Collagen II Is Essential for the Removal of the Notochord and the Formation of Intervertebral Discs

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Abstract. Collagen II is a fibril-forming collagen that is mainly expressed in cartilage. Collagen II–deficient mice produce structurally abnormal cartilage that lacks growth plates in long bones, and as a result these mice develop a skeleton without endochondral bone formation. Here, we report that Col2a1-null mice are unable to dismantle the notochord. This defect is associated with the inability to develop intervertebral discs (IVDs). During normal embryogenesis, the nucleus pulposus of future IVDs forms from regional expansion of the notochord, which is simultaneously dismantled in the region of the developing vertebral bodies. However, in Col2a1-null mice, the notochord is not removed in the vertebral bodies and persists as a rod-like structure until birth. It has been suggested that this regional notochordal degeneration results from changes in cell death and proliferation. Our experiments with wild-type mice showed that differential proliferation and apoptosis play no role in notochordal reorganization. An alternative hypothesis is that the cartilage matrix exerts mechanical forces that induce notochord removal. Several of our findings support this hypothesis. Immunohistological analyses, in situ hybridization, and biochemical analyses demonstrate that collagens I and III are ectopically expressed in Col2a1-null cartilage. Assembly of the abnormal collagens into a mature insoluble matrix is retarded and collagen fibrils are sparse, disorganized, and irregular. We propose that this disorganized abnormal cartilage collagen matrix is structurally weakened and is unable to constrain proteoglycan-induced osmotic swelling pressure. The accumulation of fluid leads to tissue enlargement and a reduction in the internal swelling pressure. These changes may be responsible for the abnormal notochord removal in Col2a1-null mice.

Our studies also show that chondrocytes do not need a collagen II environment to express cartilage-specific matrix components and to hypertrophy. Furthermore, biochemical analysis of collagen XI in mutant cartilage showed that α1(XI) and α2 (XI) chains form unstable collagen XI molecules, demonstrating that the α3(XI) chain, which is an alternative, posttranslationally modified form of the Col2a1 gene, is essential for assembly and stability of triple helical collagen XI.

Key words: collagen II • notochord • vertebral column • intervertebral disc • development

Cartilage is a structurally complex, specialized tissue composed of a dense, covalently cross-linked collagen fibrillar network and a range of other extracellular matrix components, including aggrecan, a functionally important cartilage-specific proteoglycan. Aggrecan is complexed with hyaluronan and entrapped within the collagen network, where its high negative charge density generates an osmotic swelling pressure. This swelling pressure exerts a tensile force on the collagen fibrils and enables the cartilage to resist compressive forces.

Cartilage collagen fibrils are heterotypic, composed of collagen II and the quantitatively minor collagens IX and XI. Collagen XI is buried in the fibril, and cross-link analysis suggests that it initially forms a homopolymer but eventually copolymerizes with collagen II (Eyre and Wu, 1987). This process may provide a regulatory mechanism for fibril diameter (Thom and Morris, 1991). Collagen IX is located on the fibrillar surface, where it is cross-linked to collagen II, and to itself (Diab et al., 1996), thereby mediating interactions between the cartilage collagen fibrils and the surrounding extracellular matrix (for review see Bruckner and van der Rest, 1994).
Collagen II is a homotrimer of three \( \alpha \)1(II) chains encoded by a single gene, \( \text{Col2a1} \). The \( \alpha 3(\text{XI}) \) chain of the heterotrimeric \( \text{XII} \) is also a product of \( \text{Col2a1} \), but it undergoes different posttranslational modifications (Furuto and Miller, 1983). Collagen II is predominantly localized to cartilage, where its expression is high and maintained throughout life. It is, however, also expressed in several other tissues, including notochord, fetal brain, and developing heart and eye (von der Mark et al., 1976; Cheah et al., 1991; Wood et al., 1991). The significance of collagen II expression in nonchondrogenic tissues is not well understood.

Among the tissues that express collagen II, the notochord has the most diverse functions during vertebrate development. The early notochord is a rod-like, axial structure of mesodermal origin that plays an important role in the dorsoventral patterning of both the neural tube and the somitic mesoderm. In the neural tube, it induces the formation of the floor plate (Jessell et al., 1989); in the somites, it induces the differentiation of the ventral somitic derivatives into the sclerotome (Pourquié et al., 1993). After these inductive events, some sclerotomal cells migrate toward the notochord, where they form a continuous and initially unsegmented perichordal tube. Later on, this axial mesenchyme acquires a characteristic metameric pattern of condensed and noncondensed areas. While the regularly spaced condensations represent intervertebral pattern of condensed and noncondensed areas. While the

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Materials and Methods

Mice and Antibodies

Transgenic mice carrying a null mutation in the \( \text{Col2a1} \) gene (Li et al., 1995) were used for the present study. Heterozygous females and males were mated and checked for plugs early the following morning. Fertilization was assumed to occur at midnight, and embryos were staged accordingly (noon on day 1 of plugging equals E0.5). Embryos between day 9.5 and 18.5 post coitum (E9.5–E18.5) were isolated from uterus of pregnant females and processed for analysis.
Genotyping of mice and embryos was done by PCR on DNA derived from tail tissue and yolk sac tissue, respectively. The PCR reaction was carried out in 10 μl containing 1 μl of DNA at 94°C for 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C in the presence of 1.5 mM MgCl₂. The wild-type CoLa allele was detected using primers from the 5’ (C₅₉: 5’-TTAGT ACACCTGGGTTG-CTTGGG) and 3’ (C₆₀: 5’-CGTCTGATGCGCC TAGTTCC) regions flanking exon 35 of the CoLa gene; the primer pair detecting the null allele consisted of C₁ and sequence from the neomycin gene (N₅: 5’-GCCGATTTGCTGCTGTC). Primer set C₁-C₂ yielded a 271-bp fragment, and primer set C₁-C₅ yielded a 450-bp fragment.

The following primary antibodies were used for immunohistochemistry: rabbit anti-collagen III (Col3, diluted 1:1000; obtained from Rupert Timpl, Max Planck Institute for Biochemistry, Martinsried, Germany); rabbit anti-collagen I (Col1, diluted 1:1000); rabbit anti-collagen II (Col2, diluted 1:400) and rat anti-collagen XI (Col11, diluted 1:400; both obtained from Rikard Holmdahl, Lund University, Lund, Sweden); rabbit anti-chondroadherin (diluted 1:200), and rabbit anti-cartilage oligomeric matrix protein (COMP, diluted 1:400; all obtained from Dick Heinegård and Åke Aszódi et al.).

For immunoblot analysis, the following antibodies were used: rabbit anti-collagen II and rabbit anti-collagen XI (both obtained from Gary Gibson, Henry Ford Hospital, Detroit, MI); rabbit anti-collagen IX (obtained from Mats Paulsson, University of Cologne, Cologne, Germany); and rabbit anti-collagen III (see above).

Staining of Skeletons

Skeletors of newborn mice were prepared and stained essentially as described by Braun et al. (1992).

Histology, Immunohistochemistry, and In Situ Hybridization

For histological analysis, whole embryos or trunks dissected from newborn mice were fixed in 4% fresh paraformaldehyde in PBS, pH 7.2, overnight, dehydrated in graded alcohol series, and embedded in paraffin (Paraplast X-tra; Sigma Chemical Co., St. Louis, MO). Sections were cut at 6–8 μm and stained with hematoxylin/eosin.

For immunohistochemistry, embryos were fixed in 95% ethanol/5% acetic acid overnight at 4°C, dehydrated in absolute ethanol, and embedded in Paraplast X-tra. Immunostaining was performed by the procedure described earlier (Aszódi et al., 1994). For in situ hybridization, embryos were fixed in 4% buffered parafomaldehyde overnight at 4°C, embedded in paraffin, and sectioned at 6 μm. Sections were dewaxed and hybridized to 35S[U]TP-labeled (Amersham, Buckinghamshire, UK) RNA probes. The riboprobes were generated from a 321-bp cDNA fragment specific for mouse proc(I) collagen mRNA, from a 331-bp cDNA fragment specific for mouse proc(III) collagen mRNA (Metsäranta et al., 1991), and from a 650-bp cDNA fragment encoding the NC1 domain of mouse collagen X (obtained from Björn Olsen, Harvard Medical School) using an RNA transcription kit (Stratagene, Heidelberg, Germany). Hybridization and subsequent procedures were carried out as described in detail by Moser et al. (1995).

Brdu Incorporation, 35S-labeling, and TUNEL Assay

To detect proliferating cells, three pregnant females were injected intraperitoneally with 50 μg 5-bromo-2’-deoxyuridine (Brdu) in PBS per gram body weight. After 2 h, the mice were killed, and embryos were isolated, fixed in 4% buffered paraformaldehyde, and embedded in paraffin. After sectioning, the Brdu-positive cells were detected by an anti-Brdu monoclonal antibody conjugated with alkaline phosphatase following the protocol supplied by the manufacturer (Boehringer Mannheim, Mannheim, Germany). Before mounting, the sections were counterstained with 0.3% (wt/vol) methyl green, and both the number of Brdu-labeled as well as total cell numbers were determined to calculate labeling indexes.

To determine the incorporation of sulfate into proteoglycans, pregnant mice at gestation day 13 were injected subcutaneously with 2.5 μCi of [35S]Sulfate (Amersham) per gram of body weight 6 h before killing. The embryos were removed from the uterus, fixed for 6 h in 0.1 M sodium cacodylate, pH 7.4, containing 2% glutaraldehyde and 2% paraformaldehyde, and embedded in paraffin. 6-μm sections were cut, dewaxed, and embedded into Kodak NBT-2 photoemulsion (Rochester, NY). Slides were exposed for 3–10 d at 4°C and developed in Kodak Dektol Developer.

Aptosis was analyzed by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method as described by Vaathlonkari et al. (1996).

Preparation of Cartilage Tissue for Biochemical Analysis

Cartilage samples were dissected from limbs of wild-type, heterozygous (+/−), and homozygous mutant (−/−) mice at 17.5 d post coitum and analyzed in parallel as previously described (Chan et al., 1993). Proteoglycans were extracted from the freeze-milled cartilage in a 50 mM Tris-HCl buffer, pH 7.5, containing 4 M guanidine hydrochloride (Gu/HCl), 5 mM EDTA, 0.1 mM PMSF (Sigma Chemical Co.), and 10 mM N-ethylmaleimide (Sigma Chemical Co.) for 24 h at 4°C. The extracts were desalted by dialysis against 0.5 M acetic acid and lyophilized. The cartilage residues were washed thoroughly with water, and collagens were prepared by digestion with 100 μg/ml pepsin (Sigma Chemical Co.) in 0.5 M acetic acid for 24 h at 4°C. The digests were lyophilized. In some experiments, a reduced pepsin concentration of 10 μg/ml was used to analyze abnormally pepsin-sensitive collagens (Bateman et al., 1994).

Preparation of Collagens from Chondrocyte Cultures

Chondrocytes were established from limb cartilage by collagenase digestion (Chan et al., 1995). Primary chondrocytes were initially grown as high-density monolayer cultures for 24 h. Chondrocytes were released by trypsin digestion and grown in alginate beads prepared from a suspension of 2 × 10⁶ cells/ml of alginate (Guo et al., 1989; Chan et al., 1993). Beads were suspended in DME, containing 10% FCS and 0.25 mM sodium ascorbate, for 8 wk before collagen analysis.

Collagen produced by the chondrocytes grown in alginate beads were analyzed by biosynthetic labeling for 24 h with [3H]-proline (NEN Du Pont, Bad Homburg, Germany) in fresh DME containing 10% dialyzed FCS and 0.25 mM sodium ascorbate (Chan et al., 1993). The medium, alginate, and cell-associated fractions were harvested for collagen analysis as previously described (Chan et al., 1993). Procollagens from each fraction were precipitated by adding (NH₄)₂SO₄ to 25% saturation, collected by centrifugation, and redissolved in 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl, 5 mM EDTA, 0.1 mM PMSF, and 10 mM N-ethylmaleimide. Aliquots of each sample were digested with pepsin (100 μg/ml) in 0.5 M acetic acid for 16 h at 4°C.

SDS-PAGE and Immunoblot Analysis

Collagen chains were analyzed on 5% (wt/vol) SDS–polyacrylamide gels. Electrophoresis conditions, sample preparation, Coomassie brilliant blue staining, and fluorography procedures have been described in detail previously (Bateman et al., 1984). Immunoblots were prepared by transferring the protein chains separated on SDS-PAGE to Immobilon-P membrane (Millipore Corp., Bedford, MA). The blots were subsequently hybridized with polyclonal antibodies specific for collagens II, III, IX, and XI. Bound antibodies were hybridized to horseradish peroxidase–conjugated protein G (Bio-Rad Laboratories, Hercules, CA) and detected using the BM chemiluminescence blotting substrate (PDO: Boehringer Mannheim). The procedures for antibody hybridization, membrane washings, and detection followed the protocol described by the manufacturer (Boehringer Mannheim).

Electron Microscopy

For electron microscopy, bones were removed from proximal segments of theibia and radius and from distal segments of the humerus and femur from wild-type, heterozygous (+/−), and homozygous mutant (−/−) mice at 18.5 d post coitum and analyzed in parallel. Tissue samples were fixed in 0.1 M sodium cacodylate buffer, pH 7.4, containing 2% glutaraldehyde and 2% paraformaldehyde for 3 d at room temperature, rinsed three times in isotonic sodium cacodylate buffer for 30 min, and postfixed in 0.1 M sodium cacodylate, pH 7.4, containing 1% (wt/vol) osmium tetroxide overnight. Samples were then rinsed in isotonic buffer solution, dehydrated in a graded series of ethanol, and embedded in Epon 812. Semithin (1 μm) and thin (60 nm) sections...
were cut on a Leica Ultracut S (Deerfield, IL). Sections were stained in 5% uranyl acetate for 2 h and then in a saturated lead citrate solution for 7 min. All samples were viewed in a Hitachi 7100-B electron microscope (Tokyo, Japan).

**Results**

**Abnormal Intervertebral Disc Development in Col2a1-null Mice**

Humans and mice deficient for collagen II show abnormal endochondral ossification, which leads to the formation of short and abnormally shaped long bones (Chan et al., 1995; Li et al., 1995). In the present study, the Col2a1-null mice were used to investigate the developing vertebral column. When whole-mount skeletons of normal and Col2a1-null newborn mice were compared, pronounced morphological alterations that affected the entire vertebral column became obvious (Fig. 1, A and B). In normal mice, vertebral bodies had strong alizarin red staining in their centers, representing mineralized bone tissue derived from endochondral ossification, and were surrounded by a homogenous alcian blue staining corresponding to cartilage tissue (Fig. 1, A and C). Vertebral bodies were separated from each other by IVDs, which displayed a typical discoid structure with weak alcin blue staining in the centers and strong alcin blue staining at the peripheries (Fig. 1 A). The centers correspond to the gelatinous nucleus pulposus and the peripheries to the cartiliginous part of annulus fibrosus (Fig. 1 A). In contrast, the Col2a1-null mice had abnormally shaped verteboae (Fig. 1, B and D). The vertebral bodies increased in size and showed an opposite staining pattern to the control: clear alcin blue staining in the center and alizarin red staining at the periphery, which might represent membranous ossification (Fig. 1 D). The alcin blue staining was also present between the presumptive vertebral bodies but, in sharp contrast to normal mice, was high in the center and absent or very low at the periphery (Fig. 1 B). The overall staining suggested the absence of nucleus pulposus, and the adjacent vertebral bodies were apparently connected via a soft, cartilaginous tissue (Fig. 1 B, arrows).

To determine more precisely the mutant phenotype, the vertebral column of control and Col2a1-null embryos were analyzed histologically at various stages of development (Fig. 2). Between E9.5 and E12.5, no obvious difference was found in mutant mice (Fig. 2, A and B and data not shown). At E12.5, both the formation of the precartilaginous anlage of vertebral bodies as well as the condensation of cells in the intervertebral segments occurred normally in Col2a1-null mice. The notochord appeared as a continuous structure with uniform diameter (Fig. 2 B).

At later stages of development, severe abnormalities became evident (Fig. 2, D, F, H, and J). First, the cartilage of mutant vertebrae was completely disorganized. Although hypertrophic-like chondrocytes were present in prevertebral tissues (Fig. 2, D and F), the arrangement of cartilage cells into growth plates as well as endochondral bone formation did not occur (Fig. 2, H and J). Second, the vertebral bodies became significantly enlarged, reaching approximately twice the size of normal. In the sagittal section of a wild-type thoracic vertebral body at E14.5, the average number of cells was 412 ± 38 (n = 5) compared with 424 ± 36 (n = 5) in a mutant, suggesting that the enlargement of Col2a1-null vertebral bodies was not due to an increased proliferation of mutant cartilage cells but rather the consequence of increased intercellular spaces. Third, Col2a1-null mice showed abnormal development of intervertebral discs. During normal embryogenesis, the dense intervertebral mesenchyme differentiates into the annulus fibrosus. The outermost region of the annulus fibrosus is rich in collagen I, while its inner part is hyaline cartilage expressing collagen II, which together with the vertebral bodies form a continuous cartilaginous column around the notochord (Fig. 2, C and E). After E12.5, the notochord became compressed in the centers of the vertebral bodies and notochordal cells vanished. The notochord expanded in the presumptive IVD areas (Fig. 2, C and E), and cells accumulated to form the nucleus pulposus. The acellular

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**Figure 1.** Skeletal staining of newborn vertebral columns derived from wild-type and Col2a1-null mice. Vertebral columns from wild-type (+/+) (A and C) and Col2a1-null (−/−) mice (B and C) were stained with alizarin red and alcian blue. In the wild type, a ventral view of the thoracic regions (A) showed adjacent vertebral bodies (vb) were separated by well-formed intervertebral discs (ivd) that contained a gelatinous nucleus pulposus (np) in the center and a cartilaginous inner annulus (ia) at the periphery. In contrast, the intervertebral discs of the Col2a1-null mice (B) lacked the nucleus pulposus. The neighboring vertebral bodies (vb) were connected via blue-stained cartilaginous bridges (arrows). C and D show lateral views of the fifth thoracic vertebral from wild-type and Col2a1-null mice, respectively. Vertebral arches of mutant vertebrae failed to fuse. Note that the Col2a1-null vertebral body is composed of a blue, cartilaginous tissue that is surrounded by a thin, red, bony sheath.
Collagen II and Vertebral Column Development

Aszödi et al.

Notochord remnants were visible in the vertebral bodies until birth (Fig. 2, C, E, G, and I). In Col2a1-null mice, the notochord persisted as a rod-like structure until birth without signs of compression or expansion (Fig. 2, D, F, H, and J). In addition, the diameter of the notochord increased with time along the entire vertebral column. The cells in the expanded notochord were uniformly distributed and surrounded by a very thin sheath (Fig. 2, D and F). At the intervertebral segments, both the fibrous and the cartilaginous parts of the annulus were detectable but compressed by the enlarged vertebral bodies (Fig. 2, D, F, H, and J, and Fig. 3, D, F, H, J, and L).

**Cartilage Proteins Are Expressed in Col2a1-null Tissue**

To compare the expression pattern of cartilage matrix molecules in wild-type and Col2a1-null embryos, a detailed immunohistochemical analysis was performed on

Figure 2. Comparative histological analysis of the developing vertebral column from wild-type and Col2a1-null mice. Sagittal sections of the cervical vertebral column (E12.5–17.5 and newborn [NB]) from wild-type (+/+; A, C, E, G, and I) and Col2a1-null mice (−/−; B, D, F, H, and J) were stained with hematoxylin and eosin. The sections are presented at same scale to show the size differences between normal and mutant vertebral bodies. The orientation of I and J are at right angles to the others. The segmental differentiation of prevertebrae (pv) and intervertebral mesenchymes (im) were similar at E12.5 between wild-type (A) and Col2a1-null embryos (B). In wild-type mice, the notochord gradually expanded at the intervertebral disc areas to form a nucleus pulposus (np) and vanished within the vertebral bodies (vb) (C, E, G, and I). In Col2a1-null mice, the rod-like structure of the notochord remained relatively unchanged, without any notable differential expansion or regression (D, F, H, and J). Vertebral bodies of Col2a1-null mice became double in size. Other landmarks, such as prevertebrae (pv), intervertebral mesenchyme (im), notochord (n), inner annulus (ia), and outer annulus (oa), are indicated. The arrows identify the notochordal sheath, and asterisks indicate the acellular notochordal tube. Bar, 100 μm.
Figure 3. Immunohistochemical localization of collagens in wild-type and Col2a1-null vertebral columns. Sagittal and parasagittal sections of the vertebral column from wild-type (+/-: A, C, E, G, I, and K) and Col2a1-null (-/-: B, D, F, H, J, and L) embryos were stained with specific antibodies against collagens I (Col1), II (Col2), III (Col3), IX (Col9), X (Col10), and XI (Col11). In the wild type (A), collagen II was present in the cartilage of vertebral bodies (vb), inner annulus (ia), and notochordal sheath (arrow), but absent in the outer annulus (oa) and the nucleus pulposus (np). Col2a1-null cartilage lacked collagen II (B), while collagens IX (C and D) and XI (E and F) showed a tissue distribution similar to that of wild type. The immunostaining for collagen XI was significantly weaker in Col2a1-null cartilage (F). Col2a1-null cartilage showed ectopic deposition of collagens I (J) and III (L). The distribution of collagen X was in the center of normal and in the periphery of Col2a1-null vertebral bodies (compare G and H). A–F, I, and J are sections from E14.5 embryos, and G, H, K, and L are sections from E15.5 embryos. Bar: (+/-) 200 μm; (-/-) 100 μm.
vertebral sections of various stages (Figs. 3 and 4). At E14.5, immunostaining for collagen II was high in all cartilaginous tissues (vertebral bodies, inner annulus) and in the notochordal sheath of control animals (Fig. 3 A). As previously reported, no collagen II was detected in Col2a1-null mice (Li et al., 1995 and Fig. 3 B). The distributions of collagen IX, collagen XI (Fig. 3, C–F), and non-collagenous components, including aggrecan, fibromodulin, COMP, CMP and chondroadherin, were similar in vertebral tissue of normal and Col2a1-null mice (Fig. 4, and data not shown). The level of collagen XI, however, was significantly reduced in collagen II–deficient cartilage (Fig. 3 F). By E15.5, hypertrophic chondrocytes began to differentiate, and collagen X was deposited both in wild-type and mutant mice (Fig. 3, G and H). However, the localization of collagen X was different: whereas control vertebral bodies showed strong immunostaining in the center and were negative at the periphery (Fig. 3 G), in the mutant vertebral bodies collagen X was predominantly found at the periphery (Fig. 3 H), similar to the mineralization pattern discussed above (Fig. 1 D). This unusual expression pattern of collagen X was also detectable by in situ hybridization (data not shown).

At the intervertebral level, the presence of a cartilaginous, inner annulus in Col2-null mice was confirmed by a positive immunoreaction for aggrecan (Fig. 4 B). The presence of an inner annulus could be further confirmed by the absence of the long form of collagen IX both in normal and control Col2a1-null mice (Fig. 3, C and D). In vertebrates this collagen IX isoform is expressed in the vertebral segments but not in the disc areas (Hayashi et al., 1992).

**Collagens I and III Are Expressed in Col2a1-null Cartilage**

We have reported earlier that chondrocytes of human fetuses suffering from achondrogenesis type II-hypochondrogenesis express collagens I and III and deposit these collagens into the mutant cartilage (Chan et al., 1995; Mundlos et al., 1996). To test whether these collagens are also aberrantly expressed in Col2a1-null mice, vertebral tissue was analyzed by immunohistochemistry and in situ hybridization. Tissues derived from wild-type E14.5–15.5 embryos expressed high amounts of collagen I in the outer part of the annulus fibrosus and in the surrounding mesenchyme and very low amounts of collagen I in the inner annulus fibrosus (Fig. 3 I). Collagen III was highly expressed in the notochordal sheath, the outer annulus fibrosus and the surrounding mesenchyme (Fig. 3 K). The inner part of the annulus fibrosus was weakly positive for collagen III (Fig. 3 K). A faint staining for collagen III was detectable in the pericellular regions of hypertrophic chondrocytes (Fig. 3 K). In sharp contrast, Col2a1-null embryos expressed high amounts of collagens I and III in vertebral bodies, both parts of the annulus fibrosus and notochordal

**Figure 4.** Immunohistochemical localization of non-collagenous cartilage proteins in wild-type and Col2a1-null vertebral columns. Sagittal sections of the vertebral body of E14.5 embryos from wild-type (+/+; A, C, and E) and Col2a1-null (−/−; B, D, and F) were stained with specific antibodies against aggrecan (Agn), fibromodulin (FM), and cartilage oligomeric protein (COMP). Aggrecan and COMP were similarly distributed in wild-type (A and E) and mutant (B and F) vertebral column. Fibromodulin (D) staining was decreased in Col2a1-null cartilage. The vertebral body (vb), notochord (n), and inner annulus (ia) of the developing vertebral column are indicated. Bar: (+/+ 200 μm; −/− 100 μm.)
sheath (Fig. 3, J and L). In addition, both collagens were also present inside the notochord, where they were never detected in wild-type mice (Fig. 3, I–L). This aberrant expression pattern of collagens I and III was found along the entire region of the vertebral column and at all stages analyzed.

To determine which cells in the mutant cartilage express transcripts encoding collagens I and III, in situ hybridization was performed using specific riboprobes (Fig. 5, A–D). Signals for collagens I and III were not detectable in the cartilaginous tissue of wild-type vertebrae but were present in the perichondrium, in the intervertebral disc area, and in the surrounding mesenchyme (Fig. 5, A and C). In tissue sections derived from Col2a1-null mice, both collagens I and III were expressed in the cartilage, but only by ~10–20% of all chondrocytes (Fig. 5, B and D). The intensity of hybridization signals was much stronger for collagen I than for collagen III. These data, together with the biochemical analysis of cultured chondrocytes (see below), indicate that a subpopulation of Col2a1-null chondrocytes can synthesize collagen I and III. However, we can not exclude the possibility that the immunodetectable collagen I and III in mutant cartilage may be derived partially from the perichondrium and has diffused into the cartilage matrix.

Biochemical Analysis of Cartilage Collagens

For biochemical analysis, limb cartilage tissue was isolated from normal, heterozygous, and Col2a1-null mice and sequentially extracted with Gu/HCl to remove proteoglycans and other noncollagenous proteins and with pepsin digestion to isolate the insoluble collagens. Electrophoretic analysis of these extracts showed a typical collagen solubility and composition profile in normal (Fig. 6, lanes I and 3) and heterozygous animals (data not shown), where the collagens were restricted to the Gu/HCl insoluble fraction and released only after pepsin digestion. In contrast, the collagenous component in the Col2a1-null cartilage was fully solubilized by Gu/HCl extraction (lanes 2 and 4). The collagenous component of the wild-type cartilage contained collagen II (lanes 1 and 7) and a small amount of collagen XI (lanes 1 and 9), whereas analysis of the Col2a1-null cartilage Gu/HCl extract (lane 4) and the same extract treated with limited pepsin digestion (lane 13) showed the predominant collagen to be collagen I, identified by the presence of α1(I) and α2(I) chains. Small amounts of collagen III was also detectable after pepsin digestion (lanes 6 and 13). The complete absence of collagen II in the Col2a1-null cartilage was demonstrated by an overexposed immunoblot with a collagen II antibody (lane 8). The soluble fraction also showed clear evidence for chains migrating similarly to the α1(XI) and α2(XI) chains of collagen XI (lane 4). The identity of these bands was confirmed by immunoblotting with an antibody specific for collagen XI (lane 10). Immunoblot analysis also revealed additional collagen XI chains migrating with a higher molecular weight. No positive collagen XI chains were detectable following standard limited pepsin digestion conditions (lane 11). If less stringent pepsin digestion conditions were used, partially degraded collagen XI chains were detected (lane 12), suggesting that the collagen XI present in the Col2a1-null cartilage had not formed a stable triple helical structure. The Col2a1-null cartilage contained significant amounts of collagen IX in the Gu/HCl extract (lane 4), the identity of which was confirmed by immunoblot analysis (lane 5).

The β-components (chains that are covalently cross-linked as dimers) of collagen I extracted from the Col2a1-null cartilage (lanes 4 and 13) appeared to be significantly
reduced compared with extracts of Col2a1-null skin (lane 14) and wild-type bone (lane 15) arguing against a global cross-linking defect.

**Collagen Metabolism by Cultured Chondrocytes**

To further examine the collagen synthesis of the Col2a1-null cartilage, chondrocytes isolated from limb cartilage were grown in alginate beads, which provide the three-dimensional environment necessary to maintain the chondrocyte phenotype in vitro. Wild-type cells produced and secreted collagen II, while Col2a1-null cells produced collagens I and III (Fig. 7, A and B). When the collagens extracted from the three cell culture fractions (cell-associated, alginate matrix, and culture medium) were compared, the collagen II produced by the wild-type chondrocytes was predominantly cell associated and in the immediate extracellular matrix (alginate fraction), with only a small proportion secreted into the medium. In contrast, the collagen I produced by the Col2a1-null chondrocytes was not deposited in the matrix and essentially all was found secreted into the medium, supporting the tissue extraction data.

**Cartilage of Homozygous Mutant Mice Contains Abnormal Collagen Fibrils That Cannot Form a Network**

Ultrastructural analysis of limb cartilage by electron microscopy of Col2a1-null mice revealed a reduced number of collagen fibers present in the extracellular matrix (Fig. 8, B and D). The morphology of these fibrils was abnormal: they had a large variation in diameter and were frequently arranged in groups of two to three bundles, which apparently fused with each other at many locations. The fibrillar surface was rough, and the banding periodicity was between 20–35 nm instead of the typical 65–67 nm as observed in normal cartilage tissue (Fig. 8 C). The distribution of the fibrils was irregular, and the cartilage lacked the well-organized collagen network characteristic for control hyaline cartilage (Fig. 8, A and C). The amount of the proteoglycan precipitates was significantly reduced in Col2a1-null cartilage compared with the wild-type.

**Notochordal Cells Proliferate and Are Metabolically Active within the Vertebræ of Normal Embryos**

The mechanism that is responsible for the notochordal reorganization during normal development is poorly understood. Based on studies in mammalian embryos, some studies have proposed that the growing vertebral bodies exert a mechanical pressure on the notochord, inducing cell migration to the intervertebral regions (Theiler, 1988; Rufai et al., 1995). Alternatively, others have suggested that the notochordal cells die within the vertebrae and proliferate in the disc areas (Goto and Uhthoff, 1985; Cotten et al., 1994).

To determine whether differential cell death and proliferation play a role during segmental expansion and constriction of the notochord in wild-type mice, apoptosis, proliferation, and metabolism of notochordal cells were analyzed between E13 and 14.5. During this period, notochord expansion in the IVD area was obvious but still contained cells in the vertebral body area. Cell death was analyzed using the TUNEL assay, which detects DNA segmentation occurring in apoptotic cells. At all stages ana-
lyzed, the notochord of normal mice contained no apoptotic cells in the trunk region. The result for E13.5 embryos is shown in Fig. 9A. Control sections pretreated with DNase I stained most cells (Fig. 9B). Proliferation and metabolism of notochordal cells were tested by injecting BrdU and 35S into pregnant mice, respectively. Embryos were harvested, and BrdU incorporation into proliferating cells was detected with an anti-BrdU antibody in tissue sections. The distribution of replicating cells in notochordal segments was similar within and between vertebral bodies (Fig. 9C). The labeling index (number of BrdU labeled nuclei correlated to the total number of cells) in the inter- and intravertebral areas of the notochord at E13.5 was 13.2 ± 2.8% (n = 10) and 12.2 ± 1.7% (n = 10), respectively. Although only a few cells were present in the constricted, vertebral segment of the notochord at E14.5, proliferative notochordal cells were readily detectable (Fig. 9D). The expression of radiolabeled proteoglycans was similarly high in all areas of the notochord, suggesting that cells are metabolically active at this critical stage (Fig. 9, E and F).

Discussion

In the present paper, we have analyzed the development of the vertebral column in mice lacking collagen II. We report that in the absence of collagen II, vertebral bodies are enlarged, the notochord fails to disappear within the vertebral bodies, and IVDs do not develop. This finding is surprising since such abnormalities have never been described in patients suffering from achondrogenesis type II, which is caused by the absence of collagen II and, like the Col2al-null mouse, leads to perinatal lethality characterized by micromelia, lack of endochondral ossification, and cleft palate. One possible reason for this discrepancy between mouse and man could be that these defects do not occur in humans and are specific for mice or, more likely, have escaped detection.

The Patterning of the Axial Mesoderm Is Normal in Col2al-null Mice

During vertebrate embryogenesis, somites segregate into a dorsal and ventral component, designated the dermomyotome and sclerotome. The dermomyotome differentiates into striated muscle and dermis. The sclerotome gives rise to the axial skeleton, which includes vertebrae, intervertebral discs, and ribs. Experimental evidence from the early fifties suggested that the notochord is inducing vertebral chondrogenesis. The molecule(s) produced by the notochord and responsible for chondrogenesis were termed the “inducer” (Holtzer and Detwiler, 1953; Watterson et al., 1954). The fact that isolated somites differentiate into
chondrocytes when grown on a collagen II substrate (Minor et al., 1975) and that the notochord produced collagen II (von der Mark et al., 1976) led to the hypothesis that collagen II may act as “inducer”. Therefore, we investigated the formation of the vertebral column at various developmental stages in normal and Col2a1-null mice. In the mouse, collagen II is expressed at E9.5 in sclerotome and notochord (Cheah et al., 1991; Wood et al., 1991; Ng et al., 1993). Histological analyses of the entire trunk region of Col2a1-null embryos revealed no abnormalities between E9.5 and 12.5, indicating that the patterning of somites into the dermomyotome and sclerotome and the migration of sclerotomal cells to the notochord is unaffected by the absence of collagen II. The factor(s) that induce sclerotomal differentiation are common to the notochord and floor plate since both convert an entire somite into cartilage when implanted between the paraxial mesoderm and neural tube of early chick embryos (Pourquïé et al., 1993). Recent studies have demonstrated that the signaling molecules that induce the ventralization of somites include Sonic hedgehog, a member of the hedgehog family, and Noggin, a bone morphogenetic protein antagonist, both of which are expressed by the notochord (Fan and Tessier-Lavigne, 1994; McMahon et al., 1998).

Between E11–12, the unsegmented perichordal mesenchyme acquires a metameric pattern with condensed and uncondensed areas. The condensed mesenchyme then differentiates into the annulus fibrosus of the disc anlagen, whereas the intervening, uncondensed mesenchyme develops into the cartilaginous vertebral bodies. Starting from E14.5, the annulus can be subdivided into a fibrosus outer part and a cartilaginous inner part. The latter links the disc rudiments to the developing vertebral bodies, which gives rise to a continuous cartilaginous column around the notochord (Theiler, 1988). Both the anlage of the vertebral bodies and the inner part of the annulus fibrosus develop in Col2a1-null embryos, which is consistent with a previous study showing that the collagen II is not essential for early cartilage formation (Li et al., 1995).

The Development of Vertebral Bodies and IVDs Is Dependent on the Expression of Collagen II

At around E13, notochordal cells begin to disappear from the notochord surrounded by the developing vertebral bodies and accumulate between the vertebral bodies to form the nucleus pulposus. This process is accomplished at around E16, resulting in a cell-free notochord that is visible within the cartilaginous vertebral bodies and is finally eliminated during ossification. In the absence of collagen II, the anlagen of the vertebral bodies and the intervertebral discs develop abnormally, and the notochord fails to

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**Figure 9.** Analysis of cell death, cell proliferation, and metabolic activity during normal notochord dismantling. Sagittal sections through the lumbar part of vertebral column at E 13.5 (A–C, E, and F) and E14.5 (D). Cellular death was analyzed by TUNEL assay (A and B). No apoptosis was detected either in the notochord (n) or in the vertebral body (vb) (A). Positive control section treated with DNase I stain all cells (B). Cell proliferation was monitored by BrdU incorporation assay (C and D). Proliferation rate of cells was similar in the intravertebral and intervertebral segments of notochord E13.5 (C). Dividing cells were detectable in the intravertebral segment even at E14.5 (D). Radioactive sulfate incorporations assay (E, dark field image; F, bright field image). Both intra- and intervertebral notochordal cells were metabolically active. High metabolic activity was also obvious in the cartilage of vertebral bodies. ia, inner annulus. Bars: (A–C and F) 200 μm; (D and E) 100 μm.
undergo normal morphogenesis. The vertebral bodies enlarge gradually during development in Col2a1-null mice and never initiate endochondral ossification. The increase in size is associated with a softening of the tissue and the formation of large cyst-like areas filled with fluid. These alterations are not restricted to vertebral bodies but occur in all cartilage tissues and lead, like in long bones, to an abnormal shape of the tissue (Li et al., 1995). This phenotype is most easily explained by the ability of proteoglycans to bind water, resulting in the swelling of tissue. In normal cartilage, proteoglycan swelling is counteracted by the presence of a tight collagen network (Myers and Mow, 1983), which is missing in the mutant mouse strain.

The most interesting finding, however, is the persistence of the notochord, which is still present at birth in the Col2a1-null mice. Serial sections of several vertebral columns from Col2a1-null mice revealed that along the entire body axis the notochord persists as a rod-like structure without signs of expansions or regressions. A consequence of this default notochordal morphogenesis is an arrest in the development of IVDs: the nucleus pulposus fails to form, and the anlage of the annulus fibrosus does not increase in size. These data demonstrate a crucial role for collagen II during notochord removal as well as conversion into a nucleus pulposus.

**Collagens I and III Are Expressed in Col2a1-null Cartilage**

Cartilage tissue of newborn Col2a1-null mice is soft and malformed. To test whether the abnormalities in tissue consistency result solely from the absence of the collagen II fibrillar network or result also from an altered expression of other matrix components, we analyzed many cartilage matrix proteins by immunohistochemical and biochemical techniques. The most unexpected result was that the cartilage tissue contained large amounts of collagens I and III, which, when analyzed biochemically, were present in the soluble fraction of the tissue extract. We have observed a similar default expression of noncartilage collagens I and III in patients suffering from achondrogenesis type II-hypochondrogenesis (Chan et al., 1995, Mundlos et al., 1996). Collagens I and III are usually coexpressed and expressed in many connective tissues, such as skin, bone, and tendon (Kadler, 1994). They are both expressed in precartilaginous mesenchyme and are turned off as soon as chondrogenesis begins with the expression of cartilage-specific proteins, such as collagen II, aggrecan, etc. (von der Mark, 1980). Collagen fibrils are extensively cross-linked, and in cartilage this leads to the formation of a strong fibrillar network that resists the swelling pressure of the entrapped proteoglycans. The impaired cross-linking of the abnormal collagen I and III fibrils might be one of the major reasons for the enlargement and the softening of the mutant cartilage tissue.

**Collagens IX and XI Expression in Col2a1-null Cartilage**

The stability of the collagen II network is further supported by several collagenous and noncollagenous proteins that connect the collagen fibrils with the surrounding matrix. Collagen IX is normally covalently associated with the surface of the collagen II fibrils and can interact with other collagen IX molecules (Diab et al., 1996). It is expressed at much higher levels in mutant cartilage. We do not know at present whether it is able to associate with collagen I or III fibrils. In Western assays, collagen IX appears in several sizes, suggesting that the different sizes become cross-linked with each other.

Although the absence of α1(II) chain results also in the absence of α3(XI) chain, collagen XI-like molecules are present in Col2a1-null cartilage. The importance of the α3(XI)/α1(II) chain for the stable assembly of collagen XI molecules is demonstrated by the altered sensitivity to limited pepsin digestion. Under conditions where normal collagen XI molecules from normal cartilage were resistant to pepsin digestion, the collagen XI derived from Col2a1-null cartilage was degraded. Recently, mRNAs for all three α-chains of collagen XI have been found in several non-chondrogenic tissues (Lui et al., 1995). In some tissues, the α1(XI) and α2(XI) mRNAs are expressed without α3(XI) transcript, suggesting that α1(XI) or α2(XI) homotrimers or α1α2(XI) heterotrimers can form in vivo (Lui et al., 1995). Our findings support this hypothesis but also indicate that, at least in cartilage, the Col2a1 gene is essential in the assembly of “stable” triple helical collagen.

**Collagen II Is Not Necessary for Chondrocytes to Undergo Hypertrophic Maturation**

Many proteins, including CMP, COMP, chondroadherin, and aggrecan, are specifically expressed by chondrocytes and are, therefore, lineage-specific markers for this cell type. Chondrocytes, however, can undergo a further differentiation process, referred to as hypertrophy, which results in an enlargement of the cell and the characteristic expression of collagen X (Alini et al., 1992). Hypertrophic chondrocytes are normally present in growth plates of long bones, vertebral bodies, and all other areas where cartilage is undergoing endochondral ossification.

Our data demonstrate that the vertebral chondrocytes in the Col2a1-null embryos undergo a normal sequence of differentiation, producing cartilage lineage-specific matrix components. Furthermore, histological analysis demonstrated that many of the chondrocytes present in the mutant vertebral bodies were hypertrophic in appearance, and immunostaining and in situ hybridization demonstrated the presence of collagen X, indicating the chondrocytes had also undergone further maturation and hypertrophy. These data show that collagen II is not essential for cartilage cell differentiation and hypertrophy but provide further evidence that collagen II plays a crucial role in organizing the chondrocytes into typical growth plate structures in long bones (Li et al., 1995) and in forming a centralized zone of hypertrophy in the vertebral bodies. Clearly, a collagen II matrix is essential for the structural organization and function of hypertrophic cartilage.

**Dismantling of the Notochord and Development of Intervertebral Disc Is a Collagen II–dependent Process**

The literature on the removal of the notochord within vertebral bodies and expansion between vertebral bodies is poor and only based on morphological studies. Two hypotheses have been proposed to explain the morphoge-
netic events in the notochord. One suggests that notochordal cells degenerate within the vertebral bodies and proliferate within the discs (Goto and Uthoff, 1985; Cotten et al., 1994). It is not known how the balance between proliferation and death of notochordal cells is induced, regulated, or maintained. Our experiments show unambiguously that cell death does not generally occur in the notochord and hence cannot account for the disappearance of cells within the vertebral bodies. Similarly, we found that notochordal cells proliferate evenly along the entire notochord. Therefore, we can exclude differential degeneration and proliferation as a cause for the reorganization of the notochord.

A second theory suggests that the developing vertebral bodies exert a mechanical stimulus on the notochord, which induces the migration of cells towards the presumptive IVD area (Theiler, 1988; Rufai et al., 1995). It is suggested that “squeezing forces” may increase during progressive chondrification of the vertebral body (Theiler, 1988). Studies with hyaline cartilage have shown that proteoglycans, which carry a highly negative charge because of their constituent glycosaminoglycan side chains, induce a swelling pressure that is restrained by the collagen II fibrils (Myers and Mow, 1983). Calculations with isolated tissue samples revealed that the pressure within the tissue can reach up to 0.35 MPa (Maroudas, 1976), and the internal swelling forces provide the characteristic resistance of hyaline cartilage to compressive load. This model may provide the basis for explaining the failure of IVD development in the Col2a1-null mice, implicating a mechanical mechanism in the morphogenetic process. The cartilage of the Col2a1-null mice showed increased hydration and enlargement, suggesting that the abnormal, sparse, and poorly organized collagen matrix in the cartilage, as shown by electron microscopy, is unable to effectively resist the osmotic swelling pressure exerted by the proteoglycans. This decreased internal swelling pressure is unable to force the notochordal compression that would normally induce notochordal cell migration.

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