

Domains of Type X Collagen: Alteration of Cartilage Matrix by Fibril Association and Proteoglycan Accumulation

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Abstract. During endochondral bone formation, hypertrophic cartilage is replaced by bone or by a marrow cavity. The matrix of hypertrophic cartilage contains at least one tissue-specific component, type X collagen. Structurally type X collagen contains both a collagenous domain and a COOH-terminal non-collagenous one. However, the function(s) of this molecule have remained largely speculative. To examine the behavior and functions of type X collagen within hypertrophic cartilage, we (Chen, Q., E. Gibney, J. M. Fitch, C. Linsenmayer, T. M. Schmid, and T. F. Linsenmayer. 1990. *Proc. Natl. Acad. Sci. USA.* 87:8046–8050) recently devised an in vitro system in which exogenous type X collagen rapidly (15 min to several hours) moves into non-hypertrophic cartilage. There the molecule becomes associated with preexisting cartilage collagen fibrils. In the present investigation, we find that the isolated collagenous domain of type X collagen is sufficient for its association with fibrils. Furthermore, when non-hypertrophic cartilage is incubated for a

longer time (overnight) with “intact” type X collagen, the molecule is found both in the matrix and inside of the chondrocytes. The properties of the matrix of such type X collagen-infiltrated cartilage become altered. Such changes include: (a) antigenic masking of type X collagen by proteoglycans; (b) loss of the permissiveness for further infiltration by type X collagen; and (c) enhanced accumulation of proteoglycans. Some of these changes are dependent on the presence of the COOH-terminal non-collagenous domain of the molecule. In fact, the isolated collagenous domain of type X collagen appears to exert an opposite effect on proteoglycan accumulation, producing a net decrease in their accumulation, particularly of the light form(s) of proteoglycans. Certain of these matrix alterations are similar to ones that have been observed to occur in vivo. This suggests that within hypertrophic cartilage type X collagen has regulatory as well as structural functions, and that these functions are achieved specifically by its two different domains.

DURING endochondral bone formation, hypertrophic chondrocytes and their investing matrix are removed and replaced by a marrow cavity and bony tissue. Type X collagen, a hypertrophic cartilage-specific extracellular matrix molecule, is thought to be involved in these processes, but in what way remains unclear. Several possible roles have been suggested, but at present none seems entirely satisfactory. The molecule may facilitate matrix degradation. During development, soon after type X collagen is produced, the matrix into which it is deposited is degraded (9, 25). This may be related to the observation that in vitro, type X is much more readily cleaved by vertebrate collagenase than is type II collagen (29), the most prevalent type within cartilage. The presence of type X collagen, however, does not necessarily result in matrix removal; in mature organisms, a thin seam of permanent type X-containing matrix remains subjacent to the articular cartilages at the ends of long

bones. Another role proposed for the molecule is an involvement in calcification. Here again, definitive experiments are lacking, with some studies suggesting that the deposition of type X correlates with the initiation of calcification (30), whereas others have been interpreted as suggesting that it produces a focal inhibition of calcification (20).

The type X collagen molecule itself is about half the size of a “typical” fibril-forming collagen molecule (for review see reference 26). It is comprised largely of a 138-nm-long triple helical collagenous domain, with a large globular domain at its COOH-terminal end. The globular domain has certain characteristics reminiscent of the extensions found on the procollagen, precursor forms of some collagens. If the globular domain served solely as a procollagen extension, it would be removed soon after the molecule is secreted. Data suggest, however, that it is the intact form of type X collagen which is found within hypertrophic cartilage matrix (27, 32). Thus, the globular domain is likely to be functional.

Electron microscopic studies have suggested that the type X molecule occurs in at least two different forms in hypertrophic cartilage matrix. One form appears as mats of

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filamentous material which surround the chondrocytes, forming the innermost layer of the chondrocyte lacunae (28). In the interstitial matrix, type X is observed associated with the thin type II+IX+XI heterotypic collagen fibrils found throughout hyaline cartilage matrix (20, 28). This latter association, we proposed (28), most likely represents an interaction of newly secreted type X collagen with preexisting cartilage-collagen fibrils.

Such an interaction would require the movement of type X molecules through the cartilage matrix. Recently, we (2) tested the feasibility of this model by incubating the caudal, non-hypertrophic portion of embryonic sternal cartilage (which has no endogenous type X collagen) in medium containing type X. We then used immunofluorescence and immunoelectron microscopy to evaluate the behavior of the molecule. The results demonstrated that within minutes, type X collagen molecules can pass into such cartilage matrix; within 3 h the entire sternal piece can become infiltrated. By that time much of the type X has become associated with fibrils.

Using this same system, we now report that type X collagen can, in addition, become internalized within chondrocytes. It can also alter the properties of the matrix by regulating the accumulation of proteoglycans both quantitatively and qualitatively. The triple-helical and globular domains of the molecule seem to function differently in these processes.

Materials and Methods

Purification of Type X Collagen and Its Collagenous Domain

Type X collagen was isolated from the medium of aged cell cultures of hypertrophic tibiotarsal chick chondrocytes. The material was purified by fractional salt separation as previously described (24), followed by affinity purification (18) on columns of anti-type X mAb X-AC9 (25). For affinity purification, the type X collagen (in 1 M NaCl, 50 mM Tris-HCl, 1% Triton X-100, pH 7.5) was loaded onto a column of antibody X-AC9 coupled to Sepharose CL-4B. The column was extensively washed and then the type X collagen was eluted with 2 M guanidinium chloride. Such preparations, as analyzed by SDS-PAGE, consisted solely of the intact form of type X collagen. The triple helical, collagenous domain was produced by limited pepsin cleavage of type X, followed by antibody affinity chromatography on X-AC9 antibody columns.

In Vitro Culture of Cartilage

Sterna were removed from embryonic chicks (12 d of incubation) and dissected into HBSS. The perichondrium was stripped off and pieces from the caudal 1/2-1/3 of the sternum (the "permanent" cartilaginous region which does not synthesize type X collagen) were used.

For the experimental incubations, the medium consisted of DME supplemented with or without 10% FCS and exogenous collagen. The concentration of type X collagen or of the triple helical collagenous domain of type X collagen routinely used was 60 μ g/ml. Sternal pieces (three as a group) were added to the medium in 24-well tissue culture plates (Costar, Cambridge, MA). The plates were then incubated for various times at 37°C on a Nutator rocking table to ensure gentle mixing.

Standardization of Sternal Pieces

For experiments in which quantitation of radiolabeled material was determined (described below) groups of either three or four size-matched sternal pieces were used for each determination. To evaluate the precision with which size matching could be performed, the wet weights of 24 such sternal pieces were determined with an electrobalance (model 4400; Cahn Instruments, Inc., Cerritos, CA). The pieces had a mean weight of 4.48 mg with a SD of 0.38 mg or 8.5%.

Immunofluorescence Histochemistry

After culturing, the sternal pieces were quickly rinsed in PBS and fixed in 4% paraformaldehyde for 20 min. Further processing for cryostat sectioning and for the indirect immunofluorescence procedure with anti-collagen mAbs were as previously described (7).

Before reaction with antibody, some sections were digested with either 5 mg/ml testicular hyaluronidase or 5 U/ml chondroitinase ABC (Sigma Chemical Co., St. Louis, MO) in PBS for 30 min at 37°C.

Immunoelectron Microscopy

Immunoelectron microscopy using ultrathin cryostat sections was performed as recently described (8). Briefly, the incubated pieces of cartilage were fixed in paraformaldehyde-lysine-periodate fixative, infiltrated with sucrose at 4°C, mounted on stubs and frozen. 100-nm sections were cut with an ultracytome (FC4; Reichert Jung, Vienna, Austria), placed on formvar-coated grids and reacted with antibodies complexed to colloidal gold particles. Sections were viewed in an electron microscope (CM10; Philips Electronic Instruments, Inc., Mahwah, NJ). Stereo pairs were photographed at an angle of 40°.

Radioisotopic Analysis of Type X Collagen Movement

Radiolabeled type X collagen was purified from hypertrophic chondrocytes incubated in the presence of [³H]proline. For experimental incubations, 2,000 dpm of radiolabeled type X was added along with 60 μ g/ml of the unlabeled molecule. Then, at various time points, labeled sternal pieces (size-matched) were removed from the incubation medium, and the labeled tissues were quickly rinsed to remove surface-associated isotope. The sternal pieces were solubilized in a tissue solubilizer-450 (Beckman Instruments, Inc.) and counted in Ready Organic Liquid Scintillation Cocktail (Beckman Instruments, Inc.).

Proteoglycan Radiolabeling and Analysis

Size-matched sternal cartilage pieces were incubated for 15 h at 37°C in medium containing 50 μ Ci/ml H₂³⁵SO₄ (New England Nuclear, Boston, MA). Test cultures also contained either intact type X collagen or the pepsin-resistant collagenous domain of the molecule (60 μ g/ml). After culture, the radiolabeled proteoglycans were extracted (21). Tissues and medium were processed separately. The labeled cartilage pieces were washed three times in PBS and homogenized at 4°C in extraction buffer (4 M guanidine HCl, 50 mM sodium acetate, pH 5.8, 5 mM *N*-ethylmaleimide, 0.5 mM disodium ethylenediaminetetraacetate, and 0.5 mM PMSF). After overnight extraction, the extracts and media were separately dialyzed against Tris/HCl (pH 6.8) and 1 mM sodium sulfate. The radioactivity in an equal portion of each sample was determined.

The distribution of the different classes of proteoglycans in each sample was analyzed by agarose gel electrophoresis (19). Equal portions (10%) of each sample were heated in the presence of 0.25% SDS at 37°C for 2 h and then subjected to electrophoresis through a 1% agarose gel.

Results

Association of the Collagenous Domain of Type X Collagen with Cartilage Collagen Fibrils

Pieces of non-hypertrophic sternal cartilage from 12-d embryos were incubated in either control medium (Fig. 1, *A* and *D*), medium containing intact type X collagen (Fig. 1, *B* and *E*), or medium containing the collagenous domain of type X from which the COOH-globular domain had been removed by limited pepsin digestion (Fig. 1, *C* and *F*). The cartilages were then assayed by immunofluorescence histochemistry and immunoelectron microscopy. Consistent with our previous results (2), both the type X collagen and its isolated collagenous domain completely penetrated the cartilage within 3 h, as assayed by immunofluorescence histochemistry. Therefore, the ability of the molecule to move through cartilage lies within the collagenous domain.

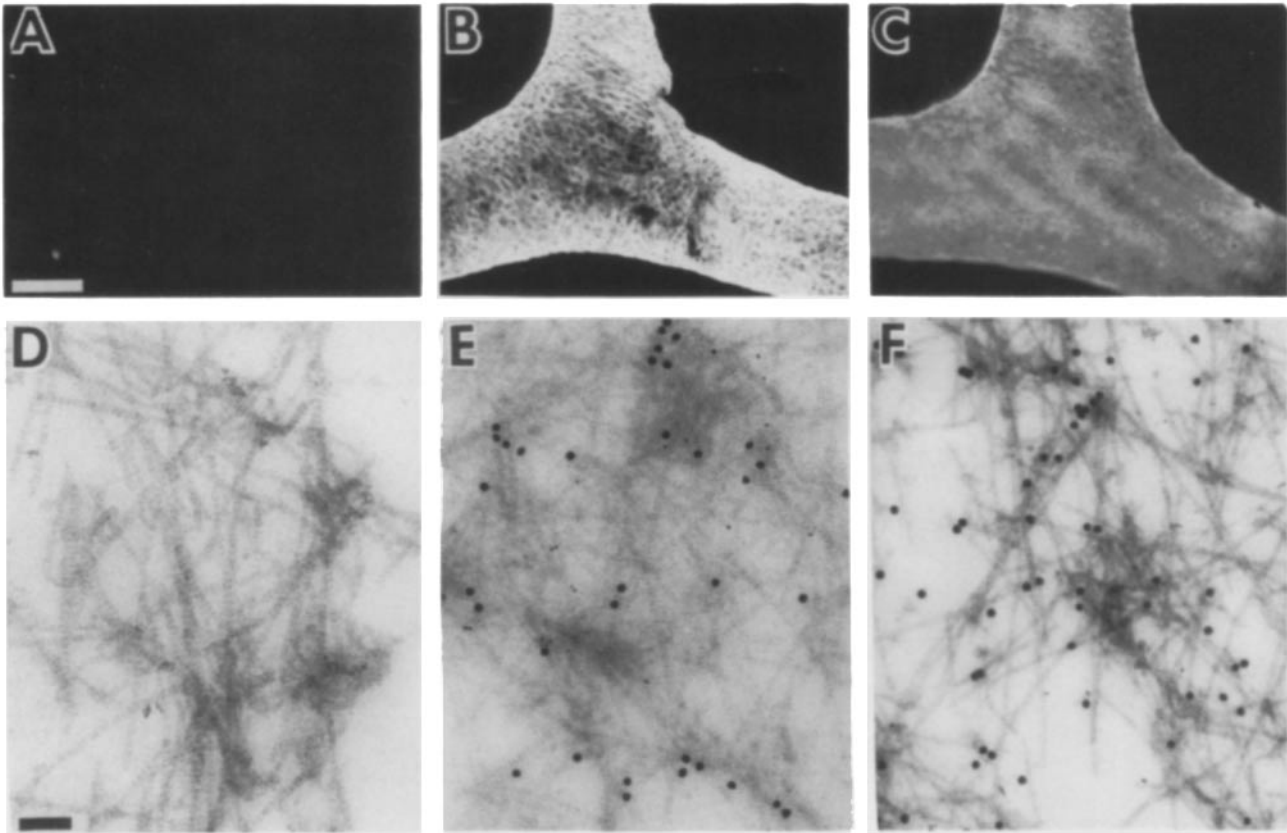


Figure 1. (A–C) Fluorescence micrographs of sections of sterna incubated for 3 h in control medium (A), medium containing type X collagen (B), or medium containing the collagenous domain of type X collagen (C). Sections were reacted with the monoclonal antibody for type X collagen and then with a rhodamine-conjugated secondary antibody. (D–F) Double-label immunoelectron micrographs of sternal cartilage that had been incubated for 3 h in medium containing type X collagen (D and E), or the collagenous domain of type X collagen (F). As a negative control, D was reacted with an anti-laminin mAb coupled to 15-nm gold and an anti-type IV collagen mAb coupled to 5-nm gold. E and F were reacted with the anti-type X collagen mAb coupled to 15-nm colloidal gold and an anti-type II collagen mAb coupled to 5-nm gold. Bars: (A–C) 100 μ m; (D–F) 100 nm.

When we examined the incubated cartilages by immunoelectron microscopy, again, as we previously reported, much of the intact type X collagen (Fig. 1 E, large gold particles) had become associated with the cartilage collagen fibrils, identifiable by the presence of type II collagen (small gold particles). In addition, we observed that the pepsin-derived collagenous domain of the molecule became associated with fibrils in a distribution indistinguishable from that of the intact molecule (Fig. 1 F). Thus, the information for type X to become associated with fibrils also resides within the collagenous domain.

Type X Collagen-mediated Alterations of Cartilage Matrix

When the incubation period for the sternal pieces was extended to overnight (15 h; Fig. 2), a difference was noted between sterna incubated with the intact type X molecule versus sterna incubated with the collagenous domain of type X. In sterna that had been incubated with the intact molecule (Fig. 2 A), the immunofluorescence for the type X molecule was substantially less than that seen at three hours (compare with Fig. 1 B). Throughout most of the sterna the fluorescence had decreased by 15 h to the point of being almost nonexistent, the exception being near the surface, where a

strong signal was still visible. In contrast, sterna incubated overnight with the isolated collagenous domain (Fig. 2 B) still retained much of the anti-type X fluorescence observed at 3 h of incubation (compare with Fig. 1 C).

We previously observed that the movement of type X collagen into cartilage does not require active cellular metabolism (2), since it occurs at 4°C and in sterna in which the cells have been killed by freeze-thawing. The diminution of the type X immunofluorescence during the overnight incubation, however, does seem to require active metabolism and also cellular secretion. Neither freeze-thawed sterna incubated overnight in type X-containing medium at 37°C nor living sterna incubated overnight in type X-containing medium at 4°C (Fig. 2 C) exhibit a decrease in the type X collagen immunofluorescence. Likewise, sterna incubated overnight in medium containing both type X collagen and monensin, an inhibitor of cellular secretion, show no decreased fluorescence signal (Fig. 2 D).

Another alteration in the sterna which occurred during the overnight incubation in intact type X collagen-containing medium was that the matrix could no longer be infiltrated by type X. When sterna were incubated overnight in type X and then transferred the next day to fresh type X-containing medium for an additional 3-h incubation, we observed little if any increase in the fluorescent signal for type X (Fig. 2 E).

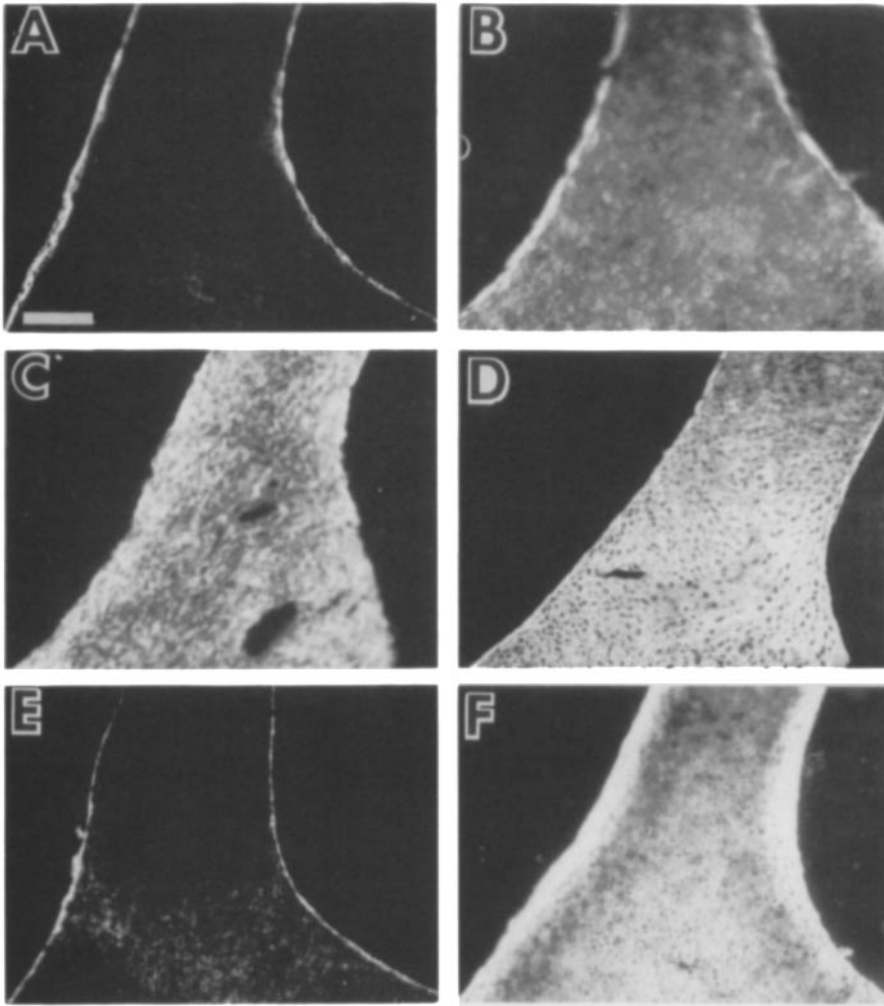


Figure 2. Fluorescence micrographs of sections of sterna incubated for 15 h at 37°C in medium containing intact type X collagen (A); the collagenous domain of type X collagen (B); at 4°C in medium containing intact type X (C); at 37°C in medium containing monensin and intact type X collagen (D); in intact type X containing medium for 15 h followed by an additional 3-h incubation in fresh type X-containing medium (E). None of these sections had been digested with hyaluronidase. F is the same as A but digested with hyaluronidase before reaction with antibody. Sections were reacted with the mAb for type X collagen and then with a rhodamine-conjugated secondary antibody. Bar, 100 μm .

This alteration in the cartilage matrix had been effected by the infiltrated type X collagen. Control sterna that had been incubated overnight in medium without type X and then transferred the next day to medium containing type X exhibited the normal inward movement of the molecule (not shown).

These observations were confirmed by similar experiments in which we assessed the ability of radiolabeled type X collagen to move into sterna which had been incubated overnight in medium with or without exogenous unlabeled type X. When such sterna were transferred the next day to medium containing ^3H -labeled type X, those which had been incubated in control medium showed appreciable uptake of the radiolabeled collagen (Fig. 3, —); those which had been incubated in type X collagen-containing medium showed only slight uptake (Fig. 3, ----).

Alterations in Matrix Proteoglycans

One possibility that would account for all of these observations would be the addition to the matrix of a chondrocyte-secreted component(s) that could antigenically mask epitopes on the type X collagen molecule and alter other properties of the matrix. Numerous studies have demonstrated that cartilage matrix proteoglycans can antigenically mask epitopes on collagens. To test whether this might be occurring in the sterna incubated overnight in type X collagen, sections of this tissue were predigested with either testicular hyaluronidase or chondroitinase ABC before reaction with the anti-type X antibody. Both treatments greatly increased the type X immunofluorescence (Fig. 2 F).

These results suggest that proteoglycan was interacting with the type X collagen, and that the COOH-terminal globular domain of the molecule is involved. The inhibition of masking by monensin suggests that it is the PG newly synthesized during the incubation period that interacts with type X collagen.

To test whether the exogenous type X was regulating the accumulation of newly synthesized proteoglycan(s) and to determine which domain of the molecule might be responsible for such activity, we examined the amounts and types of newly synthesized proteoglycans that accumulated within cultured sterna infused overnight with either intact type X or its collagenous domain. Synthesis was tested both in serum-containing medium, which produced an intrinsically high synthetic rate of the proteoglycans, and in serum-free medium, which supported a basal level of synthesis.

^{35}S sulfate incorporation into nondialyzable, macromolecular material was examined as an indicator of total proteoglycan accumulation since in cartilage $\sim 95\%$ of the sulfate is incorporated into proteoglycans (15, 16). Both the tissue-associated, 4 M guanidine-extractable fraction and the medium fraction were examined; under all experimental conditions, the great majority of the label was within the tissue fraction (Fig. 4). Only this fraction will be described. In

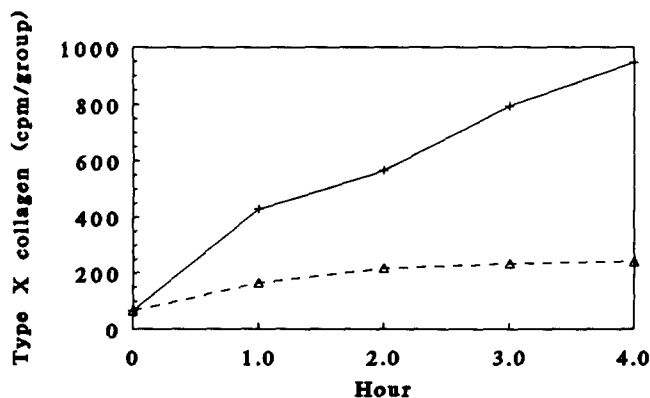


Figure 3. Uptake of ^3H -labeled type X collagen by a group of three sternal pieces that had been incubated for 15 h in type X-containing medium (---) or in control medium (—).

serum-free medium the intact type X molecule produced an 80% increase in [^{35}S]sulfate incorporation as compared to the control level (Fig. 4). In serum-containing medium, in which the basal incorporation was much greater, this increment was 20%.

When the collagenous domain of type X was tested, the results were much different. In serum-free medium the amount of labeled sulfate was almost identical to that of the control; in serum-containing medium it was 26% less than that of the control.

Cartilage contains at least two types of proteoglycans, a large one (designated PG-H), a small one (designated PG-L) (31), as well as type IX collagen, a collagen/proteoglycan. To determine which of these sulfate-containing macromolecules was being affected by type X collagen and its collagenous domain, we subjected equal proportions (10%) of each of the [^{35}S]sulfate-containing fractions to agarose gel electrophoreses, previously shown to separate these components (19). The serum-containing cultures were examined since these showed an increase with the intact type X and a decrease with the collagenous domain.

The data are shown in Fig. 5. In control cultures (lane A),

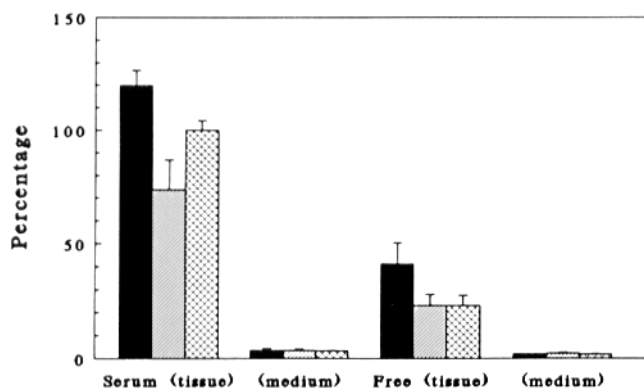


Figure 4. [^{35}S]sulfate accumulation by sternal pieces after 15-h incubation in [^{35}S]sulfate-containing control media (▨) or media containing 60 $\mu\text{g}/\text{ml}$ type X collagen (■) or with 60 $\mu\text{g}/\text{ml}$ of the collagenous domain of type X collagen (▩). Both serum-containing (serum) and serum-free (free) media were tested, as were tissue and medium fractions of each. All samples have been normalized to the samples in serum-containing control medium (designated as 100%). The vertical bars show 1 SD.

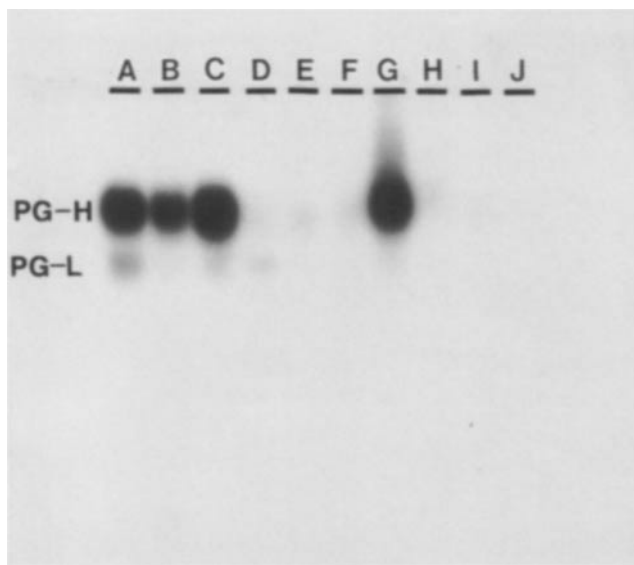


Figure 5. Autoradiogram of [^{35}S]sulfate-labeled macromolecules separated in a 1% agarose gel (typical representative of three separate experiments). (Lanes A-C) Tissue extracts of sterna incubated in control medium (A), or in medium containing 60 $\mu\text{g}/\text{ml}$ of the collagenous domain of type X (B), or 60 $\mu\text{g}/\text{ml}$ intact type X (C). (Lanes H-J) Tissue extracts (A-C), respectively, digested with hyaluronidase. (Lanes D-F) Media fractions in which sterna were incubated the same as A-C, respectively. (Lane G) Tissue extract (C) reduced with β -mercaptoethanol.

two bands are present, one corresponding to PG-H and the other to PG-L. When intact type X collagen was included in the cultures (lane C), the net increase in ^{35}S incorporation noted earlier was found to be due to PG-H, whereas PG-L was actually decreased. The net decrease in ^{35}S incorporation observed when the collagenous domain of type X was included in the cultures was found to be due to decreases in both proteoglycan species, with the PG-L being almost completely eliminated (lane B). As expected, the medium fractions of the cultures (lanes D-F) contained little material. Hyaluronidase treatment of cultures (lanes D-F) contained little material. Hyaluronidase treatment of lanes A-C eliminated the labeled materials (lanes H-J), consistent with the great majority of the ^{35}S label being incorporated into proteoglycan. Reduction with β -mercaptoethanol (lane G) did not alter the migration of either labeled component, suggesting that neither labeled component was type IX collagen, the mobility of which is increased upon reduction (1, 19).

Chondrocyte Internalization of Type X

These results suggest that the domains of type X collagen differentially affect proteoglycan accumulation. How this occurs is not yet known, but an additional observation may be related to this phenomenon. During the overnight incubation, some of the exogenously added intact type X collagen is internalized by the chondrocytes. This has been demonstrated by both immunofluorescence histochemistry (Fig. 6, insert) and immunoelectron microscopy (Fig. 6). In the immunofluorescence insert, to emphasize the punctate intracellular pattern of antibody reaction for type X collagen (arrows), the tissue section was not treated by hyaluronidase, so

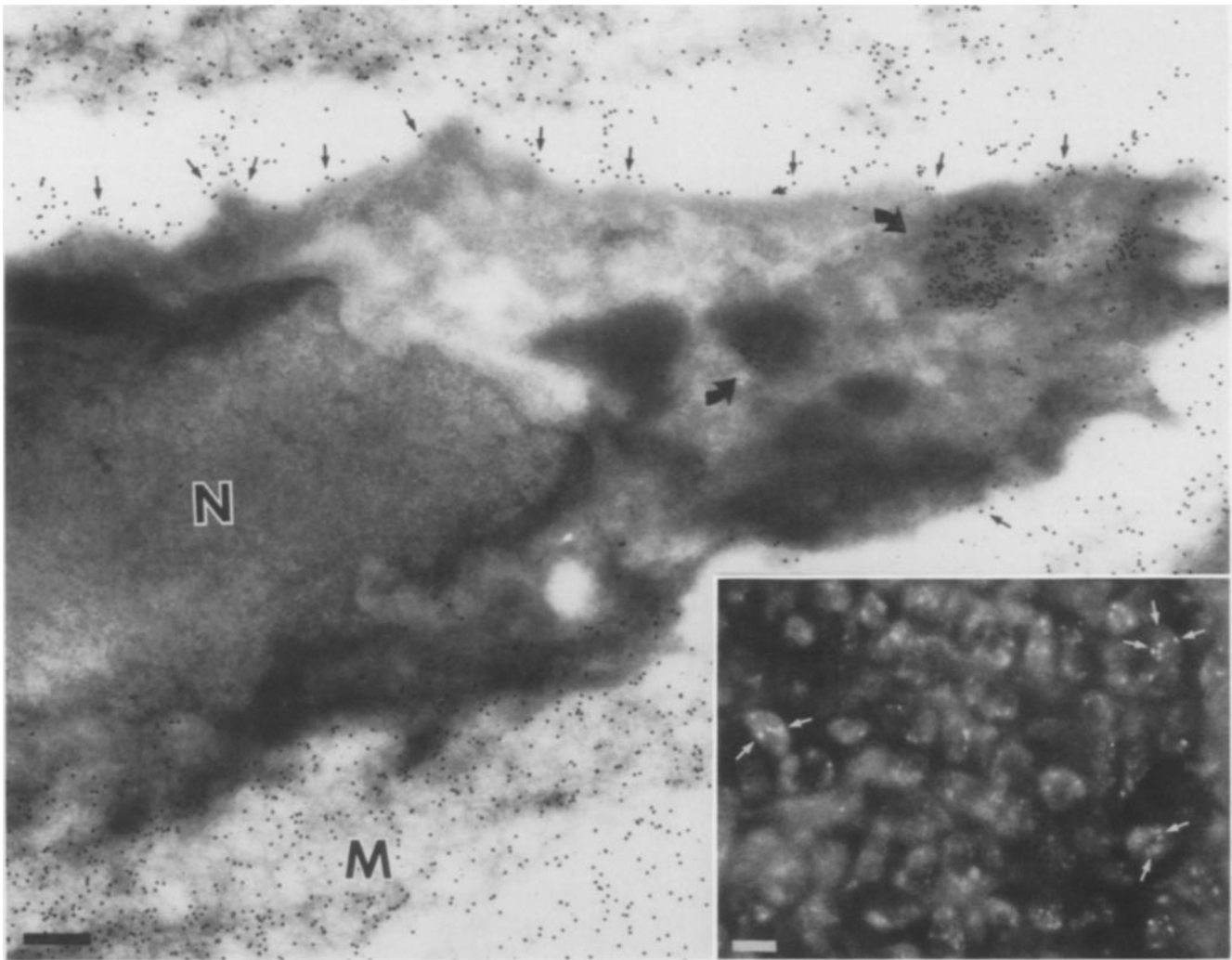


Figure 6. Immunoelectron micrograph of a chondrocyte and surrounding area from a sternal piece that had been incubated in type X-containing medium for 15 h. The section was digested with hyaluronidase and reacted with an anti-type X collagen mAb 15-nm gold complex. The small arrows designate type X collagen associated with the cell surface; the large arrows type X collagen-containing intracellular vacuoles. *N*, nucleus; *M*, cartilage matrix. (*Inset*) Fluorescence micrograph of a section of sternum that had been incubated in type X collagen-containing medium for 15 h. Section is reacted with anti-type X collagen mAb, but without hyaluronidase treatment. (*White arrows*, intracellular inclusions of type X collagen). Bars: 225 nm; (*Inset*) 4.5 μ m.

the matrix-associated type X has remained largely masked by proteoglycan.

Immunoelectron microscopy of ultrathin cryostat sections showed that some type X was closely associated with the cell surface and some was within what appear to be intracellular vacuoles (arrows). That this type X is truly intracellular was verified by visualizing sections in stereo pairs (not shown). Few of such internalization was observed in sterna incubated with the isolated collagenous domain of type X (but see Discussion). To demonstrate that the intracellular type X was truly endocytosed, and was not a result of de novo synthesis by these sternal chondrocytes, we looked for type X collagen mRNA by in situ hybridization (17). In the in vitro-incubated cartilages no mRNA for type X collagen was detected; in control hypertrophic cartilage taken directly from embryos, an intense signal for the mRNA was observed (data not shown).

Discussion

Using the same in vitro model system employed here, we previously reported a rapid and extensive movement of type X collagen through cartilage matrix. We also observed that the triple helical domain of the molecule is sufficient for this movement. In vivo it is unnecessary and unlikely that the extent of movement of type X molecules approaches that which we observe in this in vitro model system (discussed later). This model does, however, seem to mimic and offer explanations for a number of changes that occur during cartilage hypertrophy in vivo, thus facilitating studies of the mechanisms involved.

Using this system, we (2) previously observed that the fibril-associated form of type X collagen seen within hypertrophic cartilage in vivo (20, 28) could result from the association of type X with preexisting cartilage collagen fibrils. We have now observed other type X-mediated modifications, in-

cluding alterations in the accumulation of proteoglycans, alterations in the physical properties of the cartilage matrix with respect to type X collagen movement, and uptake of the molecule by the chondrocytes. Certain of these changes mirror the events that occur *in vivo* during the formation of hypertrophic matrix, and they appear to be regulated by the different domains of the type X molecule.

Fibril Association

We previously observed that the movement of type X through the matrix is a property conferred upon the molecule by its triple helical domain. As shown in the present study, this domain also seems to be sufficient for association of the molecule with the type II+IX+XI collagen fibrils already present within the matrix. This result is consistent with the proposal (28) that within hypertrophic cartilage *in vivo*, one of the fates of the newly synthesized type X is to become associated with preexisting fibrils assembled at an earlier stage of cartilage development. This association may alter such properties of the fibrils as their susceptibility to degradation, their ability to participate in calcification, and their interactions with proteoglycans (discussed later).

At least one fibril-associated collagen, type IX, is known to align along fibrils in such a way that it is in register with the underlying type II collagen molecules comprising most of the fibril. It is likely that type X collagen becomes similarly arranged. Some evidence exists that type X molecules become covalently bound to fibrils via lysine-derived cross-linking (3, 22, 24), and the formation of such crosslinks requires specific alignment of the molecules involved. Possibly the other fibril-associated collagens, such as the types IX (33), XII (5, 10, 14), and XIV (6, 14), can move through their respective matrices. If so, modification of preexisting fibrils by the addition of newly synthesized fibril-associated collagens may prove to be a common mechanism by which the properties of such fibrils become altered during development.

Alterations in Matrix Proteoglycans

Another response of cartilage to the presence of type X collagen is an alteration in the matrix proteoglycans, resulting in both antigenic masking of the type X molecules within the matrix and changes in the proteoglycans. These alterations require *de novo* cellular synthesis and secretion, and differ depending on which domain(s) of type X collagen are present.

The most dramatic proteoglycan-mediated change in the immunohistochemistry of the matrix is the antigenic masking of the type X collagen itself. This is most obvious when the intact type X molecule is tested, implicating the COOH-terminal globular domain in one or more of the processes that may be responsible for the masking.

One way in which the COOH-terminal domain may act is by promoting the binding of newly synthesized proteoglycan(s) to the type X collagen molecule. Although the epitope against which our anti-type X collagen antibody is directed is within the triple helical domain, it is located only 19 nm from the COOH-terminal domain (29), a distance easily spanned by individual PG-H monomers (11, 23), let alone the supramolecular PG-H aggregate form of the molecule which is many times larger. More work, however, will be re-

quired to determine precisely the interactions between type X collagen and proteoglycan, and to elucidate the involvement of the COOH-terminal domain.

Our data do, however, directly demonstrate a differential effect of type X collagen and its COOH-terminal domain on the accumulation of newly synthesized proteoglycans within the matrix. The intact type X molecule produced a net increase in proteoglycan accumulation, due to increased PG-H. PG-L was actually decreased. In contrast, the isolated collagenous domain produced a decrease in both proteoglycan species, with the PG-L being almost completely absent. Thus, it seems that the COOH-terminal domain enhances the accumulation of PG-H, whereas the collagenous domain inhibits the accumulation of PG-L.

We do not yet know whether these net effects on proteoglycan accumulation involve changes in synthesis, which would indicate an involvement of cellular receptors (discussed below), changes in degradation, or a combination of the two. The changes do, however, seem to mimic certain ones observed during the formation of hypertrophic cartilage *in vivo*, and they may produce physiological differences within the growth plate.

One such correlation is the distribution of the light form proteoglycan in the epiphyseal growth region. Shinomura et al. (31) using immunohistochemistry with antibodies specific for the different forms of proteoglycan, observed that one light form of proteoglycans, PG-Lb, distributed throughout the cartilage of the epiphyseal growth region except for the hypertrophic zone. PG-H, however, was present throughout both nonhypertrophic and hypertrophic cartilage. This absence of PG-Lb as well as the continuing presence of PG-H in the hypertrophic cartilage are consistent with the regulatory effects we observed for type X collagen and its collagenous domain. *In vivo*, however, it is unlikely that the entire collagenous domain (45-kD form) of type X molecule acts in regulation, since it is unlikely that this form of the molecule is present within cartilage matrix (27, 32). More likely, the activity resides in a collagenous cleavage product of the molecule. We (29) previously observed that the type X molecule is readily cleaved by vertebrate collagenase, an enzyme found within the hypertrophic zone of the growth plate (4). The major product of this cleavage is a triple helical fragment comprising approximately three-quarters of the collagenous domain of the molecule. This fragment can be excised *en bloc* from the central portion of the molecule and has sufficient thermal stability to maintain the triple helical conformation. It could perform *in vivo* as the collagenous domain does *in vitro*.

Alterations in Type X Movement into the Matrix

After exposure to the intact type X molecule, the sternal cartilage matrix no longer allows further infiltration of the molecule. This suggests a dramatic decrease, or even a cessation, of type X movement within the matrix. We do not yet know if this alteration in the physical properties of the matrix is related to the concomitant increase observed in the proteoglycan content of the matrix, but it does provide an explanation for the restricted localization of type X to the hypertrophic matrix. *In vivo*, if the *de novo* secreted type X effects a rapid change in the physical properties of the matrix surrounding individual chondrocytes, the extent of movement of the molecule would be much more limited than is observed

in our in vitro model in which a large amount of type X is rapidly made available to the tissue. This could explain why, in vivo, type X remains localized within the hypertrophic zone of the growth plate.

Internalization of Type X by Chondrocytes

We observed that some of the exogenous intact type X collagen becomes internalized by the chondrocytes. Whether this uptake is mediated by a specific receptor remains to be tested; however, one observation suggests that this may be the case. By immunoelectron microscopy, we observed internalization of the intact type X molecule, but few if any of the isolated collagenous domain. Although this suggests a specific involvement of the globular domain, negative results such as this must be interpreted with caution.

Internalization of the type X collagen may be required for the regulation of proteoglycan. If so, the apparent differences in internalization observed between the intact type X molecule and its collagenous domain might reflect the different responses these domains elicit in the behavior of proteoglycans. In addition, the internalization may indicate a feedback mechanism responsible for regulating the synthesis of the type X collagen itself (12, 13), or it may simply indicate a method of degrading excess type X. Which, if any, of these possibilities is correct, remains to be tested.

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