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## Original Articles

# Expression of type X collagen in young and old C57Bl/6 and Balb/c mice. Relation with articular cartilage degeneration

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### Summary

*Objective:* To investigate whether the development of osteoarthritic lesions in the knee joints of mice is associated with increased immunostaining of type X collagen.

*Methods:* Sections of total knee joints in combination with immunohistochemistry were used to study the distribution of type X collagen in the cartilage of young and old mice of two mouse strains, Balb/c and C57Bl/6, known to develop osteoarthritic lesions at different locations. Expression of type X collagen and PTH/PTHrP-receptor mRNA were studied by RT-PCR.

*Results:* Young adult Balb/c and C57Bl/6 mice both expressed type X collagen in the non-calcified cartilage of the tibia-femoral joint. Old mice of both strains had a strongly increased deposition of type X collagen in the patella-femoral but not in the tibia-femoral joint. The locations in the murine knee joints prone to develop osteoarthritis (OA) did not preferentially express increased amounts of type X collagen. Thus, whereas increased type X was observed in both strains in the patella-femoral joints, only Balb/c mice preferentially developed osteoarthritic lesions in these joints. Also cartilage degeneration was usually seen only in the lateral compartment of the knee joints of C57Bl/6 mice but this was not accompanied by increased type X collagen immunostaining. Increased deposition of type X collagen was not associated with elevated levels of type X collagen mRNA or with decreased levels of PTH/PTHrP-receptor mRNA.

*Conclusion:* Type X collagen expression and spontaneous OA in mice are not necessarily related since OA prone locations in the murine knee joint do not preferentially express type X collagen. © 2001 OsteoArthritis Research Society International

*Key words*: Type X collagen, Cartilage, Knee joints.

## Introduction

Articular cartilage, covering the ends of long bones, ensures almost frictionless movements of the articulating surfaces. All the structural constituents necessary to perform the specific functions of articular cartilage, such as collagens and proteoglycans, are synthesized by articular chondrocytes. Aberrations in cartilage homeostasis due to disfunctioning of articular chondrocytes will lead to impairment of cartilage maintenance and eventually to osteoarthritis (OA).

Terminal differentiation of chondrocytes (hypertrophy) has been suggested to be involved in the OA process. Hypertrophy of chondrocytes is characterized by expression of type X collagen, a homotrimer of $\alpha_1$(X) chains ordinarily not expressed in non-calcified articular cartilage. Hypertrophic chondrocytes and type X collagen are typically located in the growth plates and calcified articular cartilages[1–3]. However, limited expression of type X collagen outside these sites has been reported[4,5]. Under pathological conditions, such as human and experimental OA, strong expression of type X collagen is found in articular chondrocytes derived from non-calcified cartilage[6–10]. Moreover, chondrocyte hypertrophy and collagen type X expression in the growth plate is associated with increased matrix degradation and loss of type II collagen[11]. Similar changes are known to occur during OA. Therefore the processes involved in chondrocyte hypertrophy and OA may have features in common and might share overlapping regulatory pathways.

A molecule involved in the regulation of chondrocyte hypertrophy is parathyroid hormone-related peptide (PTHrP). In the growth plate the transition of pre-hypertrophic chondrocytes to hypertrophic chondrocytes is suppressed by PTHrP[12,13]. During the differentiation of growth plate chondrocytes from proliferating chondrocytes to hypertrophic chondrocytes, the cells express an intermediate phenotype, the pre-hypertrophic chondrocyte. These cells express indian hedgehog (ihh) and the PTH/PTHrP-receptor. It has been suggested that under the

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influence of ihh derived from the pre-hypertrophic chondrocytes the neighbouring periarticular perichondrium produces PTHrP, which then signals back to the pre-hypertrophic cells and prevents transition to complete hypertrophy[12–15]. In articular chondrocytes the role of PTHrP in the regulation of chondrocyte phenotype is unclear. However, PTHrP and PTHrP-receptor have been demonstrated in articular chondrocytes, which could imply a role for these molecules in the regulation of chondrocyte phenotype not only in the growth plate but also in articular cartilage[16].

The goal of this study was to investigate whether the development of osteoarthritic lesions in the knee joints of aged mice is associated with expression of type X collagen at these lesion sites. Since elevated immunostaining of type X collagen was indeed observed in these ageing mice we additionally evaluated whether this increased immunostaining correlated with increased expression of type X collagen mRNA and concomitant down-regulation of the expression of PTHrP-receptor mRNA.

## Material and methods

### ANIMALS

Male C57Bl/6 and Balb/c mice aged 10–12 weeks (young adult, $N=14–16$) and 1–2 years (old, $N=14–16$) were used for histological studies. Expression of mRNA was studied in male C57Bl/6 mice aged 3, 6, 10 and 18 months and in male Balb/c mice of 3, 6, 8 and 16 months. The animals were kept in cages with wood chip bedding in air-conditioned rooms at a constant temperature. They were fed a standard laboratory diet and had access to water *ad libitum*.

### HISTOLOGY

Young adult and old mice were killed by cervical dislocation. Carefully dissected knee joints were fixed in phosphate buffered formaline (pH 7.4) for 5 days, decalcified and processed in a standard automatic tissue processing apparatus. Knee joints were embedded in paraffin wax and coronal sections were prepared and stained with safranin O and fast green.

### IMMUNOLOCALIZATION OF TYPE X COLLAGEN

Type X collagen was stained with a polyclonal rabbit antibody developed by one of the co-authors (TM). This antibody was raised against a human NC1 synthetic peptide unique for type X collagen. The human and mouse sequences of this peptide have only a single amino acid difference. In earlier studies it was shown that the type X collagen polyclonal antibody specifically stained type X collagen in murine joints[17]. This antibody did not show cross-reactivity against collagen type I, II, IX and XI.

Knee joints were dissected *in toto* and decalcified in 10% EDTA (Merck, Darmstadt, Germany), 7.5% polyvinylpyrrolidone (PVP M 29.000, Serva, Amsterdam, The Netherlands) in 0.1 M Tris HCl (pH 7.4) for 2 weeks at 4°C. After extensive washing of the joints with 7.5% PVP in 0.1 M TrisHCl, knee joints were frozen in liquid nitrogen and stored at −80°C. Coronal section (7 μm) were cut on a cryostat and mounted on microscope slides pre-coated with 3-aminopropyltriethoxy-silane (Sigma, St Louis, MO). Sections were dried and stored at −80°C until use.

After thawing the sections were fixated for 5 min in 4% formaldehyde (Sigma). To enhance the permeability of the sections, extracellular matrix chondroitin sulfate and hyaluronic acid were removed by incubation with 1% hyaluronidase (type I-s, Sigma) for 30 min at 37°C. Endogenous peroxidase was then blocked with freshly prepared 1% (v/v) $H_2O_2$ in absolute methanol for 30 min at room temperature. Non-specific staining was blocked by pre-incubation of the sections with 10% normal goat serum in PBS with 1% bovine serum albumin (Sigma). The sections were incubated overnight with the anti-type X collagen antibody (dilution 1:4000), followed by incubation with the biotinylated secondary anti-rabbit immunoglobulin antibody (dilution 1:400) whereafter the remainder of the staining procedure was performed with a biotin-streptavidin detection system (Vectra elite kit, Vectra, Burlington, CA).

### ISOLATION OF RNA FROM MURINE PATELLAR CARTILAGE

RNA was isolated from fresh patellar cartilage as described[18,19]. In short, patellae were dissected from murine knee joints and immediately decalcified at 4°C in 3.5% EDTA (Sigma) for 16 h. Following decalcification the complete articular cartilage layer was stripped from the underlying bone. Total RNA was isolated using the RNeasy method as described by the supplier (Qiagen Inc, Valencia, CA, USA). In all RT-PCR experiments, cartilages from 10 patellae were pooled.

### REVERSE TRANSCRIPTASE PCR

The RNA isolated from the cartilage was treated with DNAse I (Life Technologies Inc) to remove possible contamination of the samples with genomic DNA according to the suppliers instructions. The reverse transcription (RT) reaction was performed at 39°C with moloney-murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies Inc) using oligo(dT)15 primers (Eurogentic, Liege, Belgium). Amplification of cDNA was accomplished by using Taq DNA polymerase (Life Technologies Inc) up to a maximal cycle number of 40. To estimate the relative mRNA levels, 5 μl samples were taken at increasing cycle numbers at two cycle intervals. The PCR products were electrophoresed in 1.5% agarose gels containing ethidium bromide. The cycle number at which the product was first detected was taken as a measure for the amount of specific mRNA. In the case of estimation of collagen mRNA levels, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were used as an internal control. Due to the limited amount of tissue and consequently RNA quantities, measurement of the amount of total RNA was impossible. All RT-PCR reactions were performed in duplicate. The results are expressed as relative mRNA levels whereby the expression in 3-month-old animals was arbitrarily set at 1.

The primers used in the amplification reactions were the following. Primers detecting murine type X collagen had the sequences 5′-ATACCCTTTCTGCTGCTAATGTTCTTG ACC-3′ (upper, gene position 1663-1692) and 5′-TGATA TTCCTGGTGGTCCTGGCAAC-3′ (lower, gene position 5349-5373) resulting in a product of 387 bp. Primers detecting murine PTHrP/PTH-receptor had the following sequences 5′-ATGATGGCAACAAAAAATCC-3′ and 5′-GG TGTCCACTACACCGTCTT-3′ resulting in a product of

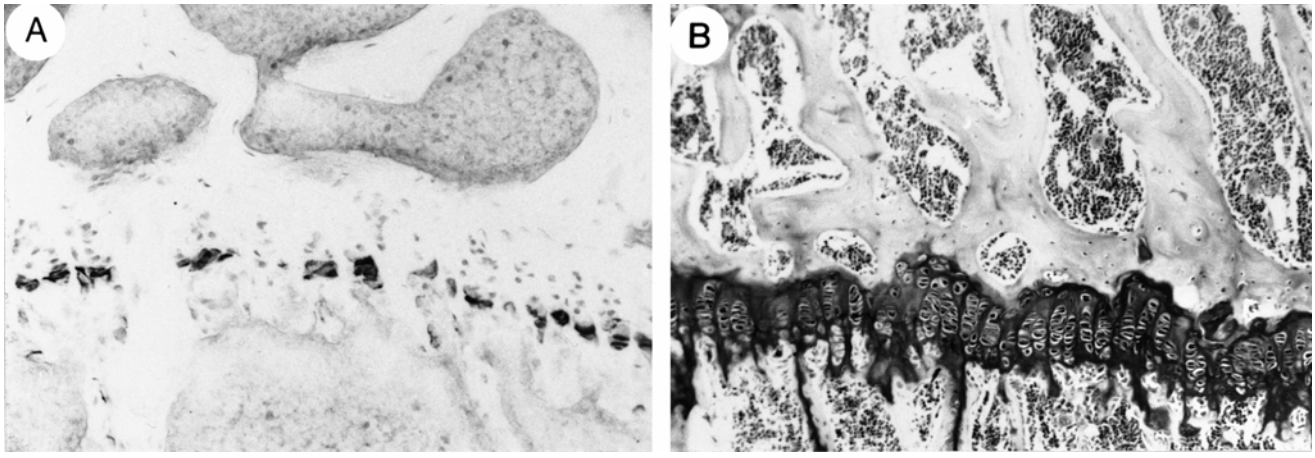


Fig. 1. Staining for type X collagen in the growth plate of young Balb/c mice. Only the hypertrophic chondrocytes in the murine growth plate showed strong type X collagen staining while the proliferating and the resting zone chondrocytes were completely negative (A—type X collagen staining, B—safranin O staining, original magnification 100×).

121 bp. To detect GAPDH the primers 5′-AACTCCCTC AAGATTGTCAGCA-3′ and 5′-TCCACCACCCTGTTGGC TGTA-3′ were used (product 553 bp). Primer sequences were selected with the computer programs Primer (Whitehead Institute, Cambridge, MA) and oligo 4.0 (National Biosciences, Plymouth, MN) and sequence data from international data banks.

## Results

With the exception of articular cartilage as described below, immunostaining of type X collagen was found only in association with hypertrophic chondrocytes, e.g. the growth plate, at the interface of bone and tendons and in calcified articular cartilage. In the growth plate, proliferating and resting zone chondrocytes were completely negative for type X collagen staining while pronounced staining was observed around the hypertrophic chondrocytes (Fig. 1). Omission of the first antibody resulted in complete absence of staining.

Table I
*Expression of cartilage degeneration and type X collagen expression in the non-calcified articular cartilages in young and old Balb/c and C57Bl/6 mice*

| Joint | Type X collagen expression | | Cartilage degeneration | |
|---|---|---|---|---|
| | Young | Old | Young | Old |
| **Balb/c** | | | | |
| Patella/femoral | − | +++ | − | ++ |
| Tibia/femoral | | | | |
| Medial | ++ | ++ | − | −/+ |
| Lateral | ++ | ++ | − | − |
| **C57Bl/6** | | | | |
| Patella/femoral | − | +++ | − | − |
| Tibia/femoral | | | | |
| Medial | ++ | ++ | − | −/+ |
| Lateral | ++ | ++ | − | +++ |

Type X collagen expression; − absent, + light, ++ moderate, +++ pronounced. Cartilage degeneration; − absent, + light, ++ moderate, +++ severe.

### IMMUNOSTAINING OF TYPE X COLLAGEN IN YOUNG BALB/C AND C57BL/6 MICE

Cartilage degeneration and type X collagen immunostaining in non-calcified cartilage in young and old mice are summarized in Table I. In the young (10–12 weeks old) Balb/c and C57Bl/6 mice cartilage lesions were absent. All cartilage surfaces were intact and there was no depletion of proteoglycans revealing the absence of cartilage degeneration in the knee joints of these young animals [Fig. 2(B),(D),(F)]. In the patella-femoral joint of young Balb/c and C57Bl/6 mice, type X collagen immunostaining was found in the calcified cartilage [Figs 2(A) and 3(A)]. Type X collagen staining was present pericellularly surrounding the hypertrophic chondrocytes. Occasionally a faint band of type X collagen immunostaining was seen at the cartilage surface in the central part of the femur [Fig. 3(A)]. The number of hypertrophic chondrocytes was higher and the layer of calcified cartilage thicker in the Balb/c than in the C57Bl/6 mice.

In contrast, on both the lateral and the medial side of the tibia-femoral joint, type X collagen was clearly observed in the non-calcified cartilage [Figs 2(C),(E) and 3(B),(C)]. All chondrocytes in the calcified cartilage stained with the type X antibody. Enlarged chondrocytes with a hypertrophic appearance expressing type X collagen were present above the tidemark in the zone of non-calcified cartilage [Figs 2(E) and 3(B)]. In addition a number of small chondrocytes also stained for type X collagen [Figs 2(C),(E) and 3(C)]. Besides the pericellular immunostaining, a band of type X collagen staining was present at the cartilage surface. No difference in type X collagen staining was observed between the medial and the lateral side of tibia-femoral joint [Figs 2(C),(E) and 3(B),(C)].

### OA LESIONS AND IMMUNOSTAINING OF TYPE X COLLAGEN IN OLD MICE

Both old Balb/c and C57Bl/6 mice developed cartilage degeneration. However, the location of osteoarthritic lesions was different between the mouse strains. The Balb/c mice preferentially developed osteoarthritic lesions in the patella-femoral joint (60% of the knee joints) while the C57Bl/6 strain showed osteoarthritic lesions mainly on the

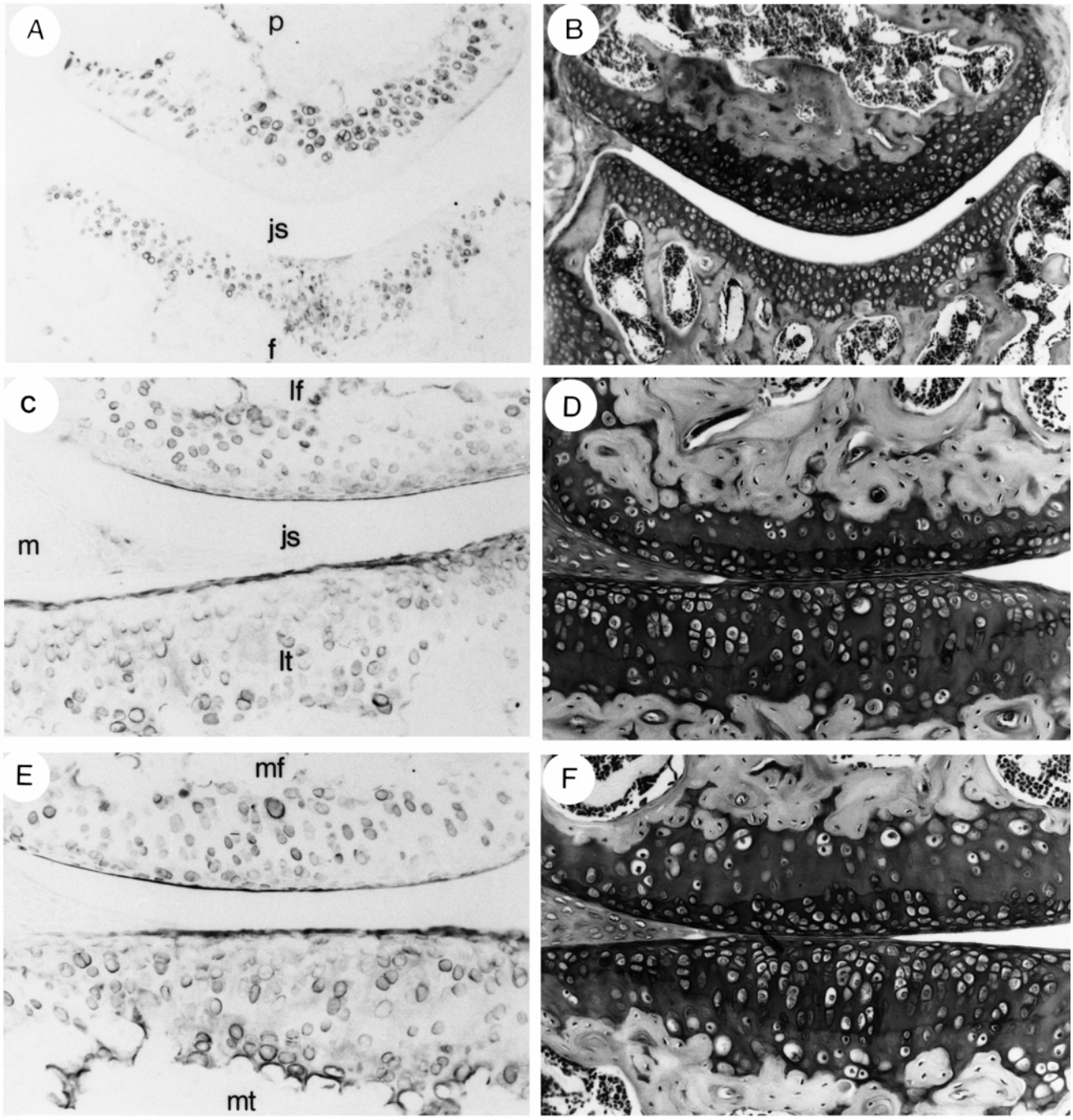


Fig. 2. Type X collagen immunolocalization and safranin O staining in the knee joints of young adult Balb/c mice. Note the almost total absence of type X collagen staining in the non-calcified cartilage of the patella-femoral joint (except sometimes at the articular cartilage surface) and the presence in the tibia-femoral joint. The cartilage surface of young Balb/c mice was intact in all compartments (A, B patella-femoral joint; C, D lateral side tibia-femoral joint; E, F medial side tibia femoral joint; A, C, E type X collagen; B, D, F safranin O; original magnification PF-joint 100× , TF-joint 200×).

lateral side of the tibia-femoral joint (63% of the knee joints) as can be seen in Figs 4(B) and 5(D). The osteoarthritic lesions were characterized by fibrillations and erosions of the non-calcified cartilage extending up to the tidemark. In animals with very severe lesions the calcified cartilage was also affected.

Type X collagen immunostaining in the patella-femoral joint was pronounced in the old mice compared with young mice of both strains, although only the Balb/c mice developed osteoarthritic lesions in this compartment [compare Figs 2(A) and 3(A) with Figs 4(A) and 5(A)]. In the young mice, staining was almost totally confined to the calcified cartilage but in the old mice type X collagen immunostaining was at least as high in the non-calcified cartilage as in the calcified. The staining in the calcified cartilage appeared to be mainly pericellular while the staining in the non-calcified cartilage of old mice was more widespread and present throughout the cartilage matrix.

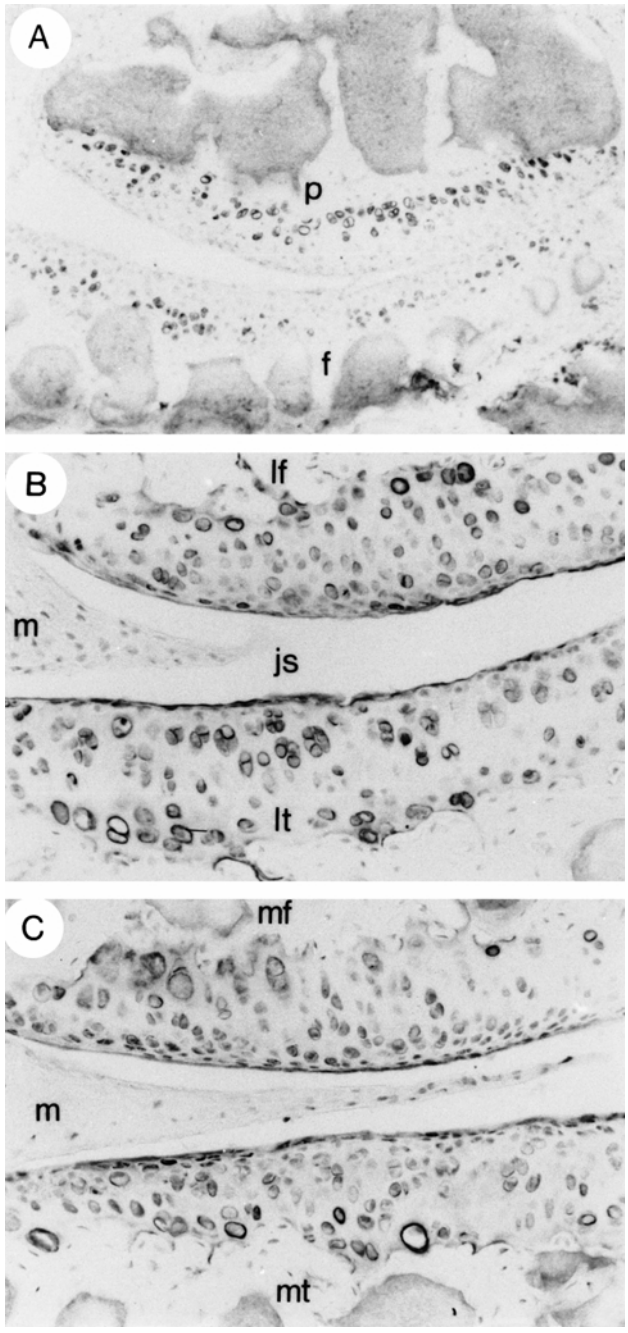


Fig. 3. Type X collagen immunolocalization in the knee joints of young adult C57Bl/6 mice. Note the absence of type X collagen staining in the non-calcified cartilage of the patella-femoral joint and the presence in the tibia-femoral joint (A, patella-femoral joint; B, lateral side tibia-femoral joint; C, medial side tibia femoral joint, original magnification PF-joint 100×, TF-joint 200×).

The increase in type X collagen staining was irrespective of cartilage damage, as could be seen in the C57Bl/6 strain compared to Balb/c mice. No increased type X collagen immunostaining was observed in sites of degeneration and loss of articular cartilage.

Although the lateral side of the tibia-femoral joint of C57Bl/6 mice preferentially develops OA like lesions, no preferential increase in type X collagen staining could be observed in the degenerate cartilage on the lateral tibial plateau of old C57Bl/6 mice [Fig. 5(C)]. Moreover, the type X collagen staining on the lateral side of the tibia-femoral joint was comparable with the staining on the medial side of this joint in the C57Bl/6 mice [Fig. 5(C), (E)]. Also, the type X collagen immunostaining in the Balb/c mice in the tibia-femoral joint was not less pronounced than the staining in C57Bl/6 mice [Fig. 4(C), (E)]. In the tibia-femoral joint neither the old Balb/c nor the old C57Bl/6 mice showed enhanced immunostaining of type X collagen compared to young mice.

### EXPRESSION OF TYPE X COLLAGEN AND PTHRP-RECEPTOR MRNA IN CARTILAGE OF YOUNG AND OLD MICE

Since a striking increase in type X collagen immunostaining was observed in the patella-femoral joint of old mice compared to young mice, expression of type X collagen mRNA and PTHrP-receptor mRNA was investigated in murine patellar cartilage of various ages. Notwithstanding the profound increase in type X collagen in the patellar cartilage, as detected by immunohistochemistry, no increase in type X collagen mRNA expression could be detected in old animals (Fig. 6). Moreover, no changes in the expression of PTHrP-receptor mRNA were found in mice from the various age groups (Fig. 6).

## Discussion

In both young and old Balb/c and C57Bl/6 mice type X collagen could be detected not only at the expected sites known to contain hypertrophic chondrocytes but also in the non-calcified cartilage of the tibia-femoral joint. During aging there appeared to be an increased deposition of type X collagen particularly in the patella-femoral compartment but not in the tibia-femoral joint. Only in Balb/c mice was cartilage degeneration preferentially expressed in the patella-femoral joint. Moreover, in C57Bl/6 mice degeneration was seen preferentially in the lateral compartment of the tibial-femoral joint, a site with no increased type X immunostaining. Thus there was no relationship between type X collagen immunostaining and degeneration in non-calcified cartilage.

Expression of type X collagen is suggested to be a specific marker for chondrocyte hypertrophy and in healthy articular cartilage it is thought to be confined to the calcified zone, although earlier studies have observed type X collagen deposition on the surface of articular cartilage[4]. Our results clearly show that in articular cartilage of young (tibia-femoral joint) and old mice (predominantly patella-femoral joint) type X collagen could be detected in non-calcified cartilage. The type X collagen enveloped chondrocytes were partly large chondrocytes, with a hypertrophic phenotype based on cell size. But small chondrocytes were also surrounded by type X collagen. However, no chondrocytes in the non-calcified cartilage stained for alkaline phosphatase (data not shown) indicating that the chondrocytes in this zone do not express all the features of a hypertrophic phenotype. These observations indicate that the expression of type X collagen in the mouse is not totally confined to hypertrophic chondrocytes.

The distribution of type X collagen in murine articular cartilage reported in this study appears to be similar to the pattern reported by Eerola *et al*.[5] These authors, who only showed data for the tibia-femoral joint, detected type X collagen immunostaining in very young mice mainly in the

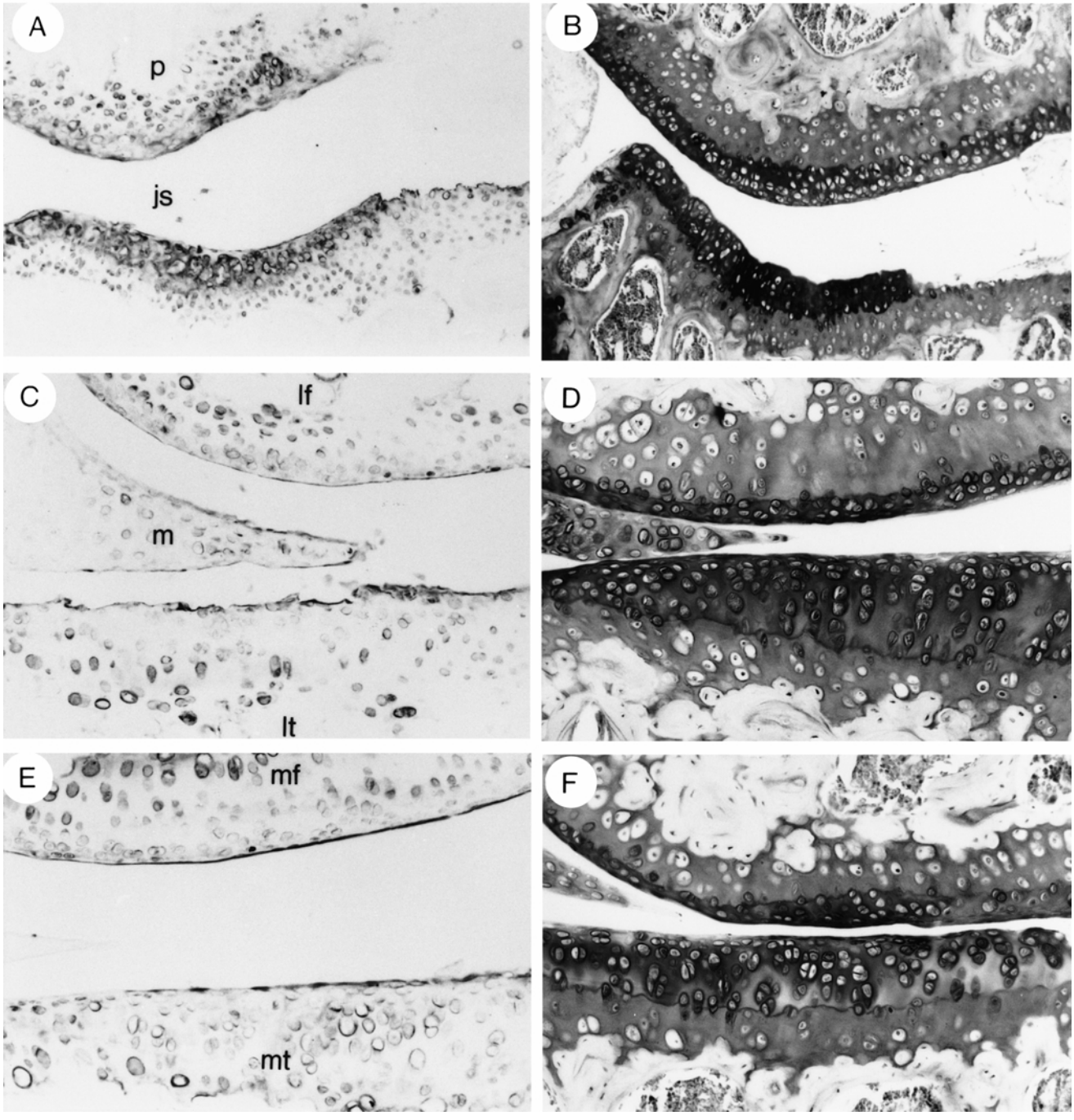


Fig. 4. Type X collagen immunolocalization and safranin O staining in the knee joints of old Balb/c mice. Note the strong type X collagen staining in the non-calcified cartilage of the patella-femoral joint and the presence of cartilage degeneration in this joint. (A, B, patella-femoral joint; C, D, lateral side tibia-femoral joint; E, F, medial side tibia femoral joint; A, C, E, type X collagen; B, D, F, safranin O; original magnification PF-joint 100×, TF-joint 200×).

transitional zone, and also in 3-month-old mice in the superficial zone. In contrast with our results, Eerola *et al*. observed the most intense staining in the tidemark area. This difference in staining pattern could be due to differences between the antibodies, either raised against recombinant type X collagen (Eerola) or a NC1 domain synthetic peptide. Moreover, differences between the mouse strains used could also account for the small dissimilarities in staining pattern.

Type X collagen expression, as a marker for chondrocyte hypertrophy, has been found in osteoarthritic cartilage and chondrocytes for osteoarthritic cartilage[6–10]. The Balb/c and C57Bl/6 mouse strains develop OA-like lesions in very different locations in the knee joint, the patella-femoral joint vs the lateral side of tibia-femoral joint, but appeared to have a strikingly similar immunostaining pattern of type X collagen in both young and old mice. Young mice of both strains had almost no type X collagen immunostaining in

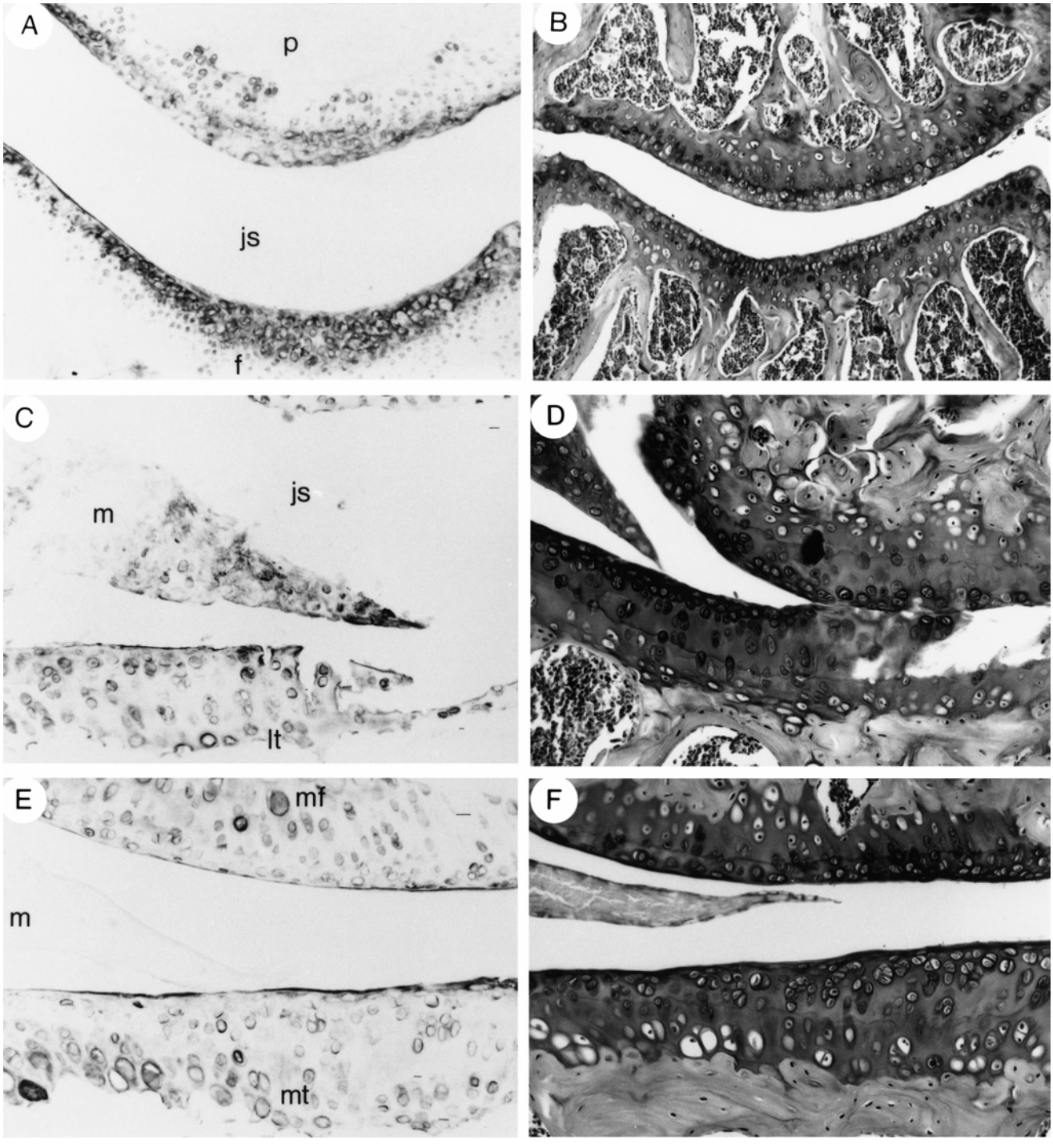


Fig. 5. Type X collagen immunolocalization and safranin O staining in the knee joints of old C57Bl/6 mice. Note the strong type X collagen staining in the noncalcified cartilage of the patella-femoral joint and the absence of cartilage degeneration in this joint. The lateral side of the tibia-femoral joint shows cartilage degeneration up to the tidemark (A, B patella-femoral joint; C, D lateral side tibia-femoral joint; E, F medial side tibia-femoral joint; A, C, E type X collagen; B, D, F safranin O; original magnification PF-joint 100×, TF-joint 200×).

the non-calcified cartilage of the patella-femoral joint, but extensive staining was observed in this area in old mice, although only the Balb/c mice develop OA in this location. Moreover, old C57Bl/6 mice develop OA on the lateral side of the tibia-femoral joint while no increased type X collagen immunostaining was observed in old mice in this compartment. These data appear to conflict with the results of Eerola *et al*.[5] using OA prone Del1 mice. Intense type X collagen staining was observed in these mice in areas with surface fibrillation, osteophyte formation and degenerating menisci. We also observed type X collagen staining in osteophytes undergoing endochondral ossification, menisci and calcifying ligaments (data not shown). However, nonosteoarthritic joints of old mice also show meniscal degeneration and ligament calcification and type X collagen expression in these tissues. The cause of staining

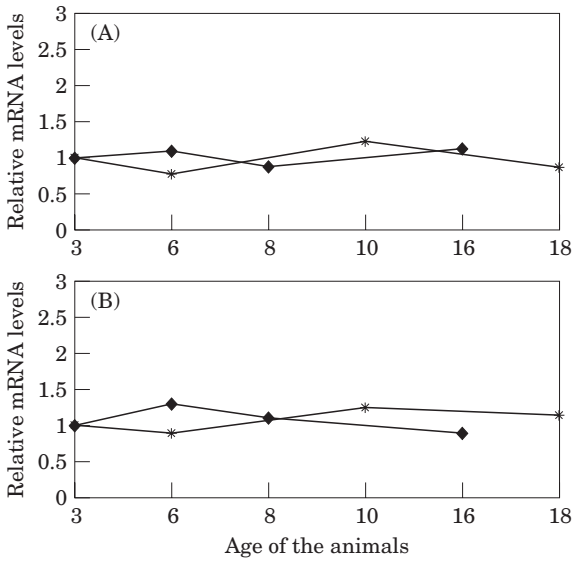


Fig. 6. Relative expression of type X collagen (A) and PTH/PTHrP-receptor (B) mRNA in Balb/c and C57Bl/6 mice of various ages. Samples were taken at increasing cycle numbers and the PCR products were electrophoresed. The cycle number at which the product was first detected was used for generating the results in this figure. The expression of GAPDH in the same samples was used as an internal control. The expression in the 3-month-old animals was arbitrarily set at 1. In all RT-PCR experiments cartilage of 10 patellae were pooled (* C57Bl/6, ◆ Balb/c).

differences with respect to the fibrillated surface of osteoarthritic cartilage remains obscure but might be related to differences in antibodies or mouse strains used.

Although a relationship between type X collagen immunostaining and OA development could not be found in old mice, cartilage of the patella-femoral joint showed a striking increase in type X collagen immunostaining in the non-calcified cartilage of old mice. Therefore expression of type X collagen and PTHrP-receptor mRNA was evaluated with RT-PCR in patellar cartilage of mice with different ages. Remarkably, in spite of the increased expression of type X collagen at the protein level, no increase in type X collagen mRNA could be detected. This observation is in concordance with studies of Eerola *et al*.[5] who found no changes in type X collagen mRNA levels in mice from 3 to 9 months old. The absence of elevated mRNA levels might indicate that the elevated immunostaining of type X collagen is not due to continued elevated synthesis of this molecule but may be caused in part by increased deposition and/or retention of type X collagen (at least the NCI domain) in the cartilage matrix. In an earlier study it was found that increased immunostaining of type X collagen in non-calcified murine patellar cartilage after intra-articular injection of papain was not accompanied by increased expression of type X collagen mRNA[17]. The absence of increased mRNA levels, the distribution of collagen type X throughout the matrix and the observation that type X collagen can diffuse long distances through chick cartilage[20] suggest that increased deposition is the basis for the enhanced immunostaining of type X collagen in patellar cartilage. The observation that PTHrP-receptor mRNA is not decreased in patellar cartilages of old mice indicates that down-regulation of the PTHrP-receptor in articular chondrocytes is not detectably involved in the increased expression of type X collagen as may be observed in the growth plates[12,13].

This study shows that young mice contain type X collagen in the non-calcified cartilages of the tibia-femoral joint but that type X collagen expression is almost absent in the non-calcified cartilage of the patella-femoral joint at this age. In old mice strongly increased expression is observed in the patella-femoral joints but not in the tibia-femoral joints. Locations in the murine joints of Balb/c and C57Bl/6 mice prone to development of OA do not preferentially express type X collagen in the non-calcified cartilage suggesting that type X collagen expression and OA development are not necessarily related phenomena.

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