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Genetics

Osteoarthritis associated with mild chondrodysplasia in transgenic mice expressing α1(IX) collagen chains with a central deletion

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ABSTRACT **Type IX collagen, containing molecules of the three distinct polypeptides α1(IX), α2(IX), and α3(IX), is an interesting hybrid extracellular matrix component in cartilage and eye tissues, with the properties of both a proteoglycan and a collagen. The α1(IX) chain has two forms, as a result of the tissue-specific utilization of two alternative promoters; the α2(IX) chain carries a covalently attached glycosaminoglycan side chain. We have introduced a gene construct controlled by a tissue-specific promoter/enhancer and expressing a truncated α1(IX) chain into mice. Examination of the offspring of two different founders revealed pathological changes similar to osteoarthritis in the articular cartilage of knee joints. In addition, mice homozygous for the transgene developed mild chondrodysplasia (i.e., mild dwarfism, anterior tonguing in the vertebral bodies, and ophthalmopathy). The relative ratio of transgene product to the endogenous α1(IX) chain was approximately one in homozygotes and less than one in heterozygotes. Therefore, the phenotypic severity correlated well with the level of transgene expression. These findings suggest that mutations in type IX collagen genes may cause certain forms of osteoarthritis and chondrodysplasia in humans.**

The highly hydrated extracellular matrix of hyaline cartilage consists of fine fibrils and "ground" substance. The fibrils are predominantly composed of staggered arrays of long, rod-like triple-helical molecules of type II collagen, which provide cartilage with high tensile strength and stiffness (1, 2). The ground substance contains large proteoglycan (aggrecan) aggregates providing strong hydrophilic properties and compressive or shear stiffness (3, 4). These macromolecular complexes, together with other components, are enmeshed with each other, and they interact to form the three-dimensional architecture of the cartilaginous models, which are subsequently replaced by bone during normal skeletal development. A portion of the cartilaginous skeleton remains as articular cartilage (or permanent cartilage) in joints and serves as the weight-bearing material whose mechanical properties are strongly dependent on the physical and biochemical properties of its matrix (2, 4).

Interactions between collagen fibrils and other matrix components are likely to provide the molecular basis for the precise three-dimensional architecture of the cartilage matrix. In addition to the major matrix components of cartilage, various other components such as type IX collagen may play a role in this interaction (5). Type IX collagen molecules are composed of α1(IX), α2(IX), and α3(IX) chains that form a long and a short triple-helical arm connected by a flexible non-triple-helical hinge (6–9). The long arm is associated with type II-containing collagen fibrils in cartilage (10, 11) and noncartilage tissues such as vitreous humor (12). The short arm projects into the perifibrillar matrix. In cartilage a large globular domain is located at the amino terminus of the short arm, and this domain could potentially participate in interactions with other matrix components (5). In vitreous humor and in embryonic chicken cornea, this large globular domain of type IX collagen is replaced by a short, alternative amino acid sequence. The difference between the cartilage and vitreous forms is due to the alternative use of two transcription start sites and RNA splice patterns in the α1(IX) collagen gene (13). In addition, type IX collagen in the vitreous humor of chickens contains an extraordinarily large chondroitin sulfate chain extending away from the fibrils (14). Thus, type IX collagen molecules on the surface of fibrils have been implicated in determining the surface properties of type II-containing collagen fibrils and their interactions with other components of the matrix (14, 15).

If this is correct, mutations affecting type IX collagen should lead to abnormalities involving cartilage and eye. To test this hypothesis and to obtain further insight into the function of type IX collagen from analysis of mutant phenotypes, we have generated transgenic mice harboring a partially deleted α1(IX) collagen gene construct. Because the presence of the repetitive Gly-Xaa-Yaa amino acid sequence is essential for the formation and stability of the triple helix of collagen molecules (16, 17), the synthesis of a shortened α1(IX) chain should interfere with stable triple helix formation and act as a "trans-dominant" mutation. In the present study, we demonstrate that transgenic mice expressing such a truncated α1(IX) collagen chain develop osteoarthritis with mild chondrodysplasia.

MATERIALS AND METHODS

Generation and Identification of Transgenic Mice. The gene construct was designed to create an in-frame deletion in the central part of cDNAs encoding the α1(IX) collagen chain. The inserts of the human cDNA clone YMh509 (18) and the rat cDNA clone pKT1643 (19) were ligated at *Bst*EII sites to produce an in-frame deletion (see Fig. 1). To achieve high level expression of this truncated cDNA in cartilaginous tissues, a rat collagen II promoter element (−977 to +110 bp) and enhancer element (1.6-kb *Bam*HI/*Bam*HI fragment) (refs. 20 and 21; kindly provided by Yoshihiko Yamada, National Institute of Dental Research, National Institutes of Health, Bethesda, MD) were ligated to the cDNA. To increase transcriptional efficiency of the cDNA construct, a 640-bp *Bam*HI/*Eco*RI fragment of rabbit β-globin intron cassette sequences (22) was inserted between the promoter sequences and transcription start site of the cDNA. The construct insert was released from the vector by digestion with *Sph* I/*Sal* I and was microinjected into fertilized eggs from C57BL/6 females mated with C57BL/6 males. The

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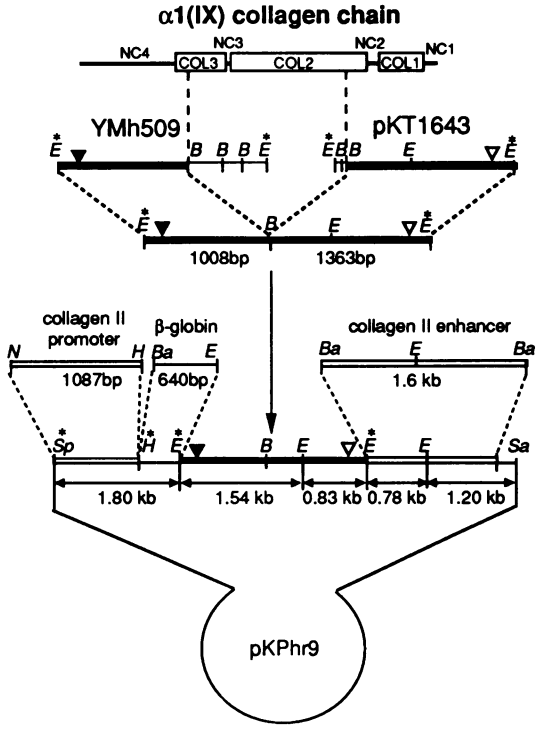

FIG. 1. Schematic description of transgene construct for microinjection. The top part of the figure shows the domain structure of the α1(IX) collagen chain, with triple-helical (COL1, COL2, and COL3) and noncollagenous (NC1, NC2, NC3, and NC4) domains (6, 8). The structural part of the partially deleted α1(IX) collagen cDNA is indicated by solid bars; it has an in-frame deletion of the sequences encoding a part of the COL3 domain, the entire NC3 domain, and part of the COL2 domain. The filled triangle in the YMh509 (18) insert indicates the translation start site. The open triangle at the 3′ end of the pKT1643 (19) insert indicates the polyadenylylation sequence. E, *Eco*RI; B, *Bst*EII; N, *Nde* I; H, *Hin*dIII; Ba, *Bam*HI; Sp, *Sph* I; Sa, *Sal* I. Restriction sites where synthetic linkers were used during cloning and ligation are indicated by asterisks.

injected eggs were then transferred to pseudopregnant ICR female mice (23). Transgenic mice were identified by Southern blot analysis of DNA extracted from tail biopsies. After obtaining F_1 progeny, transgenic mice were inbred to prepare putative homozygous F_2 offspring. Permanent homozygous lines were then generated by mating two putative homozygous mice. The homozygosity was confirmed by outcrossing the transgenic mice to nontransgenic mice, checking for 100% transmission of the transgene to the progeny.

Skeletal Morphology and Histology. The skeletons of mice were stained with alizarin red S as described (24). For light microscopy, tissues were fixed in 10% neutralized formaldehyde, decalcified in 0.3 M EDTA (pH 7.4), embedded in paraffin, sectioned, and stained with hematoxylin/eosin or safranin O. Progress of osteoarthritic change in the articular cartilage of the knee joint was evaluated on the basis of the modified Mankin's histological grading system (25). For electron microscopy, samples from 6-week-old normal and transgenic mice were prepared and examined as described (26).

Assay of Transgene Expression. To analyze the expression of the transgene and the wild-type endogenous α1(IX) collagen gene, mRNA was extracted from sternal cartilage, eye, brain, kidney, and liver, using the Micro-FastTrack kit (Invitrogen, San Diego). mRNA was reverse transcribed (Moloney murine leukemia virus reverse transcriptase; BRL) to generate cDNAs by using random hexamers. cDNAs were then used as templates for PCR with *Thermus thermophilus* DNA polymerase. Primer 1 (5′-GTCAAGCGTCGCCCCAGATTC-3′) and primer 2 (5′-CTGCAATGTAGCTGATCCCAC-3′) were used for the specific amplification of transgene cDNA. Primer 3 (5′-CCCTGGGTATCCGCAACTCTT-3′) and primer 4 (5′-AAGCCACTTGTAAGGCGTTG-3′) were used for the specific amplification of wild-type Col9a1 cDNA. PCR conditions were selected so as to have logarithmic-phase amplification of the respective cDNAs: 34–37 cycles at 94°C for 1 min, 61°C for 1 min, 72°C for 3 min, and an additional 5 min at 72°C at the end of the cycles. The identity of the PCR product was confirmed by the presence and/or absence of unique restriction sites.

Immunoblotting of α1(IX) Collagen Chains. Cartilaginous skeleton including joint was dissected from 18-day embryos and was extracted as described (27) with slight modification. Briefly, the samples were extracted in a 1 M NaCl solution buffered to pH 7.4 with 50 mM Tris·HCl, followed by precipitation with ammonium sulfate. The precipitate was resuspended in 0.5 M acetic acid containing 0.12 M NaCl. The crude collagen extract was then salt precipitated twice in 0.5 M acetic acid at 2 M NaCl, redissolved in 0.5 M acetic acid/0.12 M NaCl, dialyzed against 5 mM acetic acid, and lyophilized. A portion of the lyophilized sample was treated with chondroitinase ABC or bacterial collagenase as described (28).

SDS/PAGE was carried out with a 4% or 6% separating gel using standard conditions (29). Transfer to nitrocellulose filters by electroblotting was as described (30). The filters were allowed to react with the monoclonal antibody 23-5D1, which recognizes the NC2 domain of the human, rat, and mouse α1(IX) collagen chain (ref. 31; kindly provided by Kazushi Iwata, Fuji Chemical Industries, Toyama, Japan). The secondary antibody was horseradish peroxidase-conjugated anti-mouse IgG, and chemiluminescence was detected using ECL Western blotting detection system (Amersham).

RESULTS

Generation of Transgenic Mice. Three transgenic founder mice, one female (m29-1) and two males (m8-1 and m22-1), were obtained. Southern blot analysis indicated that they carried ≈3 to ≈10 concatameric copies of the transgene with independent integration sites in the mouse chromosome (data not shown). The female F_0 founder (m29-1) was similar in size and weight to her normal littermates. At 2 weeks postpartum, when the palpebrae opened, m29-1 was found to have opaque and irregular corneas. She was sterile due to unknown reason, and we could not obtain transgenic offspring from this line even after performing ovary transfer to C57BL/6 females. The two male transgenics (m8-1 and m22-1) apparently developed normally. However, some of the transgenic F_1 mice from these two separate lines were found to have somewhat small eyeballs. The more obvious eye phenotype developed in the F_2 offspring. In about 15% of the F_2 transgenic offspring, which were putatively homozygous for the transgene, corneas appeared opaque or irregular and were sometimes infiltrated by capillary vessels (Fig. 2 *A–C*). In addition, the mice in the permanent homozygous lines displayed mild proportionate dwarfism (Fig. 2*D*). The vertebral bodies were ovoid in shape as a result of a mild ossification defect, and the end plates in the middorsal region were irregular (Fig. 2*E*). In contrast, there was no major skeletal involvement in the heterozygous offspring (Fig. 2 *D* and *E*).

Osteoarthritic Changes in the Offspring of Transgenic Mice. All the offspring of the two transgenic strains, m8-1 and m22-1, reached maturity without showing severe skeletal deformities. However, histological analysis of the transgenics (a total of 60 mice from F_1, F_2, F_3, and F_4 generations) demonstrated early osteoarthritic changes, which were most

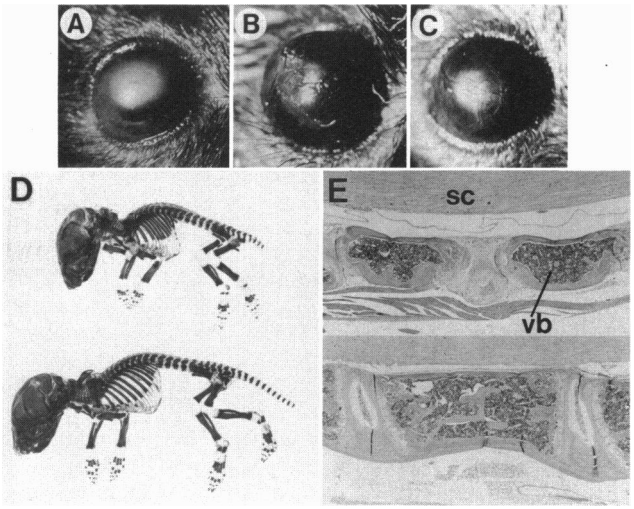

FIG. 2. Phenotypic abnormalities of transgenic mice. (*A–C*) Macroscopic appearance of eye balls in the m8-1 F_2 mice, which are homozygous for the transgene. (*D*) Skeletons of 5-day-old F_4 transgenic mice (m8-1 line) that are homozygous (*Upper*) and heterozygous (*Lower*) for the transgene stained with alizarin red S. (*E*) Photomicrograph of vertebral bodies from homozygous (*Upper*) and heterozygous (*Lower*) m8-1 transgenic mice (hematoxylin eosin; ×80). Note that the homozygote has smaller body size, deformities in the vertebral bodies, and end plate irregularities. sc, spinal cord; vb, vertebral body.

obvious in the knee joint. The articular cartilage of the knee joint looked normal by light microscopy until 6 weeks of age (Fig. 3*A*). The earliest lesion was decreased intensity of safranin O staining and a roughening or erosion of the articular surface, which was first observed at 4–6 months of age (Fig. 3*B*). Under polarized-light microscopy, the tangential layer was thinned and discontinuous (not shown). These early changes were most frequently observed in the anterior part of the weight-bearing area of the tibia, whereas femoral lesions appeared to be somewhat delayed in their onset (Fig. 3*C*). Subsequently, cartilage erosion and loss of safranin O staining progressed in both femoral and tibial condyles and were occasionally accompanied by the presence of degenerating chondrocytes or vertical fissure formation (Fig. 3 *D* and *E*). Regenerative or reparative changes were not remarkable, although clusters of small numbers of chondrocytes were observed in limited areas (Fig. 3*F*).

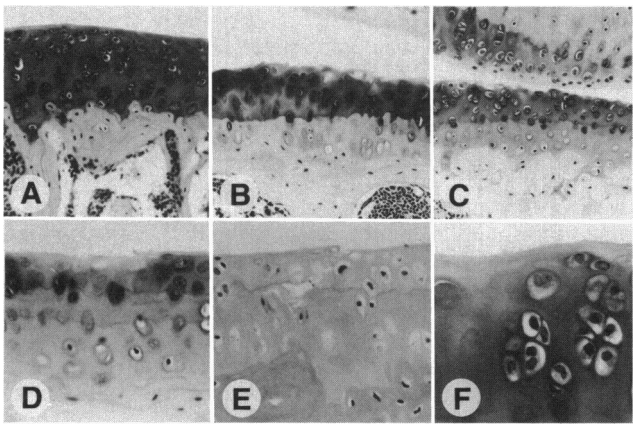

FIG. 3. Light microscopy of knee joint cartilage. (*A*) Six-week-old m8-1. (*B*) Six-month-old m8-1. (*C*) Six-month-old m8-1. (*D*) Nine-month-old m22-1. (*E*) Nine-month-old m8-1. (*F*) Eight-month-old m22-1. The histological scores for the mice in *A–F* are 0, 2, 3, 3, 5, and 4, respectively. (safranin-O staining; *A–C*, ×50; *D* and *E*, ×100; *F*, ×190.)

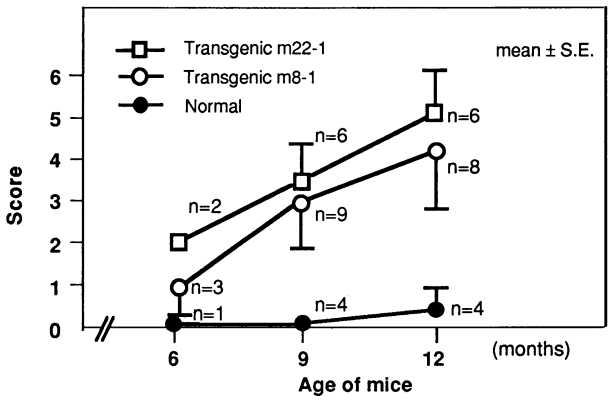

FIG. 4. Histological score of osteoarthritic changes in the knee joint. The sections were assessed with regard to three categories: cartilage surface, cellularity, and safranin O staining, each ranging from grade 0 to 3 (25). The total score ranges from 0 to 9, with 0 indicating normal cartilage and 9 indicating severe degeneration. The score was determined in the two transgenic lines (m8-1 and m22-1) and normal littermates.

Since several inbred mouse strains are known to develop osteoarthritis spontaneously, partly reflecting the aging process (32), we evaluated and compared the osteoarthritic changes of the knee joint both in transgenic and normal control mice on the basis of a modified Mankin's histological grading system (25). A total of 34 transgenic mice at 6, 9, and 12 months were scored. As shown in Fig. 4, the offspring of two independent transgenic lines showed onset of osteoarthritic changes after reaching maturity, and the changes were progressive with aging. In 12-month-old mice, the mean histological score was 4.1 and 5.2 in line m8-1 and line m22-1, respectively, indicating the presence of advanced osteoarthritic change. In contrast, the age-matched control mice scored only 0.5.

We examined the weight-bearing region of articular cartilage in the femoral condyle by electron microscopy. In the intermediate layer of normal cartilage, the collagen fibrils were mostly randomly oriented, forming tangled open meshwork structure (Fig. 5*B*). The width of the fibrils ranged from ≈30 nm to 100 nm. In contrast, in the transgenic mice the open fibrous meshwork became obscure and the fibrils were thinner than those in normal controls (Fig. 5*A*).

Transgene Expression. PCR analysis of reverse-transcribed RNA was performed to detect transgene and wild-type endogenous α1(IX) collagen gene transcripts. Primers were selected to amplify specifically the corresponding regions of the transgene and wild-type cDNAs (Fig. 6*A*). Logarithmic-phase amplification was obtained between 34 and 37 cycles of PCR (Fig. 6*B*). The analysis of the F_2 offspring, which were putatively homozygous for the transgene, demonstrated that transcripts of both transgene and endogenous α1(IX) gene

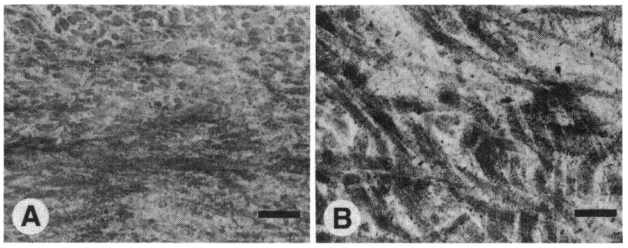

FIG. 5. Electron micrographs of articular cartilage at the femoral condyle of the knee joint. Sections were cut parasagittally. Articular surface directs to top in all figures. (*A*) Cartilage from 6-week-old transgenic mouse (m8-1). (*B*) Cartilage from 6-week-old normal mouse. (Bar = 200 nm.)

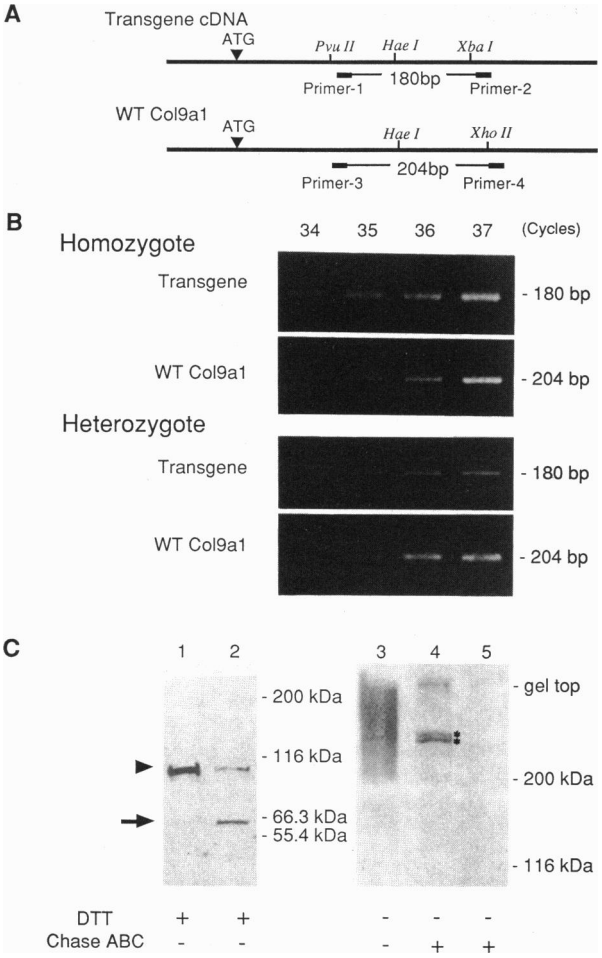

FIG. 6. Expression of the transgene. (*A*) Schematic presentation of the transgene cDNA and endogenous wild-type α1(IX) chain cDNA (WT col9a1) and the oligonucleotide primers used for the synthesis of PCR products encoding a part of the NC4 domain of the α1(IX) chain. (*B*) Reverse transcription/PCR amplification of the transgene and endogenous α1(IX) transcripts from sternal cartilage of newborn m8-1 mice that are putatively homozygous and heterozygous for the transgene. Thirty-four to 37 cycles of amplification were performed, and the PCR products were analyzed on agarose gels. (*C*) Results of Western blot analysis using an antibody against the α1(IX) chain. Samples were electrophoresed on 6% (lanes 1 and 2) or 4% (lanes 3–5) gels before and after treatment with dithiothreitol (DTT) and chondroitinase ABC (Chase ABC). Lane 1, sample obtained from normal control mouse; lanes 2–4, samples from homozygous m8-1 18-day embryos; lane 5, same sample as in lane 4, but digested with bacterial collagenase before electrophoresis. Molecular mass markers were standard globular proteins. The arrowhead indicates the position of endogenous α1(IX) chain, and the arrow indicates the shortened α1(IX) chain. Note two closely migrating bands (asterisks) at positions of 250–290 kDa in lane 4.

were present in cartilage (Fig. 6*B* and Table 1). The level of transgene expression was estimated to be roughly equal to that of endogenous α1(IX) gene expression. The transgenic mice that were heterozygous for the transgene showed lower levels of transgene expression (Fig. 6*B*). As summarized in Table 1, the transgene was preferentially expressed in cartilaginous tissues and eyes up to 28 days after birth.

Western blots of cartilage extracts revealed that the transgene was expressed as a shortened α1(IX) chain, which migrated as a band of about 65 kDa using globular molecular mass markers (Fig. 6*C*, lane 2). The endogenous α1(IX) chain was detected as a more slowly migrating band with a molecular mass of 105 kDa (Fig. 6*C*, lane 2). The ratio of the extracted shortened α1(IX) chain to the endogenous α1(IX) chain was ≈1:1 in homozygous offspring of the line m8-1. A similar ratio was found in line m22-1 (not shown). In unreduced samples, antibody specific for the α1(IX) chain reacted with higher molecular mass polypeptides, migrating as a smear (Fig. 6*C*, lane 3). After chondroitinase ABC digestion, two closely migrating bands around 250–290 kDa appeared (Fig. 6*C*, lane 4), which were sensitive to bacterial collagenase (Fig. 6*C*, lane 5). Therefore, the shortened α1(IX) chain in unreduced samples was present as a disulfide-bonded heterotrimeric molecule, which probably contained a glycosaminoglycan side chain(s).

Table 1. Transgene and endogenous α1(IX) gene expression

Line*	Copy number†	Age, days	Transgene/endogenous gene			
			Cartilage	Eye	Brain	Liver
m8-1	10 (5)	2	+‡/+	+/+	+/±	−/−
		10	+/+	+/−	−/−	−/−
		28	+/+	+/−	−/−	−/−
m22-1	6 (3)	2	+/+	+/+	+/±	−/−
		10	+/+	+/−	−/−	−/−
		28	+/+	+/−	−/−	−/−

*Homozygous F_2 mouse from $F_1 \times F_1$ inbreeding.
†Numbers in the parentheses indicate copy number in heterozygous F_1 mice.
‡Transcript detectable after 36 cycles of PCR.

DISCUSSION

The results presented here indicate that transgenic mice harboring a truncated α1(IX) collagen gene develop a mild phenotype of chondrodysplasia predisposing for early onset of osteoarthritis. The transgene was preferentially expressed in cartilaginous tissues and eyes, and the translated product was detected as a shortened α1(IX) chain that was disulfide-bonded with endogenous polypeptides to form a collagenous molecule. As reported previously (16, 17, 33), synthesis of structurally defective α chains of types I, II, and III procollagen interfere with the formation of a stable triple helix or with self-assembly of collagen into fibrils. Such interference with stable triple-helix formation causes overmodification and degradation of mutated as well as normal α chains through a suicide mechanism (16, 17). Thus, mutations in the genes for types I, II, and III procollagen that change the primary structure of the α chain act as trans-dominant mutations. Accordingly, it is likely that the introduction of the truncated α1(IX) gene acts as a trans-dominant mutation, since the transgene product, a shortened α1(IX) chain, was synthesized and incorporated into collagenous molecules, probably forming abnormal type IX collagen heterotrimers.

Evidence is now accumulating to indicate that mutations in genes for cartilage matrix components can cause skeletal dysplasias. Recent reports indicate that mutations in the type II procollagen gene in humans can cause chondrodysplasias, such as spondyloepiphyseal dysplasia and achondrogenesis type II (34, 35). In addition, a direct causal link between mutations in the type II procollagen gene and severe chondrodysplasias has been demonstrated by the generation of transgenic mice that express a mutated α1(II) collagen gene (26, 36). Depending on the nature and position of the mutations in the type II procollagen gene, however, the disease phenotype shows a wide variation. A single-base mutation in the α1(II) chain that converts the codon for arginine to cysteine has been reported to cause osteoarthritis associated with mild chondrodysplasia in humans (37). Similarly, a substitution of the codon for arginine with a stop codon was found in a family with the Stickler syndrome, which is characterized by osteoarthritis combined with ophthalmopathy (38). Thus, various mutations in the type II procollagen gene can cause various forms of chondrodysplasias (17).

The abnormalities that we have generated in transgenic mice by introducing a gene construct encoding a truncated α1(IX) chain have the features of osteoarthritis associated with mild chondrodysplasia. There was a clear difference in the phenotypic severity between the offspring that were homozygous and heterozygous for the transgene. The homozygous offspring in the two transgenic strains showed early onset and progress of osteoarthritis associated with mild proportionate dwarfism, spine involvement, and eye abnormality. In contrast, the heterozygous offspring expressing lower levels of the transgene product principally developed osteoarthritic changes with no signs of chondrodysplasia. The phenotypic severity, therefore, appears to be dependent on the level of transgene expression. Thus, the results indicate a causal relationship between the synthesis of shortened α1(IX) chains and chondrodysplasia with osteoarthritis.

Why was joint cartilage predominantly affected in transgenic mice expressing truncated α1(IX) chains? The expression of truncated α1(IX) chains apparently affects the assembly of collagen II-containing fibrils because individual fibrils in transgenic mice were thinner than those in controls. Since type IX collagen is normally associated with the surface of collagen II-containing fibrils, we speculate that the synthesis of abnormally assembled type IX collagen alters the surface properties of collagen fibrils in cartilage, thus affecting the interaction of the fibrils with other matrix components, such as proteoglycans. As a consequence of this alteration in matrix architecture, the joint cartilage may become vulnerable to repetitive and long-term mechanical stress. Indeed, biomechanical testing revealed a significant decrease in the intrinsic compressive stiffness (S. Wakitani, K.N., and T.K., unpublished observations) in the articular cartilage of the transgenic mice, even prior to the histological onset of osteoarthritis.

The abnormalities observed in the corneas may need some discussion. Although type IX collagen is a component of embryonic avian primary corneal stroma, it has not been found in mouse cornea. It is possible that the use of type II collagen promoter/enhancer may have resulted in the ectopic expression of truncated α1(IX) chain in mouse cornea. Such ectopic expression would possibly affect collagen fibrillogenesis and transparency of the cornea. The clarification of the eye phenotype, however, will need further study.

Given the close resemblance of the abnormality of transgenic mice expressing a truncated α1(IX) chain to osteoarthritis or chondrodysplasia in humans, further detailed analysis of these mice should allow insight into the functional role of type IX collagen in cartilage as well as the pathogenesis of such cartilage disorders.

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