedure is the most direct way to assess the activity of a mixture of different enzymes. In order to obtain different stimulation conditions, a variety of reagents was used: Whereas PMA is known to produce large quantities of ROS (cf. Figure 6), the extent of enzyme release is rather low in the case of this agent. On the other hand, fMLP (especially in combination with cytochalasin B) is known to induce a marked release of both, ROS and proteolytic enzymes [35]. Finally, ionomycin is one of the strongest agents to induce degranulation of neutrophils [36]. Since the release of degranulation products rises in

the same order as the extent of degradation products, we conclude that proteolytic enzymes are mainly responsible for cartilage degradation under our experimental conditions, whereas ROS have only a small impact.

On the other hand, under in vivo conditions the role of ROS may be much more important. In the inflamed joint, neutrophils may be able to secrete much higher concentrations of ROS or over longer times since the ROS are enzymatically produced. Higher concentrations may also be achieved due to the localized production of oxidants at or near the cartilage surface. For instance, Katrantzis et al. [37] have reported that under conditions of an acute inflammation the hypochlorite concentration may increase up to 340 μM. Unfortunately, the quantitative determination of highly ROS is very difficult since they are formed and consumed in a very fast process.

The formation of ROS was completely suppressed by the addition of DPI in some experiments. We had expected that cartilage degradation would be less expressed in the presence of DPI, since ROS are not able to contribute to degradation processes under these conditions. Astonishingly, the extent of cartilage degradation was obviously enhanced when the formation of ROS was suppressed by DPI. This indicates that enzymatic activity of proteases is diminished by ROS, most likely because functional groups of enzymes like thiol or amino groups react with ROS and, thus, the enzymes are reduced in their activity. This result may provide a new explanation towards the understanding of the interaction of ROS and enzymatic activity in supernatants of neutrophils. In the past, only the opposite, i.e. the activation of enzymes like collagenase by small amounts of ROS has been reported [38]. However, similar results have already been reported for the neutrophils of patients suffering from the chronic granulomatous disease (CGD) [8]. Here, higher activities of lysozyme and β-glucuronidase in comparison to neutrophils from healthy volunteers were observed. Thus, we conclude that ROS may possess important regulative properties towards the degradation of articular cartilage. This result may be of importance for the diagnosis and therapy of rheumatic diseases, which are still challenging tasks.

In this study we added the supernatants of neutrophils obtained 5 min after cell stimulation to the cartilage samples. Of course, the experimental design towards cell stimulation may be varied in a vast range. In our opinion, the experimental approach to work with stimulated neutrophils and cartilage slices fits much better the in vivo situation than the work with isolated components. However, we cannot answer the very important question which experimental design reflects the in vivo situation in the best way. This also concerns the question when the neutrophils get stimulated in the inflamed joint. Are they stimulated at the moment of entering the synovia, by the contact with soluble and insoluble antibodies in the synovial fluid or first at the moment of their contact with the cartilage surface? It is a future aim of our group to investigate the influence of ROS on enzymatic activity in more detail. It is planned to measure the activity of typical enzymes of the different granules of the neutrophils in dependence on the stimulation conditions.

One additional aspect of the present study is an analytical one. We have used different methods, but have focused our main interest on ^{1}H NMR spectroscopy to monitor the extent of cartilage degradation. In contrast to other, more sensitive and highly specific biochemical methods, ^{1}H NMR offers the advantage of an unselective method and, thus, the detection of all proton-containing compounds is realized in a single experiment [39]. Unequivocally, NMR spectroscopy is the by far best method to monitor the extent of cartilage degradation if one compares, e.g., the results obtained via protein determination by the Bradford assay and the NMR results. Whereas the Bradford assay does not yield significant differences in dependence on incubation conditions, NMR clearly monitors such differences. Like most dye-binding assays, also the Bradford assay underestimates low-molecular-weight peptides, whereas such small peptides are most easily detectable by ^{1}H NMR. The high sensitivity towards small molecules, produced by the action of less selective enzymes on cartilage, is a unique feature of NMR. Thus, we would strongly recommend that, besides common biochemical methods, ^{1}H NMR analysis should also be applied for the analysis of biological samples containing a large amount of low-mass products.

Significance

The processes of cartilage degradation during rheumatic diseases merit huge interest from the biological as well as the chemical point of view. The molecular reasons of cartilage degradation are yet unknown but seem to be closely related to the vast number of neutrophilic granulocytes in the synovial fluids from patients suffering from rheumatic diseases. In dependence on the stimulation conditions, these cells are able to release ROS as well as proteolytic enzymes. Upon their action on articular cartilage, the cartilage layer which covers the ends of bones is destroyed under the formation of low-mass products (cf. Figure 1). Although ROS and enzymes are often assumed to show a strong synergism, our study revealed that the suppression of the formation of ROS is accompanied by a higher extent of cartilage degradation, i.e. a higher enzymatic activity. We are currently confirming these results by the determination of enzymatic activities in the supernatants of PMNs in the absence and the presence of DPI.

On the other hand, the accurate assessment of the extent of cartilage degradation is a typical problem of analytical chemistry. Whereas common biochemical assays allow only

the estimation of the total amount of released carbohydrates and proteins, more sophisticated methods like NMR spectroscopy give the opportunity to assess processes of cartilage degradation in more detail. Our results clearly indicate that low-mass degradation products of cartilage are underestimated by the use of classical biochemical methods and that they are not detectable by the conventional SDS–PAGE. The sensitivity of such assays decreases when the molecular weight of the compound of interest decreases. Exactly the opposite, however, holds for NMR and MALDI-TOF.

Materials and methods

Chemicals

Chemicals for buffer preparation (KH_2PO_4, Na_2HPO_4 and glucose), all solvents (acetonitrile, methanol and dimethylsulfoxide), matrix compounds for MALDI-TOF mass spectrometry (2,5-dihydroxybenzoic acid and sinapinic acid) and trifluoroacetic acid were obtained in highest commercially available purity from Fluka Feinchemikalien GmbH (Neu-Ulm, Germany).

All chemicals for the stimulation or modulation of neutrophils (phorbol-myristate-acetate (PMA), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP); cytochalasin B, and diphenyliodonium chloride (DPI)) were obtained from Sigma (Deisenhofen, Germany).

For comparative purposes, papain from *Cacarica papaya* was purchased as lyophylisate by Fluka and used without further purification.

Cartilage preparation

Pig articular cartilage was obtained from the knee joints of juvenile pigs (about 12 months old) within a few hours after slaughter. After removing the bone, the cartilage was cut into small pieces and immediately used as substrate for the depleting agents released by PMNs. Cartilage from different animals was combined to minimize biological variability [40].

Cell preparation and oxidative activity

Polymorphonuclear leukocytes were isolated from heparinized (10 international units/ml) blood from healthy volunteers [24,41]. 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 Hank's balanced salt solution. PMNs were counted in a CASY cell counter (Schärfe System GmbH, Reutlingen, Germany) and used in a concentration of 4×10^6 cells/ml. The viability of cells was additionally assayed by flow cytometric analysis [42].

Oxidative activity of PMNs was measured by means of luminol chemiluminescence. These experiments were performed on a microplate luminometer MicroLumat LB 96 P (EG & G Berthold, Wildbad, Germany). PMNs (2×10^5 cells) were preincubated for 5 min with luminol (5×10^{-5} M final concentration) and in one case also with cytochalasin B (10^{-5} M) at 37°C. The different stimulators were added immediately before starting the measurement. To the cytochalasin B sample fMLP was added. In the experiments where the NADPH oxidase was inhibited, DPI (10^{-6} M) was added together with the luminol 5 min before measurement.

Incubation of cartilage specimens with cell supernatants

PMNs (4×10^6 cells/ml) were preincubated for 5 min at 37°C, in one case in the presence of cytochalasin B (10^{-5} M). Subsequently, the cells were stimulated for 5 min with PMA (62 ng/ml), ionomycin (10^{-6} M) and fMLP (10^{-6} M), whereby fMLP was also added to the cytochalasin B sample. The indicated concentrations were found to give the maximal response. In the experiments where the NADPH oxidase was inhibited, the cells were also preincubated with DPI (final concentration 10^{-6} M). After 3 min of centrifugation, 1 ml supernatant of the neutrophil suspension was immediately added to the cartilage slices (100 mg), mixed in plastic vessels and incubated at 37°C for 4 h. This comparably long incubation period was used in order to enhance the effect of different enzymes. After incubation, the samples were centrifuged for 10 min to remove debris and insoluble material. The resulting, clear supernatants were directly used for analysis.

NMR measurements

^{1}H NMR spectroscopy. ^{1}H NMR measurements were performed on a Bruker DRX-600 spectrometer operating at 600.13 MHz. Typically, 0.40 ml cartilage or neutrophil 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 16 K data points. This comparably low number of data points was sufficient since all resonances indicating cartilage degradation were expected to be broad. No window functions were used prior to Fourier transformation to enhance signal-to-noise ratio or resolution (line broadening or 'Gaussian' broadening). No zero filling was used. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)-1-propionate (TSP) in a final concentration of 5×10^{-4} M [43]. Although TSP is known to bind to proteins [43] resulting in a lower integral intensity of the TSP resonance, we did not find major differences, equally if TSP or for means of comparison formate as concentration standard was used. Only relative intensities are given. Resonances were identified by their known chemical shifts and by their subsequent enhancement after addition of a small amount of the corresponding pure compounds. This was especially helpful when singlets (e.g. creatine) had to be assigned.

^{13}C NMR spectroscopy. Partially relaxed ^{13}C NMR spectra were obtained at 150.94 MHz on the same spectrometer as described above. Spectra were recorded with a flip angle of 45° (90° flip angle: 4 μs) with a pulse repetition time of 2 s (SW 30000 Hz/16 K). Usually 4 K transients were accumulated under WALTZ-16 decoupling. All free induction decays were processed with a 5 Hz line broadening.

Mass spectrometry

MALDI-TOF (matrix-assisted laser desorption and ionization time-of-flight) mass spectra were acquired on a Voyager Biospectrometry workstation (PerSeptive Biosystems, Framingham, MA, USA). The system utilizes a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage used was 20 kV in the reflector modus and 25 kV in the linear modus. To enhance the signal-to-noise ratio, 128 single shots from the laser were averaged for each mass spectrum. The laser strength was kept about 10% over threshold setting to obtain the best signal-to-noise ratio.

Two different matrices were used in the present investigation: a 10 mg/ml 2,5-dihydroxybenzoic acid (DHB) solution in water containing 0.1% trifluoroacetic acid (TFA) and a 10 mg/ml sinapinic acid solution in a mixture (70:20:10, v:v) of acetonitrile, water and TFA (3 vol.%). DHB was used for carbohydrate analysis, whereas sinapinic acid was used for protein analysis [26].

Cartilage supernatants for protein analysis were directly applied on the sample plate as 1 μl droplets, followed by the addition of one drop (about 2 μl) sinapinic acid solution. Samples were allowed to crystallize at room temperature. Premixing of the matrix and the sample solution (10:1) was used for carbohydrate analysis. In this case, drying of samples with a moderate, warm stream of air improved extremely the homogeneity of crystallization.

Other analytical methods

Protein determination according to Bradford. All protein determinations were performed using a slightly modified [44] version of the dye-binding assay of Bradford [45]. The master curve was recorded using bovine serum albumin. For protein determinations of cartilage supernatants, 30 μl were mixed with 2.5 ml Bradford solution.

Alcian Blue determination of acidic carbohydrates. The determinations of acidic carbohydrates were performed using the dye-binding assay with Alcian Blue [46]. The master curve was recorded using commercially available chondroitin sulfate. For carbohydrate determinations of cartilage supernatants, 30 μl were mixed with 2.5 ml Alcian Blue solution.

Electrophoresis. A discontinuous SDS (sodium dodecyl sulfate)–PAGE (polyacrylamide gel electrophoresis) was performed according to Laemmli [47] using ready gels (10–20% gradient gels Tris–HCl, Bio-Rad Laboratories GmbH, Munich, Germany) with a BioRad Mini Protean II system. Prior to electrophoresis the samples were diluted (1:1) with sample buffer containing 4% SDS, 20% glycerol, 10% mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris–HCl, pH 6.8.

All samples were incubated for 5 min in a boiling water bath and centrifuged for 3 min at 18 000 U/min prior to the gel run. The gel was run for 1 h at ambient temperature at 140 mA and a starting voltage of 150 V. Following electrophoresis the gels were stained for 40 min with Coomassie blue (0.25% (w/v)) in acetic acid:methanol:water (2:9:9 by volume) and destained with 30% methanol 10% acetic acid solution.

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