Upregulation of type X collagen expression in osteoarthritic cartilage

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Joint destruction of articular cartilage in osteoarthritis and rheumatoid arthritis is heralded by surface fibrillation, loss of proteoglycans from the articular surface and increasing hydration and softening of the cartilage matrix (Sokoloff 1982). These initial changes are followed by chondrocyte proliferation and formation of cell clusters and extensive remodeling of the cartilage matrix, i.e. massive degradation and synthesis of new, but partially altered cartilage components.

Compared to the extensive studies published on the loss of proteoglycans and glycosaminoglycans from osteoarthritic cartilage, relatively little is known on the changes in structure and composition of the collagenous frame work of articular cartilage during arthritic degeneration. The cartilage collagen fibrils are heterofibrils consisting of about 85–90% type II collagen and 5% type XI collagen, to which type IX collagen molecules are covalently attached (for review see Mayne 1989, van der Rest and Garrone 1991). This fibrillar network is interwoven with a microfibrillar meshwork consisting of cystine-crosslinked type VI collagen molecules (Ayad et al. 1984, Keene et al. 1988).

Type X collagen, a short, network forming collagen (for reviews see Schmid and Linsenmayer, 1985, Kielty, 1993) is found exclusively in the hypertrophic cartilage in the fetal and juvenile growth plate of long bones, but also in the calcified zone of articular cartilage below the tide mark (Gannon et al. 1991). Previously, we have reported on the appearance of type X collagen in osteoarthritic articular cartilage. Conflicting reports exist on the question whether or not type II collagen is replaced by type I and III collagen in osteoarthritic cartilage (Adam and Deyl 1983, but see Goldwasser, 1982). Such discrepancies particular in the biochemical analysis of OA cartilage samples most likely result from the heterogeneity of osteoarthritic lesion within the joint, and even more among different patients and joints. Thus, in order to gain insight into the alterations in collagen expression occurring during osteoarthritic cartilage destruction, we performed immunohistological and in situ hybridization studies using collagen-specific antibodies and cDNA probes which allowed us to understand the focal changes in the chondrocyte phenotype in individual regions of the joints.

Materials and methods

1. Human cartilage samples

Human osteoarthritic articular cartilage was obtained from knee and hip endoprothesis operations in collaboration with the Orthopedic Hospital, Waldkrankenhaus St. Marien, Erlangen. The operated joints were graded clinically, and the remaining cartilage was excised focally and graded histologically according to Mankin et al. (1971). The cartilage samples were fixed in 4% paraformaldehyde for 24h at 4°, dehydrated embedded in paraffin under RNA-free conditions as described previously (Aigner et al. 1992).

2. Chondrocyte cultures

For chondrocyte cultures and PTH stimulation, epiphyseal cartilage from calf fetuses was cleaned from adhering connective tissue; the hypertrophic zone adjacent to the cartilage-bone transition zone in the growth plate was separated from the proliferating and resting zone, and both parts were separately digested with trypsin and collagenase (*Clostridium histolyticum*, CLSPA, Boehringer Mannheim) for 4h under serum-free conditions. Chondrocytes were cultured in F12/DMEM medium (1:1) containing ascorbate (50 µg/ml) for 24 hrs, and then stimulated with various PTH fragments for 24hrs as described elsewhere (S. Erdmann et al. 1995 in prep.). PTH-fragments (1–34), (3–34), (13–34), (28–48), (39–68),

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(52–84) and (64–84) were obtained from Sigma (St. Lous) MO, USA).

3. Northern blotting

For quantitative analysis of Col10A1 and Col2A1 mRNA steady state levels, chondrocytes were harvested after PTH stimulation and lysed in 4M guanidinium thiocyanate, 25 mM Na-citrate, 0.5% Na-sarcosyl, 0.7% ß-mercaptoethanol. RNA was separated by cesium chloride density centrifugation (Chirgwin et al. 1979) and subjected to formaldehyde gel electrophoresis as described (Dietz et al. 1992). The RNA was blotted onto nylon filters, crosslinked by UV and hybridized to ^{32}P-ATP and CTP-labeled collagen cDNA probes as described (Dietz et al. 1993). The cDNA probe for Col2A1 (pHCG2) which is derived from the C-propeptide was kindly provided by Drs. Sandberg and E. Vuorio (1987). The cDNA-probe for Col10A1 (pERX) is derived from the NC1 domain of human collagen type X (Reichenberger et al. 1991).

4. Preparation of human recombinant collagen type X

A full length human Col10A1 cDNA was constructed from a genomic cDNA fragment covering the entire exon 3; (Reichenberger, 1993) exons 1 and 2 as well 5'UTR sequences were synthesized by RT PCR using the 5'sequences of exon 3 as primer, and mRNA from human hypertrophic chondrocytes as template. The cDNA was ligated into the eucaryotic expression vector pCis (Gorman et al. 1990) under the CMV promoter (F. Beier et al. 1995 in prep.).

Human 293 kidney cells and HT 1080 fibrosarcoma cells were transfected with the pCMV-ColX vector and cotransfected with a vector carrying a puromycin resistance gene. Puromycin resistant, stable clones were obtained that secreted up to 10 µg/ml of human recomb. collagen type X into the medium.

5. Immunohistology and in situ hybridization

For staining of extracellular collagens, paraffin sections were deparaffinized, hydrated and pre-digested with 2 mg/ml of testicular hyaluronidase, pH 5.0, for 30 min before antibody application. Immunostaining was done as described previously (von der Mark et al. 1992, Aigner et al. 1993) using the alkaline phosphatase staining procedure.

In situ hybridization with 35S-UTP labeled cDNA probes was done as described (Aigner et al. 1992, 1993). Alternatively, non-radioactive probes labeled with digoxigenin (Boehringer Mannheim) were used. For detection of Col2A1 mRNA, pHCG2 clone was transcribed in the pGEM vector as sense and antisense RNA strand, using T7 and SPG RNA polymerase, resp. (Promega). For detection of Col10A1 mRNA, the pERX clone (Reichenberger et al. 1992) was cloned into the pGEM vector.

Results

1. Type X collagen synthesis in OA cartilage

Changes in the expression and distribution of collagen types was investigated in sections of OA cartilage obtained after endoprothesis operations. The stimulation of chondrocyte proliferation leading to chondrocyte clusters in OA cartilage (Vignon et al. 1983) suggested to us that chondrocytes might eventually reach prematurely the hypertrophic state, similar to chondrocytes in the growth plate that become hypertrophic following a proliferative phase. In fact, using a polyclonal antibody to human type X collagen (Kirsch et al. 1991), we were able to identify type X collagen deposition in the matrix of deeply fibrillated OA cartilage, in particular around chondrocyte clusters, but also in deeper zones of OA cartilage (von der Mark et al. 1992a, b). In order to confirm the immunohistological localization of ColX in OA cartilage, new poly- and monoclonal antibodies were prepared against recombinant human type X collagen (see Materials and methods). The apparent molecular weight on SDS-gel electrophoresis of the recomb. human Col X was about 75 kD (Figure 2), as compared to 66 kD of the tissue- or chondrocyte culture extracted material. The material was purified by DEAE cellulose chromatography followed by CM-cellulose chromatography; the purified material (Figure 2b) was used for immunization of mice and rabbits. Rabbit antisera were obtained showing a titer of 1:50000 in the ELISA test against recombinant human Col. X. Monoclonal antibodies were obtained that reacted with rec. Col X in the Western blot (Figure 2c), and specifically stained hypertrophic cartilage in the growth zone of a human fetal tibia epiphyses (Figure 1a). Immunohistologic studies on osteoarthritic cartilage samples using these antibodies confirmed our previous findings (Figure 1c). Chondrocytes enzymatically released from OA cartilage also synthesized significantly enhanced amounts of type X collagen in vitro compared to chondrocytes from normal articular cartilage (von der Mark et al. 1992a, b). By in situ hybridization collagen X expression was documented in hypertrophic chondrocytes of osteophytes (Hoyland et al. 1991, Aigner et al. 1993), but in articular OA cartilage only rarely significant signals were obtained, possibly due to low levels of cytoplasmic Col10A1 mRNA.

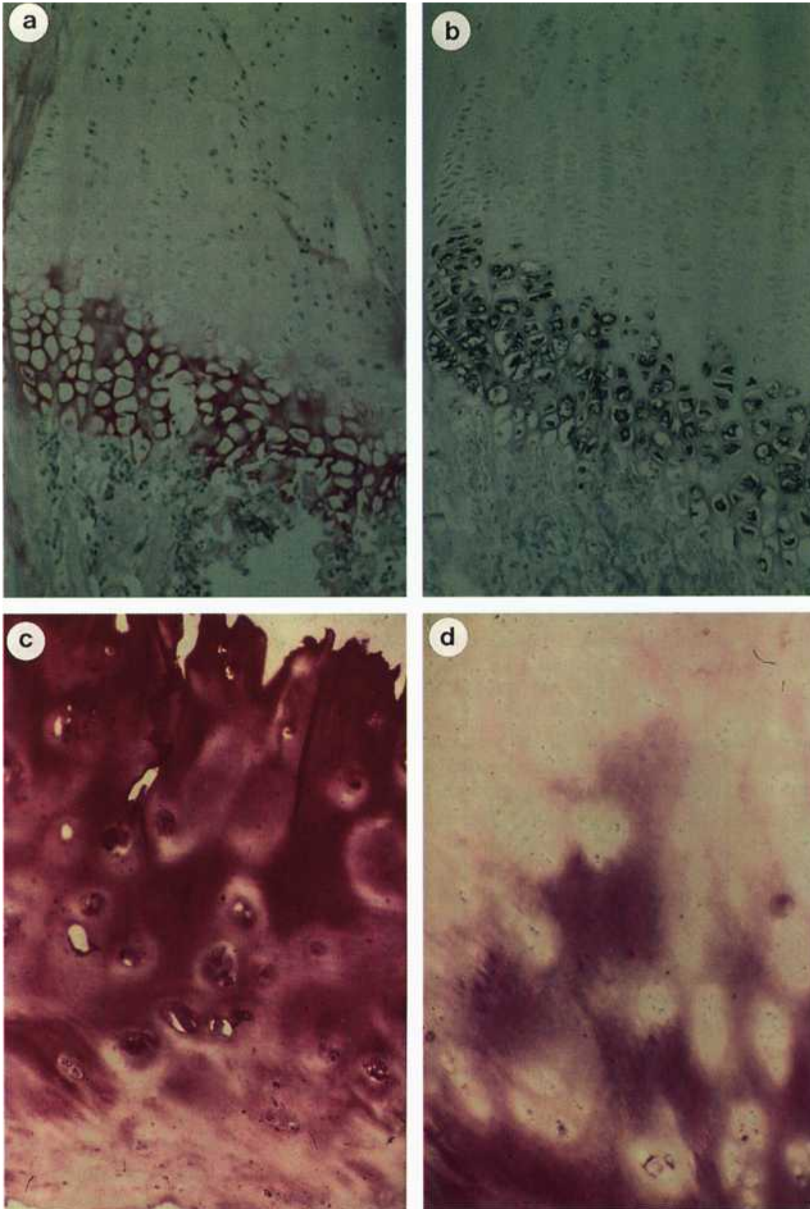

Figure 1. Type X collagen in hypertrophic fetal cartilage and osteoarthritic cartilage. a, b) Comparison of antibody staining of extracellular type X collagen in the growth plate of a human fetal epiphysis (tibia head) (28 wk) with the monoclonal ab 3B5 prepared against recombinant human type X collagen (a), and localization of cytoplasmic α1(X)mRNA by in situ hybridization (b) using the dioxigenin method. c) Antibody localization of type X collagen in osteoarthritic cartilage using a polyclonal ab. d) Osteoarthritic cartilage, stained with a monoclonal antibody against type II collagen (CIID3, Holmdahl et al. 1986), showing extensive pericellular degradation of type II collagen.

2. Regulation of collagen type X expression

The rapid onset of Col10A1 expression in a sharply delineated zone of hypertrophic chondrocytes in the fetal growth plate as indicated by in situ hybridization (Figure 1b), and the onset of type X collagen synthesis in OA cartilage raised the questions on the mechanisms regulating Col10A1 gene transcription, and on hormones or growth factors involved in the regu-

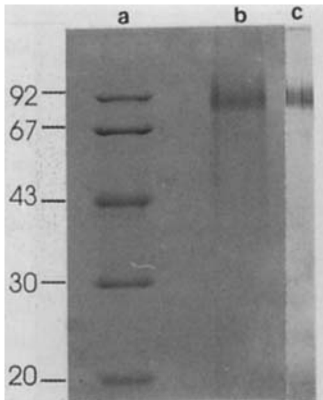

Figure 2. Preparation of monoclonal antibodies against human recomb. type X collagen. a) MW marker b) Human recomb. type X procollagen (Mr 75kd) after purification by DEAE- and CM-cellulose chromatography. c) Immunoblotting of rec. ColX with the monoclonal ab. 3B5.

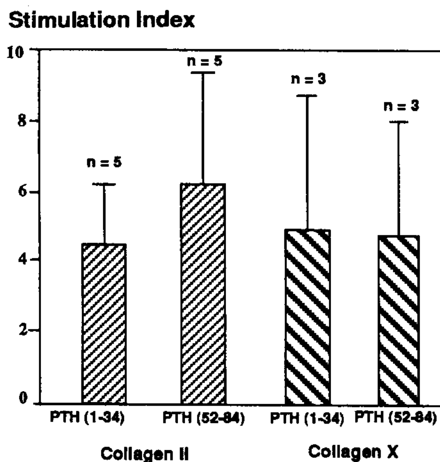

Figure 3. Stimulation of α1(II) and α1(X) mRNA levels in hypertrophic chondrocytes by various PTH fragments. Hypertrophic fetal bovine chondrocytes, freshly prepared from the growth plates of fetal calf bones in the absence of serum, were stimulated with various parathyroid fragements from the N- and C-terminal half of PTH (1 nM) for 24h. mRNA was extracted from the cell cultures, and levels of α1(II) and α1(X) mRNA were determined by Northern hybridization with specific ^{32}P-labeled cDNA probes. The stimulation index refers to the unstimulated samples.

lation. In chondrocyte cultures, besides fetal calf serum, ß-glycerophosphate (Thomas et al. 1990), high doses of Ca^{++} (10 mM, Bonen and Schmid, 1991), and thyroxin (Böhme et al. 1992) have been shown to stimulate type X collagen synthesis. Whether these factors directly regulate type X collagen expression or induce or accelerate differentiation of proliferating chondrocytes to the hypertrophic state is still open.

Recently, we have investigated the effects of various peptide fragments of parathyroid hormone (PTH) on the expression of types II and X collagen in proliferating and hypertrophic bovine fetal chondrocytes. By Northern hybridization we could show that type II collagen mRNA-levels of proliferating chondrocytes isolated in the absence of serum were stimulated severalfold after a 24h period stimulation with PTH fragments from the aminoterminal part of the molecule; the stimulatory effect was localized to res. 28–34 of PTH (Erdmann et al. in prep.). The C-terminal part of PTH had no activity in proliferating chondrocytes. In contrast, a population of hypertrophic chondrocytes isolated from the growth plate showed significantly enhanced levels of type II and of type X collagen mRNA also when stimulated with the C-terminal fragments 52–84 (Figure 3).

This finding correlated with the differential response of proliferating and hypertrophic chondrocytes to N- and C-terminal fragments of PTH in the uptake of calcium. Using Fura-2 as a fluorescent probe for intracellular calcium levels, monolayer chondrocytes were imaged in a fluorescence microscope equipped with a CCD-camera at 350 and 380 nm excitation. Individual cells responded to PTH 1–34, but not to 52–84, while others showed enhanced intracellular calcium only in response to the C-terminal PTH fragment. This indicates the presence of another yet unidentified PTH receptor on the surface of hypertrophic chondrocytes which differs from the PTH receptor on proliferating chondrocytes (Iwamoto et al. 1994).

In order to identify the PTH responsive elements in the promoter of collagen X, various fragments of the promoter region of the human type X collagen gene (Reichenberger et al. 1993) up to –3000 bp upstream of the transcription initiation site including exon 1 were coupled to the luciferase gene as reporter gene, and various chondrogenic (mouse-cfos-transformed chondrocytes, Wang et al. 1993) and non-chondrogenic cells (HT 1080; 293 cells) were transfected with the Col10A1 reporter gene constructs and stimulated with PTH fragments. Preliminary results suggest a region at –2500 to –3000 bp of the Col10A1 promoter which is responsive to the C-terminal part of PTH. Further constructs and stable transfections of other chondrogenic cells will be necessary to confirm this finding.

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References

Adam M, Degl Z. Altered expression of collagen phenotype in osteoarthrosis. Clin Chem Acta 1983; 133: 25–32.

Aigner T, Bertling W, Stöß H, Weseloh G, von der Mark K. Independent expression of fibril-forming collagens I, II, and III in chondrocytes in human osteoarthritic cartilage. J Clin Invest 1993; 91: 829–37.

Aigner T, Reichenberger E, Bertling W, Kirsch T, Stöß H, von der Mark K. Type X collagen expression in osteoarthritic and rheumatoid articular cartilage. Virchows Arch 1993; 63: 205–11.

Aigner T, Stöß H, Weseloh G, Zeiler G, von der Mark K. Activation of collagen type II expression in osteoarthritic and rheumatoid cartilage. Virchows Archiv B Cell Path 1992; 62: 337–45.

Ayad S, Evans H, Weiss J B, Holt L. Type VI collagen but not type V collagen is present in cartilage. Collagen Rel Res 1984; 4: 165–8.

Bonen DK, Schmid TM. Elevated extracellular calcium concentrations induce type X collagen synthesis in chondrocyte cultures. J Cell Biol 1991; 115: 1171–8.

Böhme K, Conscience-Egli M, Tschan T, Winterhalter K H, Bruckner P. Induction of proliferation or hypertrophy of chondrocytes in serum-free culture: The role of insulin-like growth factor-I, insulin, or thyroxine. J Cell Biol 1992; 116: 1035–42.

Chirgwin J M, Przbyla A E, MacDonald R J. Biochem 1979; 18: 5294–9.

Dietz U, von der Mark K, Bertling W. Retinoic acid induced modulation of collagen type I, II, III, IX and X expression in chick sternal chondrocytes. J Cell Biochem 1993; 52: 57–68.

Erdmann S, Müller W, Mayer H, von der Mark K, Burkhardt H. Differential effects of parathyroid hormone fragments on collagen gene expression in chondrocytes. J Cell Biol 1995; in press:

Gannon J M, Walker G, Fischer M, Carpenter R, Thompson R C, Oegema T R. Localization of type X collagen in canine growth plate and adult canine articular cartilage. J Orthop Res 1991; 9: 485–94.

Goldwasser M, Astley T, van der Rest M, Glorieux F H. Analysis of the type of collagen present in osteoarthritic human cartilage. Clin Orthop 1982; 167: 296–302.

Gorman C, Gies D R, McCray G. Transient production of protein using anadenovirus transformed cell line. DNA Protein Egng Techniques 1990; 2: 3–10.

Holmdahl R, Rubin K, Klareskog L, Larsson E, Wigzell H. Characterization of the antibody response in mouse with type IIII collagen-induced arthritis, using monoclonal anti-type II collagen antibodies. Arthritis and Rheumatism 1986; 29: 400–10.

Hoyland J A, Thomas J, Donn R, Marriott A, Ayad S, Boot-Handford R P, Grant M E, Freemont A J. Distribution of type X collagen mRNA in normal and osteoarthritic human cartilage. Bone and Mineral 1991; 15: 151–64.

Iwamoto M, Jikko A, Murakami H, Shimazu A, Nakashima K, Takigawa M, Baba H, Suzuki I, Kato Y. Changes in parathyroid hormone receptors during chondrocyte differentiation. J Biol Chem 1994; 264: 17245–51.

Keene D R, Engvall E G R W. Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network. J Cell Biol 1988; 107: 1995–2006.

Kielty C M, Hopkinson I, Grant M E. The collagen family: Structure, assembly and organization in the extracellular matrix. In: Connective Tissue and its Heritable Disorders (Eds. Royce P M, Steinmann B). New York, Wiley-Liss, 1993: 103–47.

Kirsch T, von der Mark K. Isolation of human type X collagen and immunolocalization in fetal human cartilage. Eur J Biochem 1991; 196: 575–80.

Mankin H J, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteoarthritic human hips. J Bone Joint Surg 1971; 53: 523–37.

Mayne R. Cartilage collagens—what is their function, and are they involved in articular disease. Arthritis and Rheumatism 1989; 32: 241–6.

Poole A R. Changes in the collagen and proteoglycan of articular cartilage in arthritis. In: Connective Tissue: Biological and Clinical Aspects. Basel: Karger, 1986: 316–371.

Reichenberger E, Aigner T, von der Mark K, Stöß H, Bertling W. In situ hybridization studies on the expression of type X collagen in fetal human cartilage. Develop Biol 1991; 148: 562–72.

Reichenberger E, Beier F, Luvalle P, Olsen B R, von der Mark K, Bertling W. Genomic organisation and full length cDNA sequence of human collagen X. FEBS Lett 1992; 311: 305–10.

Sandberg M, Vuorio E. Localization of types I, II and III collagen mRNAs in developing human skeletal tissues by in situ hybridization. J Cell Biol 1987; 104: 1077–84.

Schmid T M, Linsenmayer T F. Type X collagen. In: Structure and Function of Collagen types (Eds. Mayne R, Burgeson R E XX). Academic Press 1987; 223–59.

Sokoloff L. The remodelling of articular cartilage. Rheumatol 1982; 7: 11–18.

Thomas J T, Boot-Handford R P, Grant M E. Modulation of type X collagen gene expression by calcium beta-glycerophosphate and levamisole: Implications for a possible role for type X collagen in endochondral bone formation. J Cell Sci 1990; 95: 639–48.

van der Rest M, Garrone R. Collagen family of proteins. Faseb J 1991; 5: 2814–23.

Vignon E, Arlot M, Hartmann D, Moyen B, Ville G. Hypertrophic repair of articular cartilage in experimental osteoarthrosis. Ann Rheum Dis 1983; 42: 82–8.

von der Mark K, Kirsch T, Aigner T, Reichenberger E, Nerlich A, Weseloh G, Stöß H. The fate of chondrocytes in osteoarthritic cartilage: Regeneration, dedifferentiation, or hypertrophy? In: Articular Cartilage and Osteoarthritis (Eds. Kuettner et al.). New York, Raven Press Ltd. 1992: 221–34.

von der Mark K, Kirsch T, Nerlich A G, Kuß A, Weseloh G, Glückert K, Stöß H. Type X collagen synthesis in human osteoarthritic cartilage: Indication of chondrocyte hypertrophy. Arthritis Rheum 1992; 35: 806–11.

Wang, Z Q Grigoriadis, A E, Wagner E F. Stable murine chondrogenic cell lines derived from c-fos-induced cartilage tumors. J Bone Mineral Res 1993; 8: 839–47.