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Stimulation of type II collagen biosynthesis and secretion in bovine chondrocytes cultured with degraded collagen

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Abstract The functional integrity of articular cartilage is dependent on the maintenance of the extracellular matrix (ECM), a process which is controlled by chondrocytes. The regulation of ECM biosynthesis is complex and a variety of substances have been found to influence chondrocyte metabolism. In the present study we have investigated the effect of degraded collagen on the formation of type II collagen by mature bovine chondrocytes in a cell culture model. The culture medium was supplemented with collagen hydrolysate (CH) and biosynthesis of type II collagen by chondrocytes was compared to control cells treated with native type I and type II collagen and a collagen-free protein hydrolysate. The quantification of type II collagen by means of an ELISA technique was confirmed by immunocytochemical detection as well as by the incorporation of ^{14}C -proline in the ECM after a 48 h incubation. Chondrocytes in the control group were maintained in the basal medium for 11 days. The presence of extracellular CH led to a dose-dependent increase in type II collagen secretion. However, native collagens as well as a collagen-free hydrolysate of wheat proteins failed to stimulate the production of type II collagen in chondrocytes. These results clearly indicate a stimulatory effect of degraded collagen on the type II collagen biosynthesis of chondrocytes and suggest a possible feedback mechanism for the regulation of collagen turnover in cartilage tissue.

Keywords Collagen hydrolysate · Collagen secretion · Chondrocyte metabolism · Type II collagen · Cell culture (Bovine)

Introduction

Articular cartilage is a characteristic type of connective tissue. Its unique biological and mechanical properties depend on the composition of a highly specialized extracellular matrix (ECM) which can be considered as a macromolecular framework of two major components, proteoglycans and collagens. Type II collagen is the predominant collagen in cartilage (Mayne 1989). Together with smaller amounts of other collagens, type II collagen forms a three-dimensional fibrillar network which is essential for the tensile stiffness and strength of cartilage and provides the basic architecture of the tissue (Eyre et al. 1991). Aggrecans, as well as several other proteoglycans identified in articular cartilage, are embedded within the fibrous network, providing the compressibility and elasticity of this tissue (Roughly and Lee 1994).

Chondrocytes are responsible for the synthesis, organization and maintenance of the ECM (Muir 1995). Changes in the composition of the matrix affect the balance between synthetic and degradative activity of chondrocytes, leading to a continuous internal remodeling as the cells replace macromolecules lost through degradation.

Although there is still a lack of precise information about chondrocyte physiology, it is well known that normal matrix turnover depends on the ability of these cells to detect alterations in the ECM, including the presence of specific bioactive molecules, and respond by synthesizing new macromolecules (Buckwalter and Mankin 1998). Under certain pathophysiological conditions, such as osteoarthritis, the sensitivity of chondrocytes to a variety of regulatory signals seems to be decreased (Hardingham and Bayliss 1990), possibly contributing to the alterations in the matrix composition. In consequence, a progressive imbalance between matrix degradation and matrix regeneration leads to a marked decrease in type II collagen content of the ECM and eventually results in cartilage damage (Mlynarik and Tratting 2000).

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The complex regulatory mechanisms of chondrocyte function are not well understood. In recent years, various substances have been identified to influence chondrocyte metabolism and thus alter matrix turnover. Several investigations have shown that cytokines and growth hormones play an important role in the regulation of chondrocyte metabolism (Saklatvala 1986; Lotz et al. 1995; Trippel 1995; Benz et al. 2002). Cytokines may bind to cell receptors and stimulate biosynthesis of matrix macromolecules (Osborne et al. 1989; Rosier and O'Keefe 1998) as well as the production of proteolytic enzymes, such as collagenase and proteoglycanase (Pasternak et al. 1986; Eyre et al. 1991; Roughly et al. 1991). Moreover, type II collagen and collagen fragments are proposed to participate in regulatory processes that modulate metabolic activities in chondrocytes (Aycok et al. 1986; Katayama et al. 1991; Qi and Scully 1997).

The aim of the present study was to investigate the effect of degraded collagen on the metabolism of mature articular chondrocytes. To achieve this, a model system consisting of mature bovine chondrocytes in culture was treated with various concentrations of collagen hydrolysate (CH) followed by measurements on the biosynthesis and secretion of type II collagen.

Materials and methods

Isolation and cultivation of bovine chondrocytes

All cartilage samples were dissected from the joints (articulatio tarsi transversa) of steers obtained from a local abattoir within 2 h after slaughter. The cartilage was minced and extensively washed with Hanks' buffered salt solution (HBSS), containing 100 U/ml penicillin and streptomycin as well as 5 µg/ml amphotericin B. The cartilage was subsequently digested in HBSS supplemented with hyaluronidase (2,000 U/ml) and pronase (30 U/ml) for 60 min at 37°C. In the next step, the cartilage fragments were washed twice with HBSS and transferred into a collagenase solution (8 U/ml Ham F12 medium containing 10% FCS) at 37°C for 7 h with gentle shaking. After centrifugation at 400×g for 10 min, the supernatant was discarded and the pellet resuspended in 10 ml of the basal cell medium (Ham's F12, 10% FCS, 10 µg/ml gentamycin and 5 µg/ml amphotericin B). The vital chondrocytes were counted by means of the trypan blue exclusion method and seeded in 12-well culture plates at a density of 350,000 cells/cm². Chondrocytes were cultured at 37°C in an atmosphere of 5% O₂, 5% CO₂ and 90% N₂. The basal culture medium (BM) was changed every 3 days for the duration of the experiments.

Preparation of collagen hydrolysates

Collagen hydrolysate (CH) was obtained from DGF Stoess, Eberbach, Germany. For the production of the collagen hydrolysate, connective tissue of bovine skin was pretreated with NaOH and H₂O₂ at pH 12.8. After washing and neutralization to pH 7 with H₂SO₄, proteins were heat denatured for 2 h at 90°C and subsequently digested with bacterial proteases (0.02% enzymes/protein) for 2 h. The protease activity was inhibited by thermal inactivation (85°C), after which the solution was concentrated and finally spray dried.

To obtain a low molecular weight fraction (CH-F1) of the collagen hydrolysate, an aliquot of CH was filtered through an ultra-filter (Schleicher & Schuell No. 4, Düren, Germany) with an exclusion size of 5 kDa.

Type II collagen hydrolysate (CH II) was prepared from chick sternal cartilage under conditions comparable to the production of CH, as stated above. Plantasol (PLA), a collagen-free hydrolysate of wheat proteins, was obtained from DGF Stoess, Eberbach. The peptide profiles of all hydrolysates used in the cell culture experiments were analyzed by means of high performance liquid chromatography (HPLC).

HPLC

GPC-HPLC measurements were performed using a Pharmacia LKB 2150 system equipped with a UV detector operating at 214 nm and a column 2000 SW (Tosoh Biosep, Stuttgart, Germany) with a guard column (SWXL, Tosoh Biosep). An isocratic elution was carried out by a solvent consisting of 400 mmol/l sodium phosphate buffer (pH 5.3). The flow rate was 0.5 ml/min. The HPLC system was calibrated by means of well-defined human type I collagen fragments (generous gift from the Max Planck Institute of Biochemistry, Martinsried, Germany).

Stimulation experiments

Mature bovine chondrocytes were cultured as described above. After 3 days, the basal culture medium (BM) was replaced by BM supplemented with one of the following test substances:

1. CH: hydrolyzed type I collagen (mean MW 3.3 kDa)
2. CH-F1: hydrolyzed type I collagen (mean MW 1.9 kDa)
3. CH II: hydrolyzed type II collagen (mean MW 3.1 kDa)
4. PLA: collagen-free, hydrolyzed wheat proteins (mean MW 1.5 kDa)
5. Coll I: native type I collagen (Fluka, Buchs)
6. Coll II: native type II collagen (DGF Stoess)

The cells were incubated for a further 8 days in the cell media supplemented with 0.5 mg/ml of the various test substances. At the end of the stimulation period, on culture day 11, the amount of type II collagen was determined both in the supernatants and in the cell layers by means of the collagen ELISA. Chondrocytes in the control group were cultured in BM for 11 days.

In additional experiments, stimulated chondrocytes were incubated for 48 h with 400 Bq/ml [¹⁴C]-proline and the cell-associated radioactivity was determined. By the end of the culture period (day 11), the cell medium was discarded and the cells were rinsed with saline solution. The cell layers were subsequently removed from the plate and extensively washed in distilled water. After centrifugation the residual pellet was lysed in Biolute S (Zinsser Analytic) and radioactivity of the samples was determined following the addition of Aquasafe 300 Plus scintillation liquid (Zinsser) in a liquid scintillation counter (Win Spectral 1414, Wallac GmbH, Freiburg, Germany).

Quantification of type II collagen

Bovine type II collagen was determined by means of the ArthroG-CIA type II collagen capture ELISA Kit (Chondrex LLC, Redmond, USA). To solubilize the newly synthesized collagen, the cell culture medium was removed and 1.1 ml pepsin solution (0.1 mg/ml dissolved in 0.05 M acetic acid, pH 3.0) was added to the cell layer as well as to the removed medium and protein was digested for 24 h at 4°C. Subsequently, 100 µl of TSB (1.0 M TRIS, 2.0 M NaCl, 50 mM CaCl₂, pH 8) was added to stop the reaction. To monomerize the remaining polymeric collagen, 100 µl of pancreas elastase solution (1 mg/ml dissolved in 1:10 TSB) was added to the samples followed by an incubation for 30 min at 37°C. After centrifugation (10,000×g for 5 min) an aliquot of the supernatant was removed and immediately assayed for type II collagen by ELISA. The type II concentration in the digested culture medium was similarly assayed.

Immunocytochemistry

Chondrocytes were seeded on glass coverslips at a density of 100,000 cells/cm² and treated as described above. The cells were fixed with cold acetone (-20°C) for 10 min. After rinsing in TBS (0.14 M NaCl in 20 mM TRIS/HCl buffer, pH 7.4), cells were blocked in 0.2% H₂O₂-methanol for 20 min. The chondrocytes were rinsed again in TBS and incubated for 60 min with the primary monoclonal antibody against type II collagen (DBSH, University of Iowa) diluted 1:10 in TBS. After washing in TBS, the cells were incubated for 30 min with the HRP-conjugated rabbit anti-mouse immunoglobulin secondary antibody (Dako P0260) diluted 1:200 in TBS containing 10% bovine serum. Finally, the cultures were washed in TBS and the third antibody (HRP-conjugated goat anti-rabbit immunoglobulin Dako, P0448) diluted 1:150 in TBS, supplemented with 10% bovine serum, was applied for 30 min. Immunoreactivity was demonstrated by diaminobenzidine staining (DAB Kit, Vector Laboratories). Chondrocyte nuclei were counterstained with Meyer's hemalum, diluted 1:1 in distilled water.

Statistical analysis

All results are expressed as mean values \pm SD. The significance of differences in values was assessed by the Mann-Whitney U-test. Differences with $P < 0.01$ were considered significant.

Results

Characterization of the hydrolysates

Collagen hydrolysate (CH) was prepared from bovine connective tissue under the conditions described above. Subsequent to the digestion with bacterial proteases, the average size of the CH fragments was between 0.5 and 15 kDa, with a mean MW of 3.3 kDa, as determined by means of HPLC (Fig. 1).

In the low molecular weight fraction of collagen hydrolysate (CH-F1), no peptides of MW larger than 10 kDa could be detected. The calculated mean MW in this CH-F1 preparation was 1.9 kDa.

Measurements of type II collagen hydrolysate (CH II) prepared from chick cartilage, revealed peptides with a mean MW of 3.1 kDa. In agreement with the results of the determination of the MW distribution of peptides in CH, collagen fragments in the approximate range of 0.5–15 kDa were detected in CH II.

For the collagen-free hydrolysate (PLA), a MW of 1.5 kDa was specified.

Stimulation of the chondrocytes with collagen hydrolysate

The treatment of cultured chondrocytes with 0.5 mg/ml CH over a culture period of 11 days induced a marked increase in type II collagen secretion into the culture medium, with significantly higher type II collagen concentrations compared to the control cells receiving no CH supplement (Fig. 2). At the end of the culture period (11 days), type II collagen secretion was almost

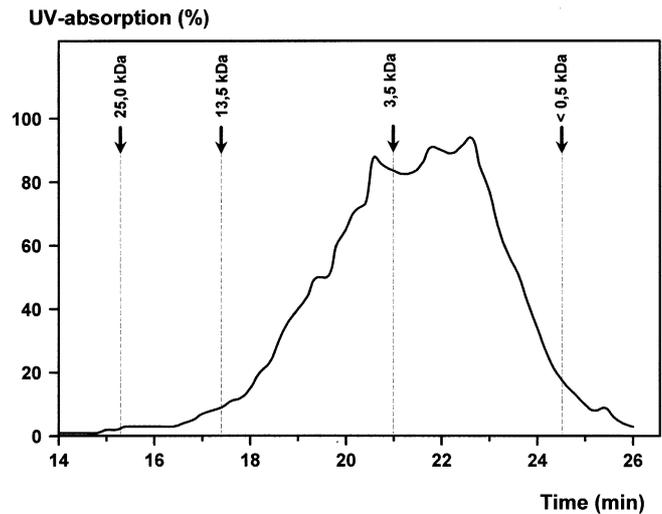


Fig. 1 Separation profile of collagen hydrolysate (CH) from the digestion of bovine type I collagen with bacterial proteases. Illustration represents one characteristic HPLC chromatogram

Type II collagen ($\mu\text{g}/10^6$ chondrocytes)

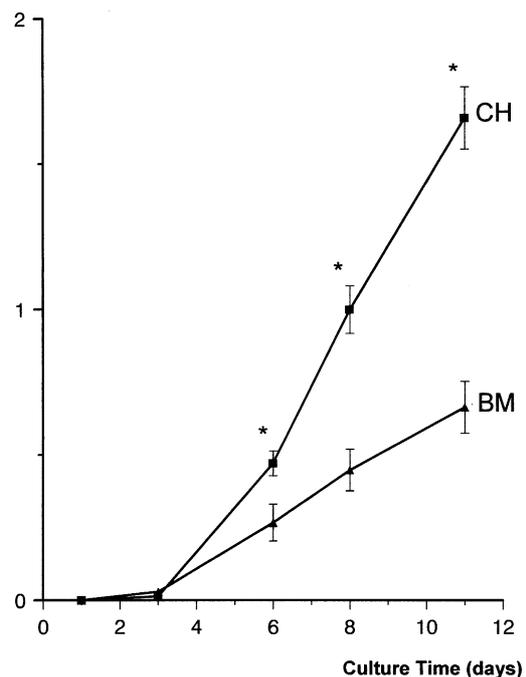


Fig. 2 Time course of type II collagen secretion into the supernatants of bovine chondrocytes cultured in basal medium (BM) or in medium supplemented with 0.5 mg/ml collagen hydrolysate (CH). Data represent mean \pm SD of four chondrocyte preparations performed in triplicate. * $P < 0.01$ compared with untreated controls

2.5-fold higher in CH-stimulated cultures in comparison with the control cells.

Increased concentrations of CH in the culture medium induced a dose-dependent stimulation in type II collagen secretion (Fig. 3). Thus, after administration of 0.1 mg/ml

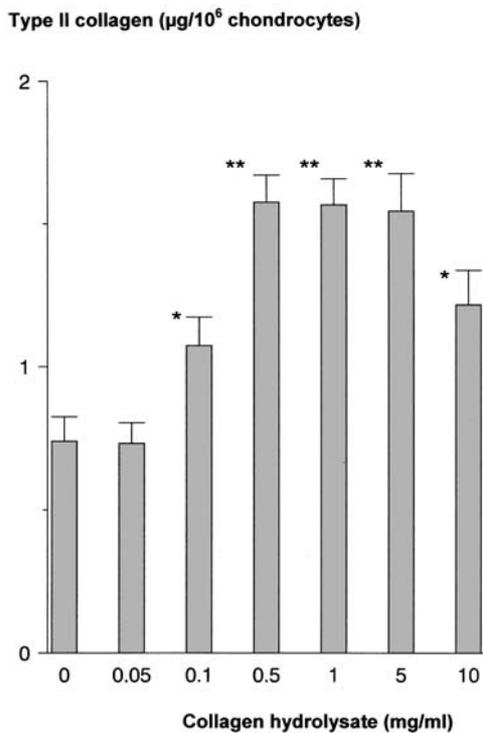


Fig. 3 Type II collagen secretion measured in the supernatants of 11-day-old bovine chondrocyte cultures after treatment with collagen hydrolysate. Data represent mean \pm SD of six chondrocyte preparations performed in duplicate. * P <0.01 compared to untreated controls. ** P <0.01 compared to treatment with 0.1 mg/ml CH

CH, an enhancement in collagen secretion of 45% could be determined compared to the control cells. Chondrocytes treated with CH at concentrations ranging from 0.5 mg/ml up to 5 mg/ml secreted a maximum of 2.2-fold more type II collagen than the untreated cells. A higher concentration of CH (10 mg/ml) resulted in a significantly decreased collagen secretion in this culture model.

The stimulation of type II collagen synthesis was also confirmed by means of immunocytochemistry (Fig. 4). Chondrocyte cultures treated with CH (0.5 mg/ml)

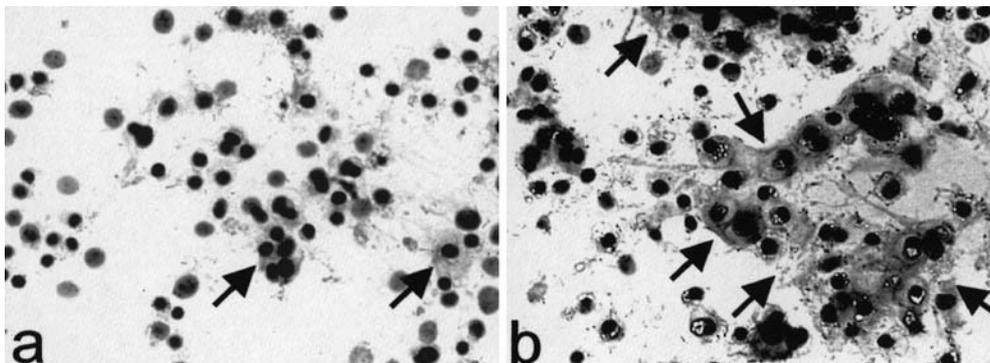


Fig. 4a, b Immunocytochemical visualization of type II collagen (note arrows) in 11-day-old bovine chondrocyte cultures (DAB staining). **a** Chondrocytes cultured in basal medium. **b** Stimulation

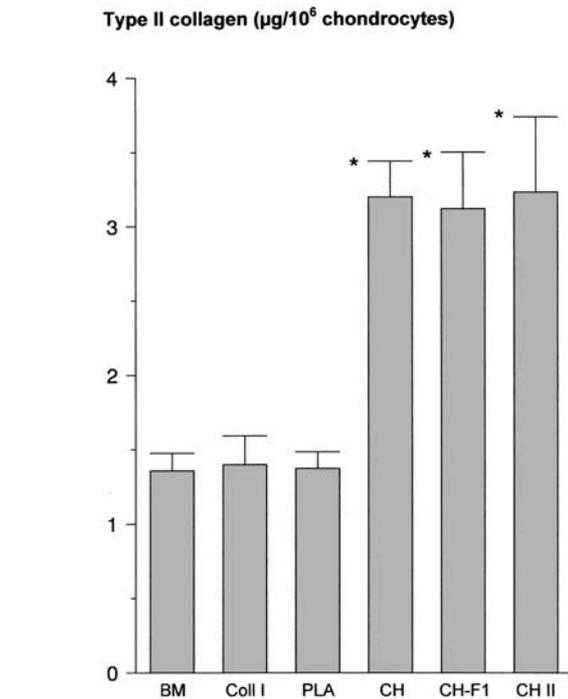


Fig. 5 Type II collagen biosynthesis measured in a bovine chondrocyte culture over 11 days after treatment with one of the following substances (0.5 mg/ml): native collagen type I (*Coll I*), collagen-free hydrolysate of wheat protein (*PLA*), collagen hydrolysate (*CH*), collagen hydrolysate fraction (*CH-F1*) and type II collagen hydrolysate (*CH II*). Cells in the control group (*BM*) were cultured in basal medium. Data represent mean \pm SD of six chondrocyte preparations performed in duplicate. * P <0.01 compared to untreated controls

deposited tight nets of collagen fibers pericellularly, whereas in the control cultures the measurable amount of cell-associated type II collagen was considerably reduced.

The specificity of the effect of CH on collagen biosynthesis in chondrocytes was investigated using native collagen and a non-collagenous protein hydrolysate. In these experiments total collagen type II formation was determined by measuring its secretion into the culture

of type II collagen biosynthesis after treatment with 0.5 mg/ml CH, as indicated by tight nets of collagen deposited around the chondrocytes. $\times 260$

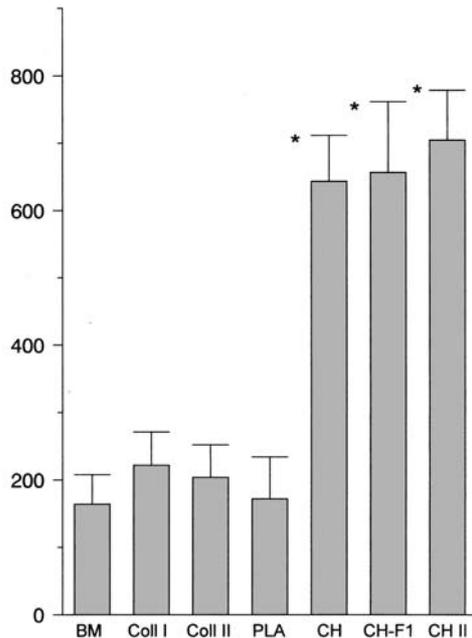
Radioactivity (dpm/10⁶ chondrocytes)

Fig. 6 Stimulation of the collagen biosynthesis in 11-day-old bovine chondrocyte cultures, as measured by incorporation of [¹⁴C]-proline (48 h incubation) after treatment with one of the following substances (0.5 mg/ml): native collagen type I (*Coll I*), native collagen type II (*Coll II*), collagen-free hydrolysate of wheat protein (*PLA*), collagen hydrolysate (*CH*), collagen hydrolysate fraction (*CH-F1*) and type II collagen hydrolysate (*CH II*). Cells in the control group (*BM*) were cultured in basal medium. Data represent mean \pm SD for six experiments performed in duplicate. * $P < 0.01$ compared to untreated controls

supernatants and its incorporation into the newly synthesized ECM (Fig. 5). After incubation with 0.5 mg/ml CH, chondrocyte biosynthesis of type II collagen was increased by more than 1.6-fold compared to the control cells. A similar increase in collagen synthesis was observed after the administration of CH-F1, representing predominantly type I collagen fragments with an MW <10 kDa. Moreover, treatment with hydrolyzed type II collagen (CH II) had the same pronounced stimulatory effect on collagen biosynthesis as compared to CH and CH-F1.

In contrast, supplementation of the cell medium with 0.5 mg/ml native type I collagen (*Coll I*) or with a collagen-free hydrolysate (*PLA*), administered in the same concentration, induced no stimulation in collagen biosynthesis in the chondrocytes. To verify these results, the stimulation of collagen formation was studied by analyzing the incorporation of [¹⁴C]-proline into the chondrocytes and the ECM respectively (Fig. 6). Supplementation of the culture medium with either CH or CH-F1 led to a pronounced increase in cell-bound radioactivity, reaching more than threefold higher values in comparison with the control cultures. After the administration of CH II, a very similar increase in collagen biosynthesis was observed, indicating no significant

differences from the chondrocytes receiving type I collagen fragments (CH and CH-F1).

In accordance with the results on the stimulation of type II collagen biosynthesis, no significant increase in [¹⁴C]-proline incorporation was observed after the administration of PLA or native type I and type II collagens (*Coll I*, *Coll II*) as compared to the untreated control cultures.

Discussion

In this work, a primary cell culture model was used to investigate the influence of collagen hydrolysate on the type II collagen biosynthesis by chondrocytes. These cells are known to change their phenotype when cultured over a longer period of time, switching over to the production of type I collagen instead of cartilage collagen type II (von der Mark et al. 1977; Benya et al. 1987; Zaucke et al. 2001). The extent and the rate of this process can be affected by numerous factors, such as seeding density, age of the cells in culture and culture medium (Elima and Vuorio 1989). Due to these changes, chondrocytes in the present study were cultured under reduced partial oxygen pressure (5% O₂) and the cell culture time was limited to 11 days. The chondrocytes treated with CH as well as the control cells retained their polygonal or rounded morphology throughout the culture period. Moreover, a persistent secretion of type II collagen could be determined in the cultures over 11 days, indicating that the majority of chondrocytes had not switched to the secretion of type I collagen. At the end of the observation period, fibroblastic cells represented less than 5% of the total cell population.

The results of our study clearly indicate that the treatment of chondrocytes with CH induced a dose-dependent increase in type II collagen secretion. The observation that extracellular collagen hydrolysate is able to stimulate collagen biosynthesis in chondrocytes suggests a possible mechanism for the regulation of collagen turnover in cartilage. Thus, an increasing concentration of collagen fragments in the ECM, due to collagen degradation processes, might induce the synthesis and secretion of type II collagen by chondrocytes. If this is the case, then only degraded peptides of collagenous origin and not intact collagen should be able to influence this process. In accordance with this theory, our results indicated that the applied collagen-free protein hydrolysate (*PLA*) as well as native type I and type II collagen failed to induce an increase in type II collagen formation, as measured by the incorporation of [¹⁴C]-proline. These results are consistent with similar investigations by Phadke and Nanda (1983) on rabbit articular chondrocytes.

In order to gain more information on the specificity of the stimulating peptides, culture experiments were performed using a low molecular weight fraction of CH, which is characterized by collagen fragments <10 kDa (CH-F1). It was shown that the marked stimulation of collagen type II synthesis induced by the CH-F1 fraction

was not significantly different to the stimulatory efficiency of CH. From these results it appears that primarily smaller collagen fragments with a MW of less than 10 kDa seem to exert a positive influence on the biosynthesis of type II collagen in chondrocytes.

In additional experiments we demonstrated that CH (predominantly hydrolyzed type I collagen) as well as CH II (hydrolyzed type II collagen) were able to stimulate type II collagen production in chondrocytes almost to the same extent. The fact that both hydrolysates exerted a stimulatory effect on the collagen biosynthesis could be explained by minor differences in the composition of fibrillar collagens (Bateman et al. 1996). It is therefore most likely that the hydrolysis of type I and type II collagen gives rise to polypeptides with identical or at least very similar structure.

To confirm the stimulatory effect of CH on chondrocyte metabolism, we investigated the type II collagen secretion by means of immunocytochemistry. Although stimulation of the chondrocytes with CH led to a marked increase in the amount of collagen secretion, it was noticeable that not all of the cells deposited collagen pericellularly. This observation could be explained by the fact that our experiments were performed on primary cell cultures consisting of a heterogeneous chondrocyte population derived from different zones of the total cartilage. Four different zones of the articular cartilage are generally recognized and investigations have shown that cells derived from the various zones, and thus from a different depth of cartilage, differ in their metabolic activity (Adyeltte et al. 1991; Muir 1995; Huber et al. 2000).

In addition to enhanced type II collagen secretion, collagen fragments induced an increased deposition of pericellular type II collagen in chondrocytes derived from normal articular cartilage. However, the exact regulatory mechanism of this metabolic activation as well as the question of how these fragments interact with the target cell, remain unclear. Although the mechanism of this metabolic activation is as yet unknown, these data provide further evidence that chondrocytes are able to detect alterations in the extracellular environment and respond by synthesizing appropriate quantities of new macromolecules in order to maintain the integrity of the ECM.

Moreover, the observed stimulation on collagen synthesis in mature chondrocytes could be of particular therapeutic relevance in the treatment of degenerative joint diseases such as osteoarthritis. The course of degenerative alterations in articular cartilage is characterized by an imbalance between matrix degradation and the regeneration of the ECM (Mlynarik and Tratting 2000; Hardingham and Bayliss 1990). Subsequent to an initial phase of activated cell metabolism, associated with increased aggrecan and collagen synthesis (Sandy et al. 1984; Aigner et al. 1997), ECM formation by chondrocytes decreases progressively (Burstein et al. 2000). This reduced cellular anabolic activity, along with an increased enzymatic degradation of ECM macromolecules, leads to a loss of cartilage stability and finally to cartilage destruction. These pathological changes are likely to be

a result of multiple factors affecting the cartilage along with an age-related reduction in chondrocyte activity (Huber et al. 2000). Since decreased collagen metabolism seems to be an important factor for the progression of osteoarthritis, it is most likely that CH treatment might achieve an improvement in the maintenance of the ECM by means of increased type II collagen secretion. This consideration is supported by clinical investigations that have demonstrated the positive influence of orally administered CH on the development of osteoarthritis (Moskowitz 2000). Based on the fact that degraded collagen is absorbed from the intestine in its high molecular form and preferentially accumulates in cartilage (Oesser et al. 1999), it might be reasonable to utilize CH as a nutritional supplement to activate collagen biosynthesis in chondrocytes. Thus, under conditions where cartilage is under considerable stress, CH might be of particular importance for the nutrition of cartilage tissue and might contribute to reduce degenerative alterations in the ECM.

To our knowledge, the current study is the first to demonstrate the stimulatory effect of degraded collagen on the type II collagen metabolism of chondrocytes. Clearly, the potential therapeutic relevance of these observations warrants further studies on the mechanisms regulating collagen biosynthesis by chondrocytes.

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