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Matrix degradation by chondrocytes cultured in alginate: IL-1β induces proteoglycan degradation and proMMP synthesis but does not result in collagen degradation

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Summary

Objective: To determine the role of interleukin-1β (IL-1β) in the degradation of proteoglycans and collagen by articular chondrocytes.

Design: Chondrocytes were cultured in alginate beads for 2 weeks to produce extracellular matrix, followed by the addition of IL-1β for 1 or 2 days. Breakdown of extracellular matrix (with and without activation of pro-matrix metalloproteinases (MMPs) by APMA) was monitored by release of glycosaminoglycans (GAG, proteoglycans) and hydroxyproline (collagen) from the beads into the medium, and by the amount of damaged collagen in the bead. Levels of (pro)MMPs in the beads were assayed by zymography and their activity was quantified fluorometrically.

Results: IL-1β induced a profound GAG release (~ 80% after 2 days at 20 ng/ml IL-1β) that was both time and IL-1β concentration dependent. Under these conditions no increase in collagen release or damaged collagen in the bead was detected. Zymography demonstrated that the synthesis of a variety of proMMPs was induced by IL-1β, without a detectable increase of MMP-activity as measured in the activity assay. After activation of the proMMPs by APMA, a time and IL-1β concentration-dependent increase in MMP-activity was found, which resulted in almost complete deterioration of collagen already after 18 h of incubation. In the presence of APMA, GAG release from IL-1β treated beads was significantly increased from 24 to 31%.

Conclusions: Our data suggest that proteoglycan and collagen degradation are regulated through different mechanisms: IL-1β induces the synthesis of active enzymes that degrade proteoglycans, such as 'aggrecanase', and inactive proMMPs. Thus, IL-1β alone is not sufficient to result in collagen-degrading MMPs. Once activated, MMPs may account for up to a quarter of the aggrecan degradation in this model.

Key words: Chondrocytes, Matrix degradation, Interleukin-1, Matrix metalloproteinases.

Introduction

Interleukin-1 (IL-1) is a cytokine alleged to play a major role in cartilage destruction in arthritis [1–6]. These processes include enhanced proteoglycan degradation and upregulation of matrix metalloproteinase (MMP) synthesis [7]. MMPs are excreted as inactive proforms that are able to degrade proteoglycans and the collagen network in cartilage after proteolytic activation. Several classes of MMPs exist (for reviews, see [8, 9]): the collagenases MMP-1, -8 and -13 cleave triple helical collagen type I, II and III, the gelatinases (MMP-2 and -9) degrade non-triple helical collagen (gelatin); active stromelysin (MMP-3) was shown to activate proMMP-1, -8, -9 and -13 [10–13] and is thought to be involved in the degradation of proteoglycans [14, 15]. Recently, the role of MMP-3 in proteoglycan degradation has been overshadowed by 'aggrecanase', an as yet unidentified proteinase that cleaves in the interglobular domain of aggrecan between Glu^{373}–Ala^{374} [16–19]. Upon IL-1 stimulation, proteoglycan degradation in cartilage explants starts within 1 day at relatively low concentrations of IL-1 [17, 20–22].

In contrast to the rapid degradation of proteoglycans, collagen release in nasal cartilage explants occurs only after long time periods ($\geq$ 3 weeks) at relatively high levels of IL-1 (50 ng/ml) [20, 23, 24]. However, maximal MMP-synthesis by chondrocytes is observed within a few days in the presence of IL-1 [25–27]. The long lag-time between synthesis of MMPs and detectable MMP-mediated

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degradation of collagen, suggests a non-IL-1 mediated activation of MMPs (e.g., by other factors or resulting from autoactivation). The present study was designed to determine the role of IL-1 in degradation of extracellular matrix upon short-term ($\leq$ 2 days) exposure of chondrocytes to IL-1 and in the absence of non-chondrocytic factors which may be present in articular cartilage, e.g., other catabolic cytokines or activating proteinases originating from other cell types like synoviocytes or inflammatory cells. For this purpose, isolated bovine chondrocytes were cultured in the alginate bead culture system, in which they produce a cartilage-like matrix [28, 29], before IL-1 treatment.

Method

CELL ISOLATION AND CULTURE

Chondrocytes from the metacarpophalangeal joint of calves (12–14 months old, local slaughterhouse) were isolated by collagenase digestion following established procedures [28, 30, 31]. Cells were suspended in 1.2% alginate (Keltone LVCR, Kelco, Chicago, U.S.A.) in 0.9% NaCl at a density of 4×10^6 cells/ml, which was passed dropwise through a 22 gauge needle into 102 mM $CaCl_2$. After 10 min of polymerization, beads were washed in 0.9% NaCl (three times) and finally in Dulbecco's modified Eagle's medium (DMEM)-glutamax (Gibco-BRL) supplemented with 100 U/ml of penicillin–streptomycin, 10% FCS (Gibco-BRL) and 50 μg/ml ascorbic acid. The cells were cultured at 10 beads per 0.5 ml medium (24 wells plate, Costar) in a humid atmosphere of 5% CO_2 in air at 37°C. The medium was refreshed twice weekly. After 14 or 21 days of culture, the beads were washed three times for 1 h with DMEM supplemented with 0.05% human serum albumin (HSA, Bloodbank, Amsterdam, The Netherlands) and transferred to a fresh culture plate. Chondrocytes were stimulated with human recombinant IL-1β (PeproTech, London, UK) for 24 or 48 h; beads and media were stored separately at −20°C until analysis.

PROTEOGLYCAN MEASUREMENTS

The amount of sulfated glycosaminoglycans (GAGs) reflecting the amount of proteoglycans in culture medium or alginate beads was determined by the dimethyl methyleneblue method using a commercial kit (Biocolor Ltd, Belfast, N. Ireland) according to the instructions of the manufacturer. Beads were digested for 2 h at 65°C in papain buffer (126 μg/ml papain in 50 mM phosphate buffer pH 6.5, containing 2 mM L-cysteine and 2 mM EDTA [32]) before the measurement. Culture media were analyzed without papain digestion. Proteoglycan degradation was expressed as the percentage of GAG released into the medium.

COLLAGEN MEASUREMENTS

After two washes, once in 0.9% NaCl containing 10 mM $CaCl_2$ and once in saline, beads were hydrolyzed in 500 ml 6 N HCl per bead at 108°C for 24 h. An aliquot (50 μl) of the hydrolysate was subjected to amino acid analysis, (see below) to determine the collagen-specific amino acid hydroxyproline. Alternatively, beads were digested in papain buffer first (see above) and then hydrolyzed.

The collagen content of culture medium was determined by amino acid analysis after acid hydrolysis (24 h at 108°C) of 100 μl of the medium with 100 μl 12 M HCl. After drying of the hydrolyzates under vacuum, the residue was dissolved in 200 μl 0.1 M borate buffer (pH 9.5). Primary amino acids were blocked by derivatization with 25 μl 0.23 M *o*-phthaldialdehyde (Sigma, St Louis, MO, U.S.A.) in acetone containing 0.23 M β-mercaptoethanol (Merck, Darmstadt, Germany), for 1 min at room temperature. After addition of 25 μl 0.45 M iodoacetamide (in acetone, reaction for 30 s) to remove excess β-mercaptoethanol, secondary amino acids (hydroxyproline and proline) were derivatized with 50 μl 6 mM FMOC-Cl (Fluka, Buchs, Switzerland) in acetone for 10 min [33,34]. Resulting samples were extracted twice with 750 μl diethylether and thereafter the water phase was diluted threefold in 0.1 M borate buffer (pH 8.0) containing 25% (v/v) acetonitrile. High-performance liquid chromatography (HPLC) analysis was performed as described earlier [35]. The amount of collagen was quantified assuming 300 hydroxyproline residues per triple helix, i.e., 1 nmol Hyp corresponds to 1 μg collagen. Collagen degradation was expressed as the percentage of total collagen released into the medium.

QUANTIFICATION OF DAMAGED COLLAGEN IN BEADS

In beads, pre-cultured under control conditions and then treated with IL-1β (0, 0.5, 5, or 20 ng/ml) for 24 or 48 h, the percentage of damaged collagen, indicative for degradation of the collagen network, was determined as described elsewhere [34]. The

assay is based on the fact that α-chymotrypsin degrades damaged or collagenase-cleaved collagen which is still covalently bound to the collagen network, thereby releasing the resulting fragments from the alginate beads. Intact triple helical collagen is unaffected by α-chymotrypsin. Briefly, proteoglycans were extracted from three beads with 250 μl 4 M guanidinium-HCl in 50 mM Tris (pH 7.6) containing 10 mM $CaCl_2$ and 10 μM BB94 (a general MMP inhibitor, also known as Batimastat or RS47,112, kindly supplied by Dr R. Martin, Roche-Syntex, Palo Alto, CA [36]) at 4°C for 6 h. After washing of the beads in the same solution without guanidinium–HCl, damaged collagen was digested by 0.25 mg/ml α-chymotrypsin (Sigma) in 250 μl/bead 50 mM Tris (pH 7.6) containing 10 mM $CaCl_2$ and 10 μM BB94 at 37°C for 3 h. Resulting supernatant and beads were hydrolyzed and subjected to amino acid analysis to determine hydroxyproline using the OPA/FMOC derivatization method described above. The percentage of damaged collagen was calculated from the amount of hydroxyproline released by α-chymotrypsin divided by the total amount of collagen present in the bead.

GELATIN ZYMOGRAPHY OF BEAD EXTRACTS AND CULTURE MEDIUM

Beads were extracted in 50 mM Tris (pH 7.5), containing 5 mM $CaCl_2$, 0.15 M NaCl, 1 μM $ZnCl_2$ and 0.1% Brij-35 for 16 h at 4°C. Extracts were mixed with an equal volume of nonreducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [125 mM Tris, pH 6.8, 0.16% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue] and incubated for 1 h at 37°C, before electrophoresis. Culture medium (50 μl) was dried under vacuum and the residue was dissolved in nonreducing SDS-PAGE sample buffer. Gelatin zymography [25, 37] was performed under nonreducing conditions using a 10% polyacrylamide gel containing 0.1% gelatin on a ATTO electrophoresis system (ATTO, Tokyo, Japan). Applied aliquot of bead extract to the gel was comparable to 0.5 bead. Since the volume of one bead is approximately 15 μl, and 10 beads were cultured in 0.5 ml of medium, the equivalent of 35 μl medium (∼ 1/20 of beads plus medium) was applied to the gel in order to compare the concentration of MMPs in beads and medium. Gels were washed in 50 mM Tris (pH 7.5) with 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 2.5% Triton X-100 in which the proMMPs and MMPs renature to active enzymes [25,37]. Subsequently, gels were incubated for 18 h in the same buffer without Triton (37°C). ProMMPs and MMPs result in white lysis zones after staining with Coomassie Brilliant Blue, due to gelatin degradation.

MMP-ACTIVITY MEASUREMENTS

Beads were extracted in 50 μl/bead 50 mM Tris (pH 7.6), 5 mM $CaCl_2$, 0.15 M NaCl, 1 μM $ZnCl_2$ and 0.05% Brij-35 at 4°C at mild agitation for 72 h. General MMP activity was measured in native (unactivated) and APMA-activated (1 mM, 2 h, 37°C) extracts with fluorogenic substrate TNO211 (Dabcyl-Gaba–Pro–Gln–Gly–Leu–Glu(EDANS)–Ala–Lys–NH_2 (5 μM in buffer as above with 0.01% Brij-35; further noted as incubation buffer) as described earlier [38]. Catalytic efficiencies of TNO211 for MMPs are (25°C): MMP-1, $18 \cdot 10^3$/M/s; MMP-2, $230 \cdot 10^3$; MMP-3, $4 \cdot 10^3$; MMP-7, $9 \cdot 10^3$; MMP-8, $29 \cdot 10^3$; MMP-9, $139 \cdot 10^3$; MMP-13, $550 \cdot 10^3$. Measurements in bead extracts (45 μl) were performed in a total volume of 180 μl in a black round bottom microtiter plate and increase in fluorescence (due to conversion of the substrate) was quantified in a Cytofluor II fluorescence reader (excitation filter: 360 nm, emission filter 490 nm; Perseptive Biosystems).

The ability of APMA to activate MMP-tissue inhibitor of metalloproteinases (TIMP) complexes was investigated as follows: 5 nM MMP-3, 1 nM MMP-13 (both generous gifts from Dr P. Mitchell, Pfizer Inc., Groton, CT, U.S.A.) and 5 nM MMP-8 (kindly supplied by Dr G. Murphy, Strangeways Labs, Cambridge, U.K.) were incubated with equimolar amounts of TIMP-1 (from Dr G. Murphy, Strangeways Labs, Cambridge, U.K.) in incubation buffer at 37°C for 2 h. This resulted in 100% inhibition of MMP activity. Thereafter, MMP-TIMP complexes were incubated with(out) 1 mM APMA for 2 h at 37°C. Resulting MMP-activity was measured by the addition of 2 μM fluorogenic substrate TNO211 (see above).

APMA ACTIVATION OF IL-1β STIMULATED BEADS

Alginate beads (cultured for 2 weeks and stimulated with 20 ng/ml IL-1β for 48 h) were incubated with 1 mM APMA at 37°C for 2 h in incubation buffer (200 μl/bead, 50 mM Tris pH 7.6, 5 mM $CaCl_2$, 0.15 M NaCl, 1 mM $ZnCl_2$ and 0.01% Brij-35) to activate proMMPs. Controls contained no APMA, or APMA in the presence of 10 μM BB94 [36]. After the activation step, the incubation medium was saved (to determine GAG and

collagen release) and 200 µl incubation buffer per bead (containing 10 µM BB94 when added during the APMA-activation step) was added to the beads. Subsequently, the beads were incubated for another 16 h at which point the percentage of damaged collagen was quantified as described above.

DATA ANALYSIS

Data are expressed as mean ± S.D. from three separate cultures. Assays were run at least in duplicate. Student's *t*-test was used for statistical analysis: $P < 0.05$ was considered as statistically significant.

Results

IL-1β INDUCED RELEASE OF GAG AND COLLAGEN INTO THE MEDIUM

Chondrocytes were cultured for 2 weeks under control conditions. After 14 days, collagen and proteoglycan levels in the beads remained constant (approximately 22 and 30 µg/µg DNA, respectively [28]). The chondrocytes were stimulated with IL-1β (0.5–20 ng/ml) for 1 or 2 days, and release of glycosaminoglycans (GAG, as a measure for proteoglycans) and collagen (measured as hydroxyproline after acid hydrolysis) into the medium was followed. A time and IL-1β dose-dependent release of GAG from the bead was seen: at 5 ng/ml (0.3 nM) of IL-1β, already 24% of GAG was released in the medium after 1 day. This was increased to 70% after 2 days [Fig. 1(a)]. At the highest concentration of IL-1β (20 ng/ml) almost all GAGs were released after 48 h (~80%). Spontaneous release was negligible: under control conditions virtually no GAGs were found in the medium. The data obtained for GAG release were in contrast to the degradation of collagen: no increase in collagen content of the medium could be observed at increasing amounts of IL-1β after 1 or 2 days [Fig. 1(b)].

CONTENT OF DAMAGED COLLAGEN IN THE BEAD

Stimulation of MMP-activity by IL-1β does not necessarily result in release of collagen into the medium, i.e., degraded collagen may stay in the matrix due to intermolecular cross-linking; after 2 weeks of culture, a considerable level of collagen cross-links are present in the beads [28, 29]. Therefore, the amount of α-chymotrypsin degradable damaged collagen was quantified. By this method, unwound collagen resulting from proteolytic degradation is further cleaved by α-chymotrypsin and released from the collagen network, whereas triple helical collagen is not degraded [34]. Beads stimulated with IL-1β for 1 or 2 days revealed no increase in the percentage of α-chymotrypsin-degradable collagen [Fig. 1(c)].

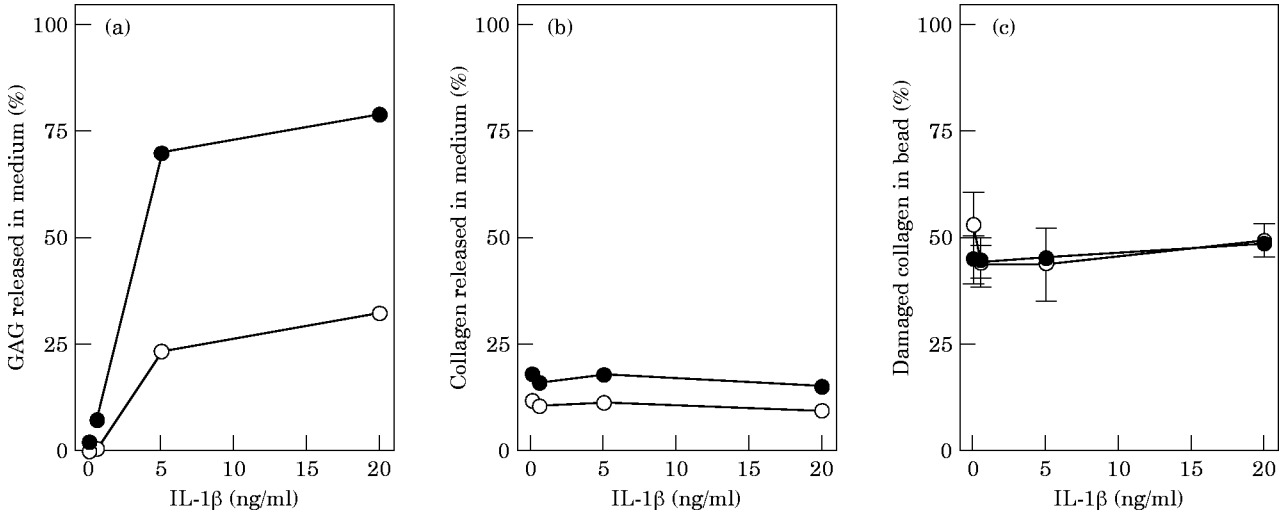

FIG. 1. IL-1β induced matrix degradation by bovine chondrocytes in alginate. Chondrocytes were cultured for 2 weeks under control conditions and subsequently stimulated with IL-1β (0, 0.5, 5 and 20 ng/ml) for (○) 24 or (●) 48 h. Proteoglycan degradation [panel (a): percentage of GAGs released into the medium], collagen degradation [panel (b): Hyp released], and the amount of damaged collagen in the bead [panel (c): the percentage of α-chymotrypsin degradable collagen] were monitored as described in Method.

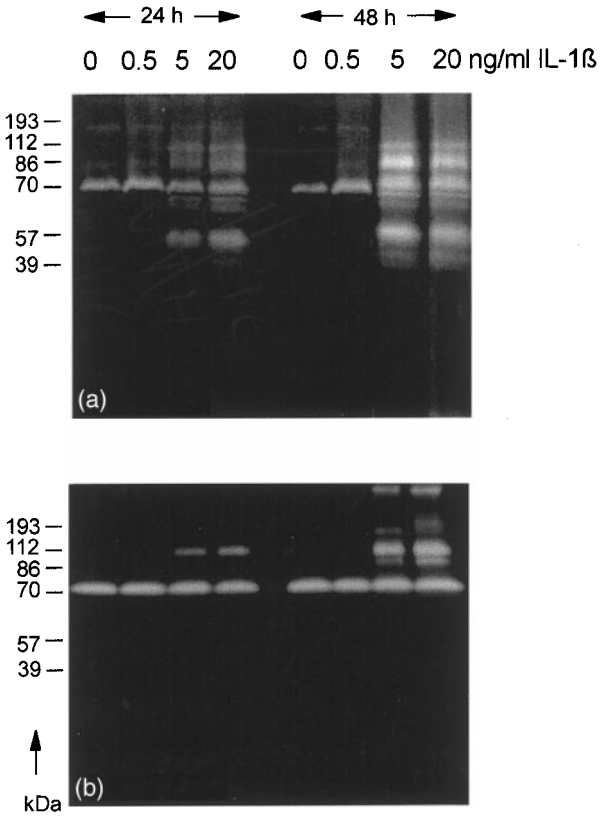

FIG. 2. Gelatin zymography of bead extracts and medium. Chondrocytes cultured in alginate for 2 weeks under control conditions were subsequently stimulated with IL-1β for 24 and 48 h. Beads extracts (a) and medium (b) were applied to a 10% polyacrylamide gel containing 0.1% gelatin. IL-1β concentrations and are indicated at the top of the gel; molecular weights shown on the left. Lysis zone at ~72 kDa is likely to correspond to proMMP-2, and at ~90 kDa to proMMP-9.

IL-1β INDUCTION OF MMP PRODUCTION: GELATIN ZYMOGRAPHY

To rule out the possibility that IL-1β does not lead to MMP synthesis by chondrocytes in the alginate bead system, extracts or culture medium from IL-1β treated alginate beads were subjected to zymography. In the non-stimulated culture only proMMP-2 (72 kDa) is observed in the beads and the medium (Fig. 2). Treatment with IL-1β resulted in a clear IL-1β dose- and time-dependent induction of proMMP synthesis. In more detail, strong induction of several lysis bands between 40 and 60 kDa [bead extracts, Fig. 2(a)] and at ~90 kDa (proMMP-9) was observed, whereas the level of proMMP-2 was not or only marginally increased. The 45–60 kDa bands are likely to correspond to multiple forms of stromelysin (MMP-3) or the collagenases MMP-1, -8 and -13 (different degrees in glycosylation and autolysis of proMMP into smaller forms [8, 9, 25, 39]). The lysis zone between proMMP-2 and -9 ((80 kDa) may represent active MMP-9 and the band at (60 kDa) may result from the active from of MMP-2 [Fig. 2(a)].

In the medium from nonstimulated cultures, only proMMP-2 is present and it is not induced by IL-1β [Fig. 2(b)]. The stimulation of proMMP-9 synthesis is comparable to that found in the bead extracts. Since other (pro)MMPs are not present in the culture medium, we conclude that besides proMMP-2 and proMMP-9, the majority of the MMPs synthesized by the chondrocyte upon IL-1β treatment are retained in the bead.

MMP-ACTIVITY IN BEAD EXTRACTS; EFFECT OF APMA

For proteolytic cleavage of extracellular matrix, proMMPs have to be activated after excretion. To investigate whether the large increase in synthesis of MMPs upon IL-1β stimulation [zymography Fig. 2(a)] results in net MMP-activity, extracts of alginate beads were analyzed with a sensitive activity assay with the fluorogenic MMP substrate, TNO211 [38]. This substrate is rapidly cleaved by all MMPs tested so far (MMP-1, -2, -3, -7, -8, -9, and -13). No increase in MMP-activity was observed after IL-1β stimulation up to 48 h at 20 ng/ml IL-1β. This seems to be in contrast to zymography [Fig. 2(a)] which indicates the induction of a variety of MMPs. Therefore, it is most probable that the lysis zones originate from proMMPs or MMPs which are inactivated by complexation with TIMPs. It is known however, that induction of TIMP upon IL-1 treatment in chondrocytes is absent [25, 51]. Such MMP/TIMP complexes are known to dissociate in the denaturing zymography buffer environment [40], resulting in lysis zones corresponding to active MMPs in zymography.

To investigate whether the lysis zones in zymography belong to proMMPs, bead extracts were activated with APMA. This resulted in a time and IL-1β dose-dependent increase in MMP-activity [Fig. 3(b)] measured with TNO211, consistent with the relative increase in (pro)MMP-levels seen in zymography [Fig. 2(a)]. MMP-activity in beads exposed to 20 ng/ml IL-1β for 48 h was 15-fold higher than that in controls beads (without IL-1β). Addition of the selective MMP-inhibitors TIMP-1 (10 nM) or BB94 (10 nM) totally suppressed the enzyme-activity (data not shown). The APMA-induced elevation of MMP-activity was not due to dissociation of MMP/TIMP complexes: APMA-treatment *in vitro* of MMP-3/TIMP-1, MMP-8/TIMP-1 or MMP-13/TIMP-1 complexes did not

result in active enzyme, when measured with TNO211 (data not shown).

Therefore, the detected MMP-activity must result from APMA-activated proMMPs, which were not neutralized by free TIMPs in the bead. Furthermore, the lysis zones seen in zymography are most likely to correspond to proMMPs and not active MMPs.

ROLE OF MMPS IN GAG RELEASE AFTER ACTIVATION BY APMA

Beads, treated for 1 day with 20 ng/ml IL-1β still contain approximately 75% of the GAGs [Fig. 1(a)], and high levels of proMMPs [Fig. 2(a)]. To examine whether active MMPs (once activated) are able to degrade proteoglycans, the beads were incubated for 2 h (37°C) with 1 mM APMA to activate proMMPs. In the absence of APMA, approximately 24% of the GAGs (still present in the bead after IL-1β-stimulation) is released, most likely resulting from aggrecanase-activity [Fig. 4(a)]. When treated with APMA, the GAG release was increased to 31% ($P < 0.05$). This additional degradation of aggrecan indicates that activated MMPs may play a significantly role in proteoglycan degradation. No significant effect of APMA on proteoglycan release was seen using beads which were not stimulated with IL-1β [Fig. 4(a)].

EFFECT OF ACTIVATION OF PROMMPS ON COLLAGEN DEGRADATION, AND DAMAGED COLLAGEN CONTENT IN THE BEAD

To monitor the effect of proMMP activation on collagen degradation, IL-1 stimulated beads (2 days, 20 ng/ml) were incubated with APMA. This resulted in a threefold increase in collagen release from the bead in as little as 2 h in comparison with non-APMA activated beads [Fig. 4(b)]. This demonstrates that the APMA-mediated increase in MMP-activity as determined with the fluorogenic activity assay [Fig. 3(b)] indeed results in rapid degradation of the collagen network. In non-IL-1β stimulated beads APMA treatment caused no increase in collagen release [Fig. 4(b)], indicating that a negligible amount of proMMPs are present in the control situation. The collagen release after IL-1 and APMA-activation could be totally abolished by the MMP-inhibitor BB94 [10 μM; Fig. 4(b)]. Similar findings are shown for the fraction of damaged collagen in IL-1 stimulated beads after exposure to APMA: an increase upon APMA-activation and blocking of degradation by BB94 down to basal levels of damaged collagen [IL-1, no APMA; Fig. 4(c)]. Hydroxyproline release [Fig. 4(b)] together with the amount of damaged collagen [Fig. 4(c)] indicate that after APMA activation the IL-1 induced MMPs are able to rapidly degrade the collagen network.

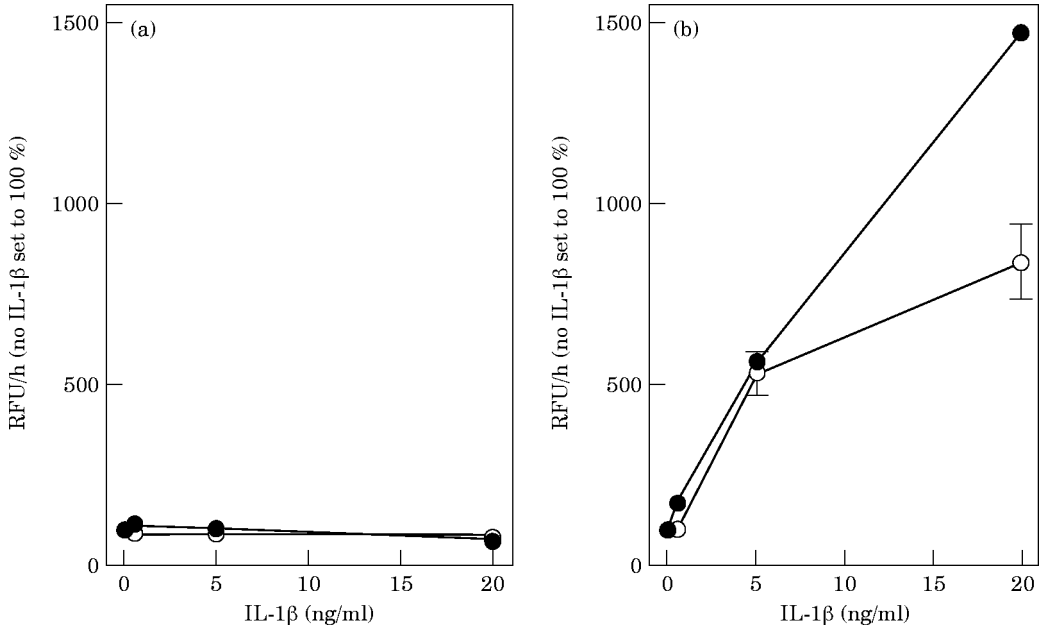

FIG. 3. MMP-activities in extracts of IL-1β stimulated beads. Chondrocytes cultured in alginate for 2 weeks under control conditions were subsequently stimulated with IL-1β (0, 0.5, 5, and 20 ng/ml) for 24 and 48 h. MMP-activity in the extracts with and without prior activation by APMA was determined using the fluorogenic MMP substrate TNO211, see Method for details.(a) Non-activated; (b) APMA activated; (○) 24 h; (●) 48 h.

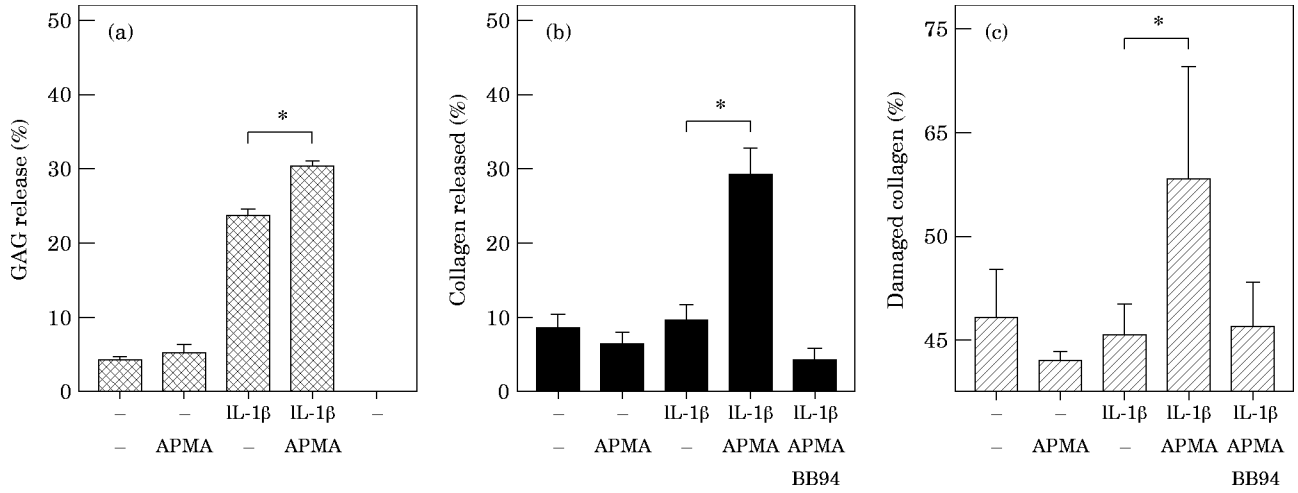

FIG. 4. Effect of proMMP-activation by APMA on proteoglycan (a) and collagen (b) degradation, and the percentage of damaged collagen in the bead (c). Chondrocytes containing alginate beads were cultured for 2 weeks and subsequently stimulated with IL-1β for 24 h (proteoglycans) or 48 h (collagen), followed by an incubation for 2 h in the presence of 1 mM APMA. The percentage of damaged collagen in the bead (α-chymotrypsin degradable collagen) was determined after another incubation of 16 h in the absence of APMA. * Significantly different: $P < 0.05$ (Student's *t*-test).

Discussion

The alginate culture system was previously shown to be an excellent model to study matrix synthesis by chondrocytes [28, 29, 41]. In the present study we have been able to apply established methodologies such as degradation of proteoglycans, collagen release, and quantification of damaged collagen content in this model system to study the direct effect of IL-1 on matrix degradation by chondrocytes.

Topographical differences in susceptibility of chondrocytes to IL-1 in cartilage explants [42] have been eliminated in the alginate culture system for all beads contain an identical cell population. As such, more reproducible results can be expected with the alginate culture system than with articular cartilage explants.

Upon stimulation with IL-1β extensive proteoglycan depletion occurred: at only 5 ng/ml more than 70% of the GAGs was released within 2 days. This in good agreement with the rapid proteoglycan degradation observed with cartilage explants [20, 21, 23, 24, 43–45]. The IL-1 mediated release of proteoglycans was attributed to 'aggrecanase' based on the release of specific aggrecan fragments [17, 43]. In our studies, we show that APMA-treatment of IL-1β stimulated beads resulted in a nearly 30% increase of GAG release. This suggests that MMPs (when activated) can contribute to proteoglycan degradation in a cartilage-like extracellular matrix. This corroborates the findings that MMPs can degrade isolated aggrecan [14, 19, 46–49] and implies that MMPs can play a role in proteoglycan turnover *in vivo*. To which extent MMPs are involved in aggrecan degradation in cartilage remains unknown until quantitative methods become available to monitor both the neoepitopes generated by MMPs and aggrecanase. In our model system, after activation of proMMPs, 25% of the total GAG release was likely to result from MMP-activity. This suggests that *in vivo*, where only a small fraction of the MMPs is activated, their involvement in aggrecan degradation will be small compared to aggrecanase-mediated turnover.

No increase in MMP-activity in alginate beads was detected after 2 days of stimulation by 20 ng/ml IL-1β. Therefore, aggrecan degradation can not be related to MMP-activity, which corroborates recent findings that 'aggrecanase' is not a known MMP [50]. The absence of MMP-activity in the bead corresponded to the absence of hydroxyproline release into the medium (treatment with 20 ng/ml IL-1β, 2 days). As local degradation of the collagen network in the bead does not result *per se* in release of collagen fragments into the medium, the amount of degraded collagen in the bead was quantified. However, also no significant increase in degraded collagen was observed which supports the finding that no active MMPs are present after stimulation with IL-1β. Clearly, this was not due to a lack of MMP synthesis: upregulation of MMP synthesis was observed in zymography as new lysis zones which are likely to correspond to MMP-1, -3, -8 or -13 (45–60 kDa) and MMP-9 ($\sim$90 kDa), whereas MMP-2 expression was not or only marginally induced. In

zymography, the sensitivity to detect the gelatinases MMP-2 and MMP-9 is about 100-fold higher than for other MMPs [49, 51]. Since the intensity of the lysis bands in the region corresponding to the molecular weights of MMP-1, -3, -8 and -13 were comparable to those of the gelatinases, our findings indicate that gelatinases constitute 10% or less of the total MMPs.

The excreted MMPs upon IL-1β treatment were still in the latent form, as concluded from experiments where APMA was used to activate proMMPs: the collagen matrix in the alginate bead rapidly deteriorated which was paralleled by an increase in MMP-activity measured with the fluorogenic substrate TNO211. Together with the findings that the MMP-inhibitor BB94 inhibited enzyme-activity and collagen degradation, this suggests that the latent MMPs induced by IL-1 are able to rapidly degrade the collagen matrix once activated. This presence of active MMPs after APMA treatment in combination with the observation that APMA did not activate MMP-TIMP complexes, indicates that the increase in enzyme-activity must be a result from an excess of active MMPs over TIMPs. Hence, our enzyme activity data are consistent with immunological findings that MMP/TIMP ratios in cartilage are increased upon IL-1 treatment [25, 42, 52].

Our collagen degradation data correspond to recent findings from others with bovine nasal cartilage explants: collagen degradation was only found after exposure to high amounts of IL-1 for 3 weeks [20, 23, 24]. The late onset of degradation in nasal explants was putatively attributed to a change in chondrocyte phenotype during the culture period (3 weeks) [20]. Another possible explanation is that prolonged stimulation of cartilage explants with IL-1 results in a accumulation of latent MMPs, which may lead to autoactivation of proMMPs due to high local concentrations as described for proMMP-2 [53]. Taken together, investigations by others and the present study indicate that IL-1 effectively increases proMMP synthesis and that activation of these proMMPs requires other factors than IL-1 for collagen degradation of cartilage to occur.

The actual presence of active MMPs in cartilage from arthritides was shown immunohistochemically: MMP-generated neoepitopes in collagen and aggrecan were observed [54–58]. This implies the presence of MMP-activating factors in cartilage *in vivo* which may include catabolic cytokines and activators of proMMPs. Several enzymes have been suggested to play a role in proMMP activation. Plasmin [59,60], kallikrein [61], mast cell proteinases [62] activate proMMPs *in vitro*, but are not produced by chondrocytes. Elevated levels of the plasminogen activator/plasmin system and kallikrein have been found in synovial fluid from arthritis patients [63, 64]. Besides proteolytic activation of latent MMPs, other mediators can be involved like hypochloric acid produced by neutrophils that is able to activate proMMP-8 and -9 [65, 66]. In addition, a decrease in pH is known to activate proMMP-3 [67, 68] and may be local phenomenon, but the involvement in cartilage degradation is still unknown.

In this model, we have shown that a pool of proMMPs can be activated by the artificial activator APMA resulting in matrix degradation. For investigation of putative proMMP activators, the alginate culture system for chondrocytes seems a suitable and convenient model, and can be helpful in screening drugs for their ability to counteract induced matrix degradation. In contrast to cartilage explants, interfering constituents from synovial fluid which may confound the direct effect of a cytokine on matrix degradation are lacking in alginate beads.

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