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OSTEOARTHRITIS and CARTILAGE

Type X collagen biosynthesis and expression in avian tibial dyschondroplasia

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Summary

Objective: Tibial dyschondroplasia (TD) is an abnormality of growth plate cartilage characterized by the presence of non-vascularized, non-mineralized tissue. The objective of this study was to examine structural and functional alterations of the growth plate-specific type X collagen in TD cartilage.

Design: Collagen biosynthesis was examined in organ cultures and in cultured chondrocytes from normal growth plate and TD cartilage. Thermal stability of type X collagen extracted from normal and TD cartilage organ cultures to protease digestions by trypsin plus chymotrypsin or bacterial collagenase was determined. The expression of collagen genes was examined in cultured normal and TD chondrocytes.

Results: Synthesis of total collagen and of type X collagen was greater than threefold higher in organ cultures from the TD lesion compared with normal growth plate. The increase in type X collagen synthesis in the lesion was compensated by a reduction in the relative proportions of types II and XI collagens. The thermal denaturation and collagenase cleavage properties of purified types II and X collagens from TD cartilage were normal. The expression of type X collagen gene was threefold higher in cultured TD chondrocytes compared to chondrocytes from normal growth plate. Normal growth plate chondrocytes in primary cultures synthesized predominantly type X collagen (80% of total collagen). In contrast, TD chondrocytes synthesized mainly types I and II collagens and type X collagen represented only 22% of total collagen. TD cells initiated the synthesis of type I collagen within 5 days of primary culture, whereas normal chondrocytes did not synthesize this collagen during the same culture period. Although type X collagen synthesis was reduced in TD chondrocytes, the mRNA levels for type X collagen were substantially higher than in normal chondrocytes.

Conclusion: Accumulation of type X collagen in TD cartilage results from its increased biosynthesis which is due largely to increased expression of the gene for this collagen, although, the chondrocyte culture studies suggest the possibility of postranscriptional defect in type X collagen synthesis or processing in TD lesion. Moreover, the TD chondrocytes in contrast with normal chondrocytes display evidence of prompt loss of their specific phenotype during short-term primary cultures.

Key words: Chondrocytes, Growth plate cartilage, Type X collagen, Type II collagen, Avian dyschondroplasia.

Introduction

Chondrocytes in growth plate cartilage are responsible for the development and growth of long bones. During the process of bone growth, proliferating chondrocytes within the growth plate undergo a precisely coordinated process of temporal and spatial changes in their growth and differentiation programs associated with a concomitant reorganization of the pre-existing extracellular matrix (ECM). Some of the most dramatic changes in the biosynthetic program of the cells within this tissue include the initiation of type X collagen gene expression and the appearance of alkaline phosphatase activity [1–4]. The increase in type X collagen synthesis is accompanied by a decrease in the synthesis of other cartilage collagens [4, 5]. The temporal and topographical distribution of type X collagen, which is restricted to a subset of chondrocytes embedded in a matrix undergoing calcification, vascular invasion, and degradation, have implicated a structural and/or functional involvement of this molecule in endo-

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chondral bone formation. Kwan *et al*. [6] showed that type X collagen appears to assemble into an hexagonal lattice *in vitro*. This lattice may play a structural role by participating in the formation of a network with other components of the hypertrophic cartilage matrix that may represent a prerequisite modification of the ECM necessary for the transition of cartilage to bone that occurs during endochondral bone formation. Disruption of the precise pattern of differentiation of chondrocytes in the growth plate and of the expression of their biosynthetic products is observed in a number of conditions, for example, rickets [7–8], and tibial dyschondroplasia (TD). TD is a common skeletal disease of rapidly growing broiler chickens [9], turkeys [10–11] and certain mammals [12]. Avian TD is characterized by the appearance of an avascular, opaque cartilage mass in the growth plate resulting from an accumulation of prehypertrophic chondrocytes embedded within a matrix that fails to undergo mineralization [9, 13, 14]. Electron microscopic studies by Hargest *et al*. [13] showed that chondrocytes within the dyschondroplastic lesions failed to achieve normal hypertrophy and some underwent necrosis. Although matrix vesicles were present throughout the affected tissue they became calcified only at the distal edge [14].

Numerous studies have examined the ECM of affected TD cartilage [15–18]. Bashey *et al*. [15] showed no differences in the total collagen content and extractability between normal and TD lesions. However, the content of type X collagen in the TD cartilage was twice when compared to age matched normal hypertrophic cartilage. Chen *et al*. [16] employing immunochemistry showed that type X collagen was present in TD tissues, however, mRNA for type X collagen was not detected. Since type X collagen is synthesized exclusively by growth plate cartilage hypertrophic chondrocytes and its expression is strictly confined to tissues evolving from chondrogenesis to osteogenesis, it has been postulated that this collagen may be fundamentally implicated in the process of endochondral ossification [1]. In an attempt to clarify further the role that type X collagen may play in the initiation of endochondral bone formation we examined the biosynthetic expression of type X collagen in organ cultures of TD cartilage, a tissue that fails to undergo mineralization, in comparison to normal growth plate cartilage. In addition, primary chondrocyte cultures were established from TD and normal growth plate cartilage to examine whether there are differences in the expression of collagen genes between these two tissues.

Materials and Methods

ORGAN CULTURES OF NORMAL GROWTH PLATE AND TD LESION CARTILAGE

Cartilage from the tibiotarsal growth plate of 25-day-old normal male Plymouth Rock (Gallus domesticus) chicks and from chicks with TD lesions was obtained from the Poultry Science Department of the Pennsylvania State University (University Park, PA, U.S.A.). For collection of tissues, the proximal end of the tibia was split longitudinally. For normal tissue, the articular cartilage was removed and the epiphyseal growth plate excised with a scalpel. For TD lesions, they were carefully excised from the adjacent tissues such as growth plate, trabecular bone and cortical bone by sharp disection. The lesion tissue obtained with this procedure does not contain growth plate or trabecular bone tissue. The cartilage from normal growth plate and from TD lesions was incubated in Dulbecco's modified Eagle's medium (MEM) containing 10% FCS, ascorbic acid (50 μg/ml), β-aminopropionitrile (β-APN) (2.5 mM) and 1% streptomycin and was labeled for 24 h with 5.0 μCi/ml [^{14}C]-proline. Metabolically labeled tissues were homogenized with a Polytron homogenizer for 3 min at 4°C in a buffer containing 1.0 M NaCl, 50 mM Tris/HCl pH 7.4, and the following protease inhibitors: 5.0 mM disodium EDTA, 0.2 mM phenylmethylsulphonyl fluoride, 5.0 mM N-ethylmaleimide and 1.0 mM p-aminobenzamidine hydrochloride, and extracted in the above buffer for 72 h at 4°C. An aliquot of the original homogenate from normal growth plate and TD cartilage was assayed for DNA content [19]. The solubilized material was clarified by centrifugation and the insoluble material was sequentially extracted as previously described [20]. Briefly, this procedure involved sequential extraction first with 1.0 M NaCl, then with 0.5 M acetic acid, then with 0.5 M acetic acid containing 1 mg/ml pepsin, followed by extraction with 50 mM Tris/HCl pH 7.4, containing 0.15 M NaCl, and 25 mM dithiothreitol (DTT) and finally with 0.5 M acetic acid containing 1 mg/ml pepsin. The residue was resuspended in 1.0 M acetic acid and boiled for 5 min. Each extraction was carried out for 48 h at 4°C, ultimately resulting in complete solubilization of the tissue. Following centrifugation, the supernatants were dialyzed exhaustively against 0.15 M NaCl, 50 mM Tris/HCl, pH 7.4 and aliquots taken for detection of ^{14}C radioactivity. The amount of collagen extracted from normal growth plate and TD cartilage was determined by a bacterial collagenase assay [21] and the relative proportion

of collagen was calculated by the method of Breul *et al*. [22].

COLLAGEN PRODUCTION AND mRNA ANALYSES OF PRIMARY CULTURES OF CHONDROCYTES ISOLATED FROM NORMAL GROWTH PLATE AND TD CARTILAGE

Cartilage from normal growth plate and from the TD lesion was utilized for preparation of freshly isolated chondrocytes which were obtained as described previously [23]. Briefly, the cartilage was finely minced with a scalpel and submitted to sequential enzymatic digestion with 0.1% trypsin in Hank's medium for 20 min followed by 300 U/ml hyaluronidase in Hank's medium for 1 h and finally 0.2% crude bacterial collagenase (type II) in Earle's balanced salt solution for 1 h. After isolation, primary chondrocyte cultures were established by plating the cells at a density of 5×10^5 cells/cm^2 on plastic culture dishes. The isolated chondrocytes from normal growth plate comprise cells present in all zones of the growth plate, whereas, chondrocytes isolated from the TD lesion may represent only the viable hypertrophic chondrocytes remaining in the lesion [13]. Chondrocytes were cultured for 5 days in DMEM containing 10% fetal bovine serum (FBS) and antibiotics. At day 6, the medium was replaced with fresh DMEM containing 10% FBS, 5.0 μCi/ml [^{14}C] proline, ascorbic acid (50 μg/ml), and β-APN (2.5 mm), and the experiment was terminated 24 h later. The media were removed and the cells were washed with Hank's solution and immediately solubilized in 4 M guanidinium isothiocyanate. An aliquot of the 4 M guanidinium isothiocyanate-solubilized cells was used for DNA assay [19]. The collagenous proteins from both media and cell layers were analyzed by sodium dodecyl sulfate polacrylamide gel electrophoresis (SDS-PAGE). For Northern hybridizations total RNA was isolated in a $CsCl_2$ discontinuous gradient and 10 μg aliquots were denatured in formaldehyde, electrophoresed in 0.8% agarose gels, and then transferred to nitrocellulose filters. cDNAs specific for chicken α1(II) procollagen [24], chicken type X collagen (clone AR10C 121; Reginato *et al*. unpublished observations) and 18S ribosomal protein [25] were nick-translated with [^{32}P]-dCTP to a specific activity of greater than 10^8 cpm/μg DNA as described by Rigby *et al*. [26]. The filters were hybridized for 24 h in 50% formamide and washed under low stringency conditions. Filters were exposed to X-Omat AR film (Eastman Kodak, Rochester, NY, U.S.A.) and the relative proportions of each transcript determined by laser densitometric analysis of underexposed autoradiographs to assure a linear correlation between the levels of each transcript and the intensity of the corresponding band.

GEL ELECTROPHORESIS OF ^{14}C CONTAINING COLLAGENS FROM ORGAN AND MONOLAYER CULTURES

The labeled proteins synthesized in the organ cultures or in monolayer cultures were examined by SDS-PAGE in 8% polyacrylamide slab gels under reducing conditions. In certain experiments, samples were digested with pepsin (100 μg/ml) at 15°C for 16 h before electrophoresis to convert type II procollagen into collagen and to remove the non-collagenous domains of types IX, X and XI collagens. After electrophoresis the gels were processed for fluorography with Enhance (New England Nuclear, Boston, MA, U.S.A.) and exposed to X-Omat AR film (Eastman Kodak).

QUANTITATIVE ANALYSIS OF THE RELATIVE PROPORTIONS OF NEWLY SYNTHESIZED COLLAGENS IN ORGAN CULTURES OF NORMAL GROWTH PLATE AND TD CARTILAGE

In order to examine the relative proportions of the various cartilage collagens synthesized in the organ cultures, the tissues were extracted as described above, aliquots from each extract were electrophoresed in 8% SDS-polyacrylamide gels under reducing conditions before and following limited pepsin digestion, and the gels submitted to fluorography. The fluorographs were scanned in a linear-drive densitometer at 540 nm and the areas under each peak quantified. The densitometric analyses were carried out in slightly underexposed fluorographs to assure linearity between the calculated areas and the absolute amount of radioactivity in each band. The relative proportions of type IX and type XI collagens were calculated as follows: for type IX collagen, it was assumed that the prominent 69 kDa band observed following the pepsinization/reduction conditions used, represented one-third of the heterotrimeric type IX collagen molecules [27]. Therefore, the values obtained from densitometric scanning of the 69 kDa band were multiplied by three to obtain an estimate of the relative proportion of triple helical type IX collagen. For type XI collagen the values obtained from densitometric scanning of the well separated and distinct α1(XI) and α2(XI) collagens were added and divided by two to

estimate the amount of the α3(XI) collagen band which co-migrated with the α1(II) collagen chain. The values for the three chains were added to obtain the relative proportion of the triple helical type XI collagen. For type II collagen, the calculated value of the α3(XI) collagen chain was subtracted from the densitometric value of the co-migrating α1(II) and α3(XI) collagens. In this manner, any error introduced in these calculations would be a consistent and systematic one, because all samples were treated in an identical fashion. All values were expressed as percentages of the total collagen in each sample.

Fractional NaCl precipitation of the extracted collagens

To obtain purified type X collagen, fractional NaCl precipitation of the extracted collagens from acidic solutions was performed as described previously [15, 28]. For this purpose, portions of the DTT extract, which contained greater than 75% of total tissue collagen were dialyzed against 0.5 M acetic acid containing progressively increasing concentrations of NaCl. Fractions precipitating at 0.8 M, 1.2 M, and 2.5 M NaCl were obtained. Type X collagen which precipitated with the 2.5 M NaCl buffer was collected by centrifugation at 40 000 *g* for 30 min and suspended in 0.15 M NaCl, 50 mM Tris-HCl, pH 7.4 and dialyzed against the same buffer.

Thermal stability of α1(II) and α1(X) collagens from normal growth plate and TD cartilage organ cultures

The thermal stability of the newly synthesized collagens was determined by brief protease digestions at increasing temperatures with a combination of trypsin and chymotrypsin as described previously [29]. The 1.0 M NaCl extracts from the organ cultures of normal growth plate and TD cartilage were preincubated at the specified temperature for 10 min. The digestion was carried at the same temperature for 2 min. For the digestion, a 0.1 volume of 0.15 M NaCl, 50 mM Tris-HCl buffer, pH 7.4 containing 1 mg/ml trypsin and 2.5 mg/ml chymotrypsin was added. The digestion was stopped by adding a 0.1 volume of 5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co. St. Louis, MO, U.S.A.), and aliquots of each sample were analyzed by SDS-PAGE and fluorography. The thermal stability of the collagens was calculated as described [29].

Thermal denaturation of vertebral collagenase-cleaved type X collagen from normal growth plate and TD cartilage organ cultures

For this purpose samples of purified Type X collagen from normal growth plate and TD cartilage were digested with vertebral collagenase. Collagenase was activated as described previously [30] and added to a final concentration of 4 μg of activated collagenase per 100 μl of sample and the digestion was performed at 18°C for 15 h. The reaction was stopped by addition of EDTA to a final concentration of 15 mM. To digest the collagenase-treated samples with a mixture of trypsin and chymotrypsin the samples were dialyzed against modified Krebs II buffer at 4°C for 15 h. The enzymatic digestion was carried out by adding a 1/10 volume of the modified Krebs buffer containing 1 mg/ml trypsin and 2.5 mg/ml chymotrypsin. Each sample was preincubated at the temperatures indicated for 10 min, and the digestion was carried out at the same temperature for 2 min. The digestion was stopped by adding 1/10 volume of 5 mg/ml soybean trypsin inhibitor (Sigma). Samples were analyzed on SDS-PAGE electrophoresis and fluorography.

Results

Protein and collagen biosynthesis by organ cultures of normal growth plate and TD cartilage

Table I shows the incorporation of ^{14}C-proline into total protein and collagen by organ cultures of normal growth plate and TD cartilage and the amounts present in each extractant. The results were normalized for the DNA content of each sample. TD cartilage synthesized 3.8-fold greater amounts of ^{14}C-collagen than normal growth plate cartilage. Approximately 10–15% of the newly synthesized collagen appeared in the culture media from the two tissues. Substantial differences were noticed in the relative proportion of collagen extracted with neutral salt and acetic acid solutions. In normal growth plate cartilage, 38% of the total collagen synthesized was extracted with these solutions. In contrast, neutral salt and acetic acid solubilized only 24% from the TD cartilage. Pepsin was ineffective in solubilizing newly synthesized collagen from either tissue since less than 6% of additional radiolabeled collagen was extracted. Subsequent extraction with DTT resulted in essentially complete solubilization of all remaining newly synthesized collagen. The DTT

Table I
Total protein and collagen biosynthesized by organ cultures of normal growth plate and tibial dyschondroplasia (TD) cartilages

	Normal		TD	
	Total protein	Collagen	Total protein	Collagen
	(dpm×10^{-3}/μg DNA)		(dpm×10^{-3}/μg DNA)	
Total	25.7 ± 0.21	17.5 ± 1.6	90.3 ± 0.8	65.7 ± 1.5
Medium	6.4 ± 0.4	2.6 ± 0.2	12.8 ± 0.07	4.4 ± 0.1
Extracts				
1.0 M NaCl + 0.5 M acetic acid	8.8 ± 2.1	6.0 ± 1.7	24.2 ± 0.2	16.0 ± 1.0
Pepsin	0.52 ± 0.1	0.35 ± 0.05	3.6 ± 0.1	2.1 ± 0.2
DTT	9.0 ± 0.4	7.8 ± 0.07	46.0 ± 1.0	40.4 ± 2.0
Pepsin after DTT	0.13 ± 0.03	0.09 ± 0.01	1.7 ± 0.1	1.6 ± 0.2
Final residue	0.85 ± 0.07	0.65 ± 0.01	2.1 ± 0.07	1.2 ± 0.07

Organ cultures from normal growth plate and TD cartilage were minced and then labeled with 5.0 μCi [U-^{14}C]-proline for 24 h as described in Methods. After labeling, the tissues were homogenized in 1.0 M NaCl, 50 mM Tris-HCL, pH 7.4 buffer and sequentially extracted as previously described [4]. The extracts were dialyzed and aliquots taken for total radioactivity and collagen was determined by a collagenase assay as described by Peterkofsky and Diegelman [21]. Each value represents the mean ± S.D. of triplicate extractions. The collagenase assays of each extract were performed in duplicate. Aliquots of the homogenates were used for determination of DNA [19].

fraction represented approximately 42% of newly synthesized collagen from the normal growth plate collagen and 62% from the TD lesion. Very little additional radioactivity was solubilized with subsequent pepsin digestions. The entire procedure was extremely efficient in extracting all the newly synthesized collagen since the final residue contained less than 3% of newly synthesized collagen from either tissue.

SDS-PAGE of newly synthesized collagens from organ cultures of normal growth plate and TD cartilage

Figure 1 shows a representative fluorogram of the newly synthesized proteins present in the media, pooled neutral salt plus acetic acid, pepsin, DTT, and pepsin following DTT extracts, and the final residue of cartilage from the normal growth

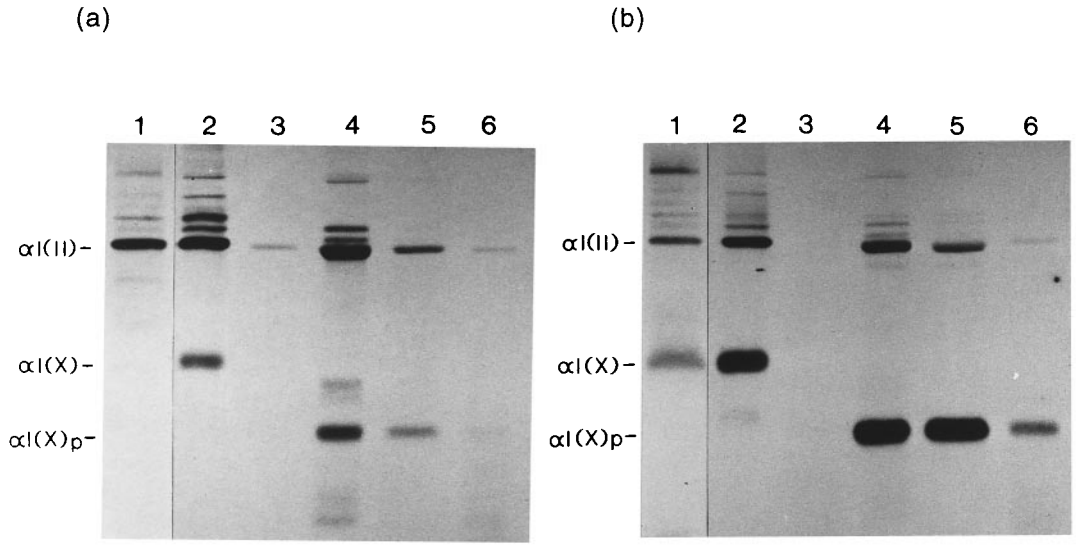

FIG. 1. SDS/PAGE fluorograph of newly synthesized collagens from (a) normal growth plate, and (b) tibial dyschondroplasia cartilage organ cultures. The tissues were labeled with ^{14}C-proline and extracted as described in Methods. The samples were processed and electrophoresed under reducing conditions on an 8% SDS-polyacrylamide gel and processed for fluorography. Lane 1, media; lane 2, pooled 1.0 M NaCl and acetic acid extracts; lane 3, pepsin extracts; lane 4, DTT extracts; lane 5, pepsin after DTT extracts; lane 6, final residues.

plate (a) and from TD (b) organ cultures. The media samples of the two tissues [Fig. 1(a) and (b): lane 1] showed a prominent band corresponding to α1(II) collagen and a faint 59 kDa band corresponding to intact type X collagen. In addition, several bands appeared above the α1(II) collagen chain which represent unprocessed and partially processed chains of α1(II) procollagen. The sample containing the pooled 1.0 M NaCl and acetic acid extracts [Fig. 1(a) and (b): lane 2] showed a prominent band corresponding to α1(II) collagen and several bands above it. Two of the bands migrating above the α1(II) chain correspond to the intact type XI collagen chains. A prominent single band was seen migrating with an apparent Mr of 59 000 in samples from the two tissues. This band corresponds to intact α1(X) collagen chains. The intensity of the band corresponding to α1(X) collagen chains was much higher in the extract from the TD lesion [Fig. 1(b): lane 2] compared with normal growth plate cartilage [Fig. 1(a): lane 2]. The electrophoretic pattern of the labeled proteins solubilized with pepsin showed a faint band corresponding to α1(II)p collagen chains found only in the normal growth plate cartilage [Fig. 1(a): lane 3]. Samples from the DTT extracts from the normal growth plate [Fig. 1(a): lane 4], and TD lesion [Fig. 1(b): lane 4] showed a band corresponding to the co-migrating α1(II)p and α3(XI)p collagen chains, and two bands migrating above corresponding to the α1(XI)p and the α2(XI)p of type XI collagen. Small amounts of α2(I)p collagen chain migrating immediately below the α1(II)p chain were present in the DTT extracts of the TD lesion [Fig. 1(b): lane 4]. Furthermore, a prominent 45 000 Mr band corresponding to pepsin cleaved type X collagen was observed in the two tissues. This band was much more intense in the DTT extract of the TD lesion than in the extract from normal growth plate. The normal growth plate showed, in addition to the type X collagen band, two fainter bands of Mr of 54 kDa and 36 kDa migrating above and below type X collagen which correspond to two chains of type IX collagen. These bands were not present in samples from the TD lesion. The pepsin after DTT extract of the two tissues contained bands corresponding to pepsin-cleaved type II and type X collagen chains (lanes 5). The α1(X) p chain band was several-fold more intense in TD than in normal growth plate cartilage. The final residue of the normal growth plate cartilage and the TD lesion contained faint bands of α1(II)p collagen and the pepsin- cleaved type X collagen (lanes 6).

Table II

Relative proportions of cartilage-specific collagens synthesized by organ cultures of normal growth plate and tibial dyschondroplasia (TD) cartilages

	Normal ($dpm \times 10^{-3}/\mu g$ DNA)		TD ($dpm \times 10^{-3}/\mu g$ DNA)	
Total	17.5		65.7	
Type I	ND	(0%)	1.3	(2.0%)
Type II	7.2	(40.6%)	18.3	(27.8%)
Type IX	1.3	(7.9%)	ND	(0%)
Type X	3.3	(19.0%)	41.7	(63.5%)
Type XI	5.7	(32.5%)	4.4	(6.7%)

The values shown were calculated from densitometric analyses of pepsin digested 1.0 M NaCl plus acetic acid pool, DTT, and pepsin after DTT extracts, and the final residue as described in Methods. The values in parenthesis are the percentage of each collagen chain from the total extracted collagen. ND = not detected.

RELATIVE PROPORTIONS OF COLLAGENS SYNTHESIZED IN NORMAL GROWTH PLATE AND TD CARTILAGE ORGAN CULTURES

Table II shows the relative proportions of cartilage-specific collagens produced by organ cultures of chick normal growth plate and TD lesion cartilage. The major collagen in the normal growth plate was type II collagen and its relative proportion was higher in the normal growth plate (40.6%) compared with the TD lesion cartilage (27.8%). Similarly, the proportion of type XI collagen was higher in the normal growth plate (32.5%) compared to the TD lesion cartilage (6.7%). In contrast, in the TD lesion, the major collagen was type X collagen. In the normal growth plate, type X collagen represented only 19.0% of the total collagen, whereas, it was more than threefold greater in the TD lesion (63.5%). The TD lesion contained a small amount of type I collagen (2.0%). Type IX collagen was only present in the normal growth plate and represented about 7.9% of the total collagen.

THERMAL STABILITY OF NEWLY SYNTHESIZED TYPE II AND TYPE X COLLAGENS FROM NORMAL GROWTH PLATE AND TD CARTILAGE

To define whether cartilage from the TD lesion synthesized structurally unstable type II or type X collagen molecules, the thermal stability of these collagens was examined by rapid proteolytic digestion with a mixture of trypsin and chymotrypsin at increasing temperatures [39]. Fig. 2 shows the SDS-PAGE electrophoretic patterns of proteolytically-cleaved collagens from the normal growth plate [Fig. 2(a)] and TD lesion [Fig. 2(b)]. The thermal denaturation of the two collagens was

calculated from the electrophoretic analysis of the samples [Fig. 2(c)]. There was no significant difference in the thermal stability of type II or X collagen synthesized by normal growth plate and the TD lesion. The thermal stability for type X collagen calculated from these studies was 43°C in

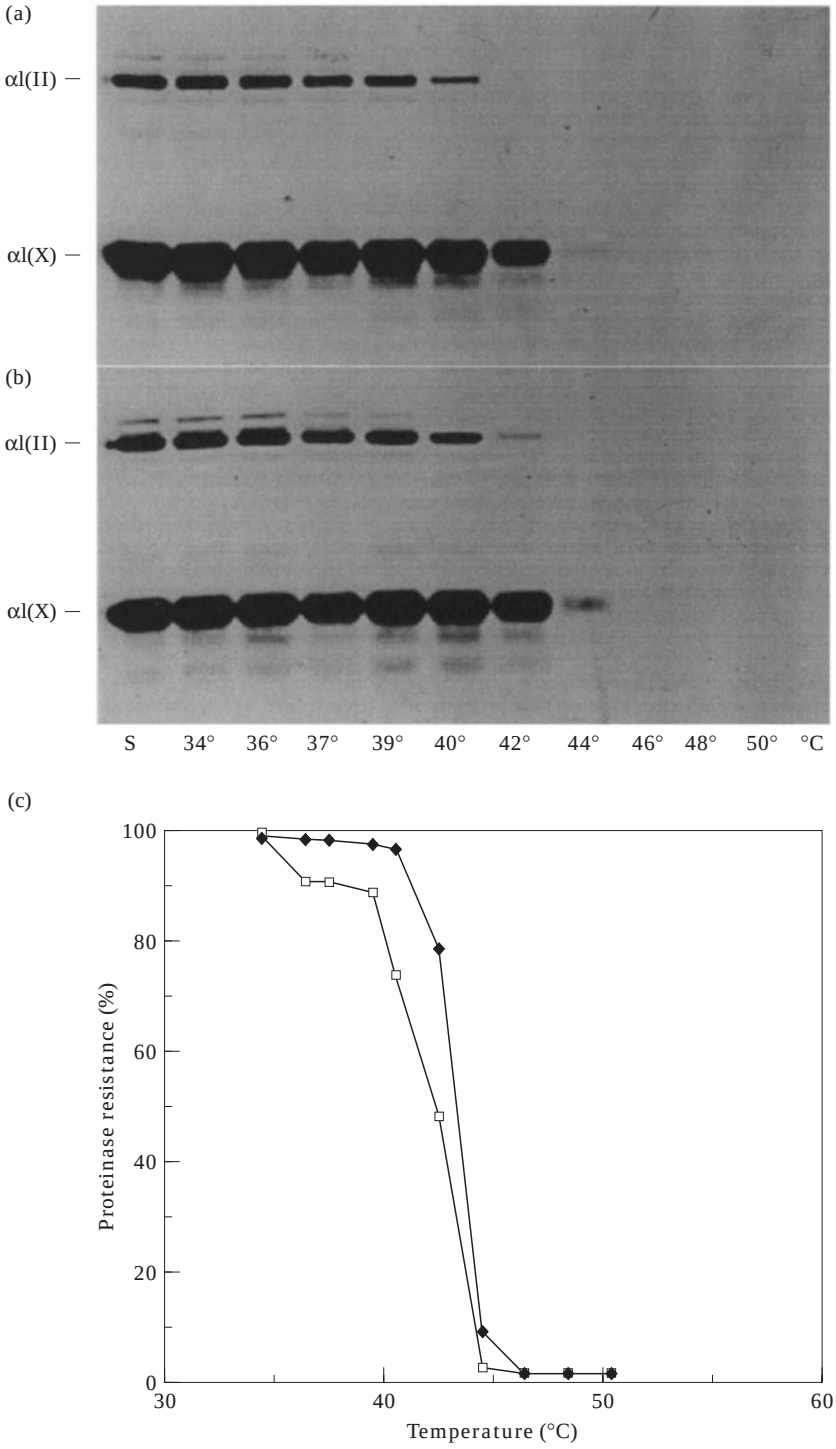

FIG. 2. Thermal stability to brief protease digestion of type II and type X collagen. The tissues were labeled with [^{14}C]-proline, and pooled 1.0 M NaCl and acetic acid extracts were digested with a mixture of trypsin and chymotrypsin at the temperatures indicated as described in Methods. The samples were then separated by SDS/PAGE on an 8% polyacrylamide gel sample. (a) normal growth plate, and (b) tibial dyschondroplasia cartilages. S denotes start of the reaction (c) thermal stability plot for type X collagen from normal growth plate (□) and tibial dyschondroplasia (■) organ cultures. The band corresponding to type X collagen was calculated from densitometric analysis and expressed as per cent proteinase resistant and ploted against each temperature.

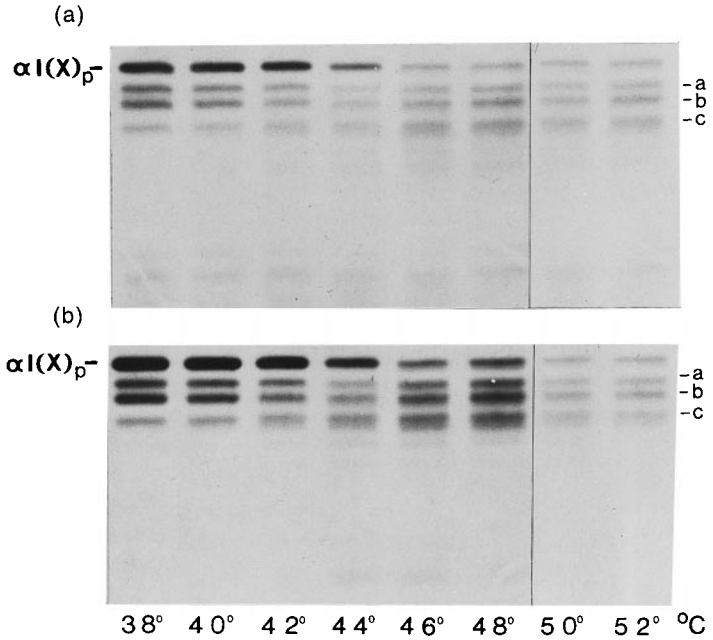

FIG. 3. Thermal stability of mammalian collagenase cleaved fragments of type X collagen. Type X collagen was fractionally precipitated under acetic conditions in 2.5 M NaCl and was digested with mammalian collagenase as described in Methods. The sample was treated with mixture of trypsin and chymotrypsin at the temperature indicated. The digests were subsequently separated by SDS/PAGE on a 10% polyacrylamide gel under reducing conditions and processed for fluorography. (a) Normal growth plate and (b) tibial dyschondroplasia organ cultures. a, b, c, α1(X)p collagen fragments.

normal growth plate cartilage and about 44°C in TD cartilage.

THERMAL STABILITY OF MAMMALIAN COLLAGENASE-CLEAVED FRAGMENTS OF TYPE X COLLAGEN FROM NORMAL GROWTH PLATE AND TD CARTILAGE

To further examine possible structural alterations in the molecular structure of type X collagen from the TD lesion, type X collagen synthesized by organ cultures of normal growth plate and TD lesion cartilage was purified by fractional NaCl precipitation and submitted to mammalian collagenase cleavage followed by determination of the thermal stability of the resulting fragments. As described previously [30–32] collagenase treatment of type X collagen resulted in three smaller fragments (a, b, and c in Fig. 3). The thermal stability of the collagenase fragments obtained with purified type X collagen from normal growth plate [Fig. 3(a)] and TD lesion [Fig. 3(b)] were essentially identical.

COLLAGEN BIOSYNTHESIS AND STEADY STATE mRNA LEVELS OF TYPES II AND X COLLAGEN IN PRIMARY CULTURES OF NORMAL AND TD CARTILAGE CHONDROCYTES

In order to confirm the differences in the proportion of type X collagen synthesized by normal growth plate and TD lesion cartilage organ cultures described above, and to examine whether these differences were reflected at the mRNA level, we established primary cultures of chondrocytes from normal growth plate cartilage and from TD cartilage. Collagen biosynthesis and determination of type II and type X collagen mRNA levels were examined in parallel in these samples. Total mRNA was prepared from the primary cultures and Northern hybridization with chicken cDNAs specific for types II and X collagens were performed. Fig. 4(a) shows the SDS-PAGE of newly synthesized collagens present in samples of pooled media plus cell extracts. Normal growth plate cartilage chondrocytes synthesized α1(II) and α1(X) collagen chains (lane 1). Lane 2 shows the biosynthetic products of chondrocytes from TD lesion. A marked decreased in the intensity of the band corresponding to type X collagen was observed in the samples from TD chondrocytes (lane 2). An additional band, below the α1(II) band corresponding to the α2(I) chain of type I collagen was observed [arrowhead in Fig. 4(a): lane 2] indicating that type I collagen was produced in the primary cultures of TD chondrocytes. Quantitation of the amounts of type X, type II, and type I collagens produced by normal growth plate and TD chondrocytes are shown in Fig. 4(b). The results demonstrate that the major biosynthetic product of

normal growth plate chondrocytes in primary culture is type X collagen representing 80% of the total collagen with no detectable type I collagen. In contrast, in samples from primary cultures of TD chondrocytes, type X collagen represented only 22% and type I collagen was the most predominant collagen species synthesized in these cultures representing 41% of the total collagen biosynthesized.

To determine whether these changes at the biosynthetic level corresponded to the steady state mRNA levels, Northern hybridization was performed in the same samples. Fig. 5 shows Northern blots of total RNA from normal growth plate and TD chondrocytes with type II and X collagens cDNAs. Northern hybridization revealed an elevated level of Type X mRNA in the sample from the TD lesion as compared to normal growth plate chondrocytes. The relative proportion of the steady state mRNA levels for type II and Type X collagen showed that type X collagen mRNA was 3.2-fold higher in the TD samples compared to samples from normal growth plate. The ratio Type X/Type II steady state mRNA levels was also greater in the TD lesion (3.4:1).

Discussion

The main objective of this study was to further examine by metabolic studies whether biosynthetic and structural alterations in the expression of type X collagen are present in TD cartilage. The biosynthetic studies of TD cartilage organ cultures performed here showed increased biosynthesis of Type X collagen compared to normal growth plate cartilage. These findings are in agreement with previous observations which showed twice the amount of type X collagen in TD when compared to normal growth plate cartilage from age matched chicks [15]. A simple explanation for the higher levels of type X collagen synthesis in the TD lesion explants is that the TD tissue contains essentially pure hypertrophic cartilage which although heterogeneous in their morphology synthesize large amounts of type X collagen, whereas, chondrocytes isolated from the normal growth plate contains cells in various stages of differentiation and the hypertrophic chondrocytes are accompanied by resting and proliferative chondrocytes. A similar increment in type X collagen in TD lesion has been reported by Wardale and Duance [18]. However, immunohistochemical studies using a specific type X collagen antibody showed a decrease in matrix associated type X collagen in TD tissues in contrast to normal growth plate which exhibit an abundant extracellular matrix is rich in Type X collagen [16, 21]. These studies further demonstrated that type X collagen was retained intracellularly in TD chondrocytes and accumulated within these cells [16, 21]. Since the matrix in the TD cartilage lesion fails to calcify and contains high amounts of type X collagen, the results of these studies indicate that the presence of type X collagen is not sufficient for initiation of

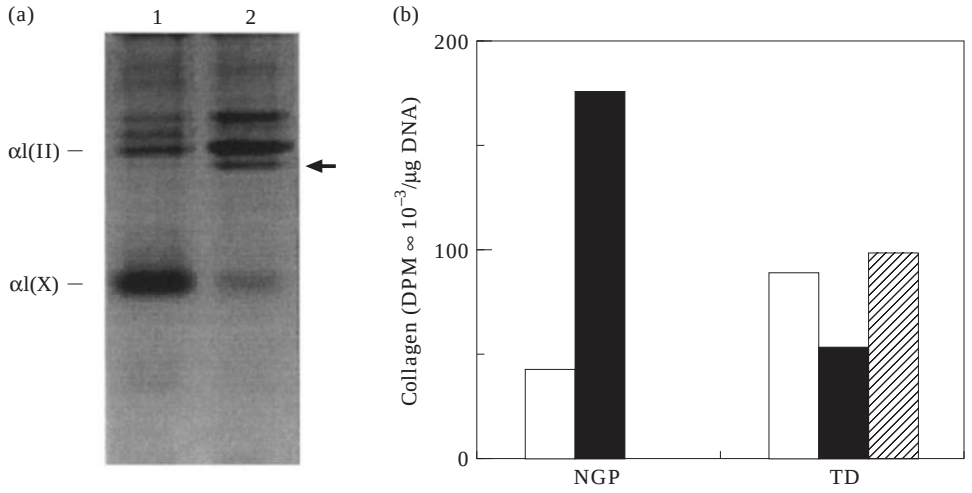

FIG. 4. SDS/PAGE fluorograph and quantitation of type I (▨), II (□) and X (■) collagens synthesized by chondrocytes in primary cultures. Chondrocytes were isolated from normal growth plate and tibial dyschondroplasia and plated in primary cultures for 5 days. The cultures were labeled with [^{14}C] proline for 24 h as described in Methods. After labeling the pooled media and cell extracts were electrophoresed in SDS-PAGE on an polyacrylamide gel and processed for fluorography. (a). SDS/PAGE. Lane 1: normal growth plate chondrocytes; Lane 2, tibial dyschondroplasia chondrocytes. Arrowhead shows the α2(I) collagen chain. (b) Quantitation of collagen types. The values for each collagen type were calculated from densitometric analysis of underexposed fluorographs and normalized for the DNA content of the samples. NGP: normal growth plate; TD: tibial dyschondroplasia.

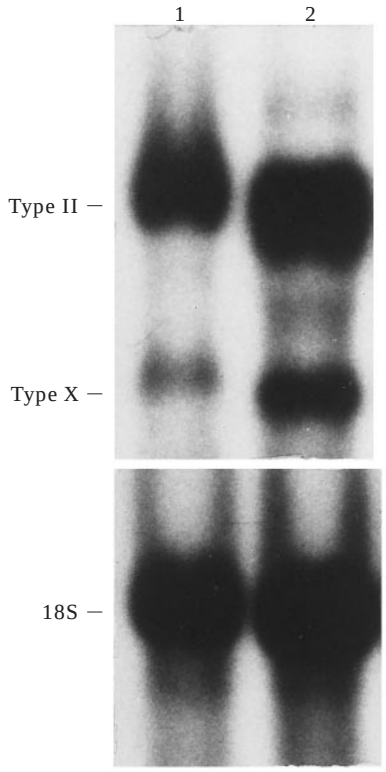

FIG. 5. Northern hybridization of types II and X collagen mRNAs from normal growth plate and tibial dyschondroplasia chondrocytes. The chondrocytes were maintained for 1 week in primary cultures and total RNA was isolated in a CsC1 discontinuous gradient. Ten micrograms of total RNA isolated from each sample was electrophoresed and blotted onto nitrocellulose as described in Methods and hybridized to chicken types II and X collagens and 18 S cDNA probes. Following flurography, the relative proportions of type II and type X collagen mRNA was calculated. Lane 1: normal growth plate chondrocytes; lane 2: tibial dyschondroplasia chondrocytes.

calcification. In order to determine whether the type X collagen synthesized in the TD lesion is structurally abnormal, we examined the thermal denaturation of purified types II and X collagen from TD cartilage organ cultures. The results showed no significant differences between the type X collagen synthesized by normal and TD cartilage. These findings confirm our previous data which showed that type X collagen in TD has a normal amino acid composition and cyanogen bromide peptide pattern [15].

Isolated chondrocytes from normal growth plate and TD lesion were established in primary culture in monolayers and were examined for their ability to acquire the morphologic and biosynthetic features and characteristic of hypertrophic chondrocytes. Normal growth plate chondrocytes cultured for 5 days retained a polygonal morphology and became hypertrophic. These cells also showed an increased synthesis of type X collagen which represented its main biosynthetic product. These findings are in agreement with prior studies showing that avian sternal chondrocytes [33] and bovine chondrocytes [34] are able to reach hypertrophy and produce type X collagen when maintained in monolayer cultures. However, the TD lesion chondrocytes failed to reach the high level of type X collagen synthesis observed with normal growth plate chondrocytes even though a similar total amount of total collagen was produced in chondrocytes from both tissues. These data suggest that in monolayer, chondrocytes cultured from the TD-lesion fail to reach and acquire the characteristics of hypertrophic chondrocytes [13, 15]. Indeed, these cells promptly initiated the expression of type I collagen, suggesting a more rapid tendency of the TD chondrocytes to become de-differentiated in culture and to express collagenous products that are characteristic of non-chondrogenic cell lineages. Similar phenotypic changes in the expression of types X and I collagen were observed by Wardale and Duance when TD lesions chondrocytes were grown as monolayer on plastic culture dishes [18].

When we compared the mRNA levels for type II and X collagen with the synthesis of the corresponding proteins, a discrepancy was noted as the mRNA level for type X collagen was 3.2-fold greater in TD chondrocytes despite the lower level of production of the corresponding protein. This finding may suggest that the low levels of type X collagen observed in TD chondrocytes in primary culture may be due to a defect in secretion, incorporation into the extracellular matrix or increased degradation of the protein. These findings confirm similar conclusions reached by other investigators [16, 18].

Chen *et al*. [16], using a monoclonal antibody directed against the NH_2-terminal-telopeptide of the α1(II) chain showed a strong reactivity within the non-hypertrophic regions of the growth plate cartilage, but reactivity was absent in the hypertrophic zone. The distribution of this antibody was abnormal in the TD-lesion and showed increased reactivity in the affected tissues. They also found decreased immunofluorescence for type X collagen within the lesion. These observations are similar to those in sections from embryos in which collagen cross-linking was inhibited by the lathyrogen β-APN, suggesting indirectly that TD cartilage exhibits an abnormal cross-linking. However, in the experiments reported here the TD organ and cell cultures were incubated in the presence of β-APN to inhibit cross-linking, thus, excluding a major participation of abnormal

cross-linking in the pathogenesis of the TD lesion. Direct analysis of collagen cross-links, however, showed a six- to 10-fold increment in lysyl derived cross-links in TD lesions [17, 18].

In summary, the data presented here and the results of our previous studies clearly show important abnormalities of type X collagen metabolism in avian TD. The amount and proportion of type X collagen is substantially increased in the lesion when compared with normal growth plate cartilage from age matched chicks. The increment in type X collagen in TD lesion is due to its increase biosynthesis as observed by organ culture studies presented here. Although the chondrocyte primary culture studies showed increased mRNA levels for type X collagen in TD, this elevation was not reflected at the protein level, which was substantially decreased compared to similar cultures of normal growth plate chondrocytes. These results suggest that establishment of TD chondrocytes in primary cultures uncovers a defect in either the secretion or incorporation of type X collagen into extracellular matrix. Moreover, the rapid initiation of expression of type I collagen by primary cultures of TD chondrocytes suggests that these cells have become de-differentiated to an earlier stage in their development and probably evolve from prehypertrophic cells that display a greater plasticity in their cellular phenotype. Clarification of the intimate mechanisms responsible for these abnormalities may provide valuable clues for the understanding of the structure and function of normal and diseased growth plate cartilage.

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