REVIEW ARTICLE

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The role of type X collagen in facilitating and regulating endochondral ossification of articular cartilage

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Structured Abstract

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Objective – This review was compiled to explore the role of type X collagen in growth, development and remodeling of articular cartilage by elucidating the linkage between the synthesis of this protein and the phenotypic changes in chondrogenesis and the onset of endochondral ossification.

Design – The current studies closely dedicated to elucidating the role of type X collagen incorporating into chondrogenesis and endochondral ossification of articular cartilage were assessed and analyzed to allow for obtaining the mainstream consensus on the bio-molecular mechanism with which type X collagen functions in articular cartilage.

Results – There are spatial and temporal correlations between synthesis of type X collagen and occurrence of endochondral ossification. The expression of type X collagen is confined within hypertrophic condrocytes and precedes the embark of endochondral bone formation. Type X collagen facilitates endochondral ossification by regulating matrix mineralization and compartmentalizing matrix components.

Conclusion – Type X collagen is a reliable marker for new bone formation in articular cartilage. The future clinical application of this collagen in inducing or mediating endochondral ossification is perceived, e.g. the fracture healing of synovial joints and adaptive remodeling of madibular condyle.

Key words: type X collagen; hypertrophy chondrocytes; endochondral ossification

Introduction

Type X collagen has been much of interest of many studies and researches because of its specific distribution in articular cartilage and its integral association with endochondral ossification. The clarification of the mechanism might help further justify its clinical application in detecting and mediating endochondral bone formation.

Collagen forms extracellular framework of cartilage and underlies tensile strength. At least 14 different types of collagen molecules, based on at least 25 different gene products, make up the collagen superfamily of extracellular matrix proteins. Each of these collagen types exhibits a degree of tissue specificity. The collagenous matrix of hyaline cartilage displays a rich assortment of collagen molecules, several of which are specific to cartilaginous tissues (1). Articular cartilage is a morphologically and biochemically heterogeneous tissue. The two major constituents of articular cartilage are collagen and proteoglycan, with collagen type II and aggrecan being the most abundant (2,3). Proteoglycans withstand compressive loading and collagen provides the tissue with strength to withstand tensional forces. These mechanical properties can largely be ascribed to the major molecular species of aggrecan and collagen type II, respectively. What is less well understood are the contributions of the minor collagen and proteoglycan species beyond their role in matrix integration and organization. It is evident, however, that some of these minor species form distinct spatial patterns within the tissue which can vary during development, growth, aging and the onset of degenerative disease (4–6).

Type X collagen is a member of the family of short-chain collagens. It consists of a homotrimer of three α1 (X) chains, with a short (38 aa) non-helical amino terminus (NC2), a triple helix of 463 aa and a COOH-terminal highly conserved non-collagenous domain (NC1) of 161 aa. Supramolecular aggregates of type X collagen formats in the territorial matrix of hypertrophic chondrocytes *in vivo* (7), and like type VIII collagen, type X collagen trimers assemble into hexagonal matrices *in vitro* (8). In contrast to the genes coding for fibrillar collagens (e.g. collagen types I, II, III, V and XI) which contain multiple small exons encoding long polypeptide chains, genes of the short chain collagen family (collagen types X and VIII) characteristically contain a large exon encoding almost the entire polypeptide chain (9,10). The comparison of the combined nucleotide and deduced amino acid sequences of mouse col10α1 with those of chicken, bovine and human collagen X genes showed a high degree of similarity, indicating conservation of this gene throughout evolution (9,11).

Type X collagen is specific to cartilage and is developmentally regulated. It is synthesized by terminally differentiating chondrocytes, i.e. hypertrophic chondrocytes (4). In the hypertrophic region of the epiphyseal plate in the maturing mammal, up to 18% of collagen synthesis may be type X collagen. The biological function of type X collagen is thought to facilitate the process of calcification possibly through matrical organization changes (8). The expression of type X collagen is restricted in the matrix of hypertrophic chondrocytes, suggesting that it is of major importance in endochondral bone growth and development. There has been much discussion of the role of type X collagen in matrix mineralization and endochondral ossification, together with the expectation that mutations in type X collagen may be responsible for some chondrodysplasias in humans and mice (5,7,12). Type X collagen serves as a marker of the terminally differentiated (hypertrophic) chondrocyte phenotype, and detection of the type X collagen gene transcript and translation product are useful for studies of chondrocyte growth and differentiation (13).

The specific location of type X collagen in articular cartilage

During the phenotypic conversions of articular chondrocytes from resting to proliferating and to hypertrophic cells, there are concomitant changes in the synthesis and deposition of new extracellular matrix components. In the proliferative zone, chondrocytes actively synthesize new matrix by secreting a mixture of types II, IX, and XI collagen and proteoglycans. In the hypertrophic zone, the chondrocytes become enlarged and begin synthesizing type X collagen, which is one of their major biosynthetic products (4,14,15). Type X collagen constitutes 45% of the total collagen produced by mature hypertrophic chondrocytes, and therefore is a major secreted protein product of this cell type. Examination of immunostained hypertrophic cartilage

revealed that type X collagen was localized in the capsule or membrane-like configuration around each hypertrophic chondrocyte (16). The expression of type X collagen has been considered a characteristic feature of hypertrophic chondrocytes and the transcriptional regulation of this gene has been thought to be dependent on cellular hypertrophy (17). It is also found in the extracellular matrix in the vicinity of collagen II-containing fibrils (7,12). The hypertrophic chondrocyte-specific transcriptional activation of the collagen X gene is accompanied by decreases in the levels of both collagen IX mRNA and collagen II Mrna (16).

The mechanism of type X collagen's specific location in hypertrophic chondrocytes has been mapped out by determining which portions of the gene are involved in hypertrophic chondrocyte-specific promoter activity (18). The results demonstrate that the 4200-bp 5′ flanking fragment (fragment ABC) of the chicken collagen X gene limits CAT activity to mature hypertrophic chondrocytes. These data strongly suggest that multiple, diffuse upstream negative regulatory elements in the type X collagen gene act in an additive manner to restrict transcription of the collagen X gene to hypertrophic chondrocytes (18).

Type X collagen and endochondral ossification

Endochondral ossification is the major process leading to the formation and growth of most of the skeleton in vertebrates during skeletogenesis. This process involves gradual replacement of the primordial cartilaginous model by bony tissue through temporally and spatially coordinated events of chondrocyte proliferation, maturation, and hypertrophy. During the final stages of this process, calcification of hypertrophic cartilage occurs, the vasculature invades, and bone matrix is deposited, replacing the cartilaginous matrix at the chondro-osseous junction of the growth plate (19–21). The cartilaginous chondrocytes are packed in form of zonation which reflects the progressive stages from chondrogenesis to endochondral ossification. The resting zone consists of small, spherical cells. These cells give rise to proliferating chondrocytes which appear as flattened cells within the proliferating zone. These chondrocytes become round and enlarge into hypertrophic chondrocytes within the zone of maturation. The cells finally enter the upper and lower hypertrophic zones, the latter being distinguished from the former by the presence of calcifying zones of cartilage (22,23).

The invariable distribution of type X collagen in hypertrophic zone of cartilage indicates that this particular collagen type could play an important role in endochondral ossification. Grant *et al.* (24) studied type X collagen expression in experimental fractures created in the chicken humerus. Biosynthetic analysis using [^{14}C] proline incorporation demonstrated that type X collagen synthesis occurred during endochondral ossification in the fracture callus. Type X collagen synthesis occurred in the areas of cartilaginous callus composed of hypertrophic and degenerative chondrocytes that are associated with increased vascularity and matrix mineralization. Synthesis of this collagen type was not detected in either skeletal muscle or bone. The synthesis of type X collagen by fracture callus is further evidence supporting its close association with the process of endochondral ossification. To confirm its involvement in the events of endochondral bone formation, Hoyland *et al.* (25) observed type X collagen mRNA expression in normal and osteoarthritic human cartilage. Osteoarthritis (OA) is a disorder characterized by new endochondral bone formation. A 700-bp restriction fragment encoding most of the C-terminal non-collagenous domain and part of the 3′ untranslated region of the human collagen X gene has been used for *in situ* hybridization studies on human OA joints removed from hip and knee replacement operations. The results were compared with immunohistochemical localization of type X collagen gene product. Type X collagen gene expression was detected in chondrocytes present in OA tissue in areas where there appeared to be a re-initiation of the endochondral bone formation (25). In an effort to clarify the temporal relation between type X collagen expression and endochondral ossification, the pattern of stage-specific expression of the mouse α1 (X) gene was preliminarily assessed by reverse-transcription polymerase chain reaction assays (RT-PCR) using total RNA from 10.5, 11.5, 12.5, 13.5, 16.5 and 18.5 day old mouse fetuses, and from a 0.5 day-old mouse neonate. RT-PCR assays indicate that the mouse collagen X gene is first expressed at 13.5 days post-coitum, temporally preceding the onset of endochondral ossification (26).

The temporal correlation between synthesis of type X collagen and initiation of endochondral ossification

was further confirmed by Rabie *et al.* In their study, mandibular condyles of SD rats were stretched from the fossae by using functional appliances. The peak of type X collagen mRNA and molecular expression in hypertrophic chondocytes of condylar cartilage occurred before the peak of new bone formation in the erosive cartilage (27,28). These finding are fully supportive to the statement that type X collagen expression is closely associated with endochondral ossification and invariably precedes the onset of ossification (9,29,30). Further evidence for the association of type X collagen with endochondral ossification was demonstrated by Chung *et al.* (17), who investigated type X collagen expression in developing rat Meckel's cartilage. *In situ* hybridization of newborn rat condylar and angular cartilages undergoing endochondral ossification showed restricted labeling with the α1(X) collagen probe in the hypertrophic chondrocyte layer. In contrast, the α1(X) collagen probe totally failed to label the major distal portion Meckel's cartilage even in the hypertrophic cartilage zone. lmmunohistochemistry using the anti-type X collagen mono-specific antibody consistently failed to recognize the epitope in the corresponding portion of Meckel's cartilage throughout the experimental periods of gestational day 17, newborn, and postnatal day 7, while the strictly localized positive staining was found in the posterior part of Meckel's cartilage which gave rise to malleus and incus.

Type X collagen expression in mandibular condylar cartilage

Much of the knowledge about type X collagen has been derived from the chick model, growth plate cartilage, and articular cartilage other than condylar cartilage. A few studies have been dedicated to identifying type X collagen in condylar cartilage (31–33). The temporomandibular joint is a specially adapted synovial joint. It is distinguished from other synovial joints by its unique histological, developmental, and functional characteristics (34,35). The difference between condylar and other articular cartilage is clearly manifested in their bio-molecular pathway of growth and development. Primary cartilage growth starts with the cartilage cells within the central layer of an epiphyseal plate (20). Apparently cell divisions are taking place in the middle part of an epiphyseal plate of the long bone. This type of growth in which new material is formed within existing tissue is interstitial growth (35). Condylar cartilage, or the secondary cartilage growth starts with the mesenchymal-like tissue covering of the prenatal or postnatal condyle. The new members of the cartilage family therefore have been added without the mitosis of existing cartilage mother cells, but through mitosis of undifferentiated mesenchymal cells. This mode of growth in which new cells are added from the exterior is appositional growth (34,36). The difference between the two types of cartilage is also highlighted by their ability of adaptation. Both condylar cartilage and other articular cartilages are present throughout postnatal life. However, articular cartilage remains unchanged with regard to morphological and biosynthetic features (37) and do not take part in the endochondral ossification after growth ceases (38). Therefore, articular cartilage is not adaptive to the changes or stimuli exerted on it. Condylar cartilage, on the other hand, has a special multidirectional capacity for growth and remodeling, therefore, is adaptive to the mechanical or positional changes via chondrogenesis and endochondral ossification.

In a study aiming to identify the distribution of cartilage collagens in neonatal mice condylar cartilage, Silbermann and von der Mark (31) found intense type X collagen expression in hypertrophic chondrocytes within the mineralization zone, but in no other zone. The authors assumed that this type of collagen represents a transient and developmentally regulated collagen which appears to be synthesized by hypertrophic chondrocytes. Another study relating to type X collagen expression in condylar cartilage in response to mastication forces was reported by Salo *et al.* (32). Types X and VI collagen and fibrillin were localized by *in situ* hybridization and immunohistochemical methods in the mandibular condyles of rats, and the response of these molecules to post-weaning diets of soft food, ordinary pellets, or hardened pellets was studied. Type X collagen was synthesized, particularly in conditions of soft food consistency, by cells in the perichondrium-periosteum and in the bone and by cells at the erosion front between cartilage and bone. Type X collagen synthesis diminished under higher compression forces due to mastication and with increasing age. The invariable expression of type X collagen in hypertrophic chondrocytes of condylar cartilage has further been evidenced by a spree of recent studies in rats, where new bone

formation in erosive zone of condylar cartilage occurred after type X collagen had expressed itself in response to forward positioning of the mandible (39–42).

Type X collagen gene mutation

Gene defects often provide informative insights into the relationship between the structure and function of a protein. Since collagen X is a developmentally regulated gene product, a better understanding of the key function of this collagen during the transitional phase from cartilage to bone, may be derived from studies on the phenotypic consequences of type X collagen gene mutations (26). The mouse provides a good model for such studies because of the wealth of knowledge of its development and genetics. As an initial step for such studies, Thomas *et al.* (11) have isolated a genomic clone containing the complete mouse collagen X gene from a mouse cosmid library using a bovine cDNA clone that encodes the C-propeptide of type X protein as a probe.

The spatio-temporal restriction of type X collagen to the hypertrophic cartilage zone implies a functional involvement of type X collagen in the process of endochondral ossification. To address its role during endochondral ossification, transgenic mice for type X collagen were generated (43). The hypertrophic chondrocytes from these mice expressed transgenes consisting of chicken α1(X) cDNA with in-frame deletions flanked by chicken type X regulatory elements. The histologic defects of these mice primarily affected hypertrophic cartilage and were manifested by day 21 after birth as compressions in the growth plates and a reduction in bony trabeculae. At the ultrastructural level, the primary defect in hypertrophic cartilage localized to a pericellular network around hypertrophic chondrocytes, which was disrupted in the genotypically positive mice. Chung *et al.* (33) investigated the consequences resulting from the interference of type X collagen function on the growth and development of the craniofacial skeleton through analysis of transgenic mice with a dominant interference mutation for type X collagen. Genotypically positive mutant mice showed significant craniofacial skeletal abnormalities, including the underdevelopment of the chondrocranium and mandible. Mean radiographic optical densities of the mutant condylar cartilage and the subchondylar areas were 32% less than the corresponding areas of normal mandibles. Histologically, transgene-positive mice revealed compressed hypertrophic cartilage zones and reduced trabeculae in the mandibular condyle. In the normal condyle, type X collagen was immunolocalized in the whole hypertrophic cartilage layer; in the mutant condyle, immunoreactivity to type X collagen was only seen sporadically.

Kwan *et al.* (44) have generated collagen X deficient mice, which shows that deficiency does have phenotypic consequences such as abnormal trabecular bone architecture. Other consequences of the mutation are reduction in thickness of growth plate resting zone and articular cartilage, altered bone content, and atypical distribution of matrix components within growth plate cartilage. It is, therefore, proposed that collagen X plays a role in the normal distribution of matrix vesicles (MV) and proteoglycans within the growth plate matrix. Type X collagen deficiency impacts on the supporting properties of the growth plate and the mineralization process, resulting in abnormal trabecular bone.

Mechanism of type X collagen regulating endochondral ossification

Because of its specific association with hypertrophic chondrocytes in the calcifying zone of growing cartilage, type X collagen has been proposed to be important for endochondral bone formation (29). Despite the wealth of information about collagen X, the precise function of this protein and its role in endochondral ossification has remained the unresolved subject. Proposed mechanisms include providing an easily resorbed fabric for the deposition of bone matrix during endochondral growth of long bones (4); providing support as the cartilage matrix is degraded during endochondral ossification (45); or regulating the calcification process during endochondral ossification (12,46).

Regulating mineralization

Matrix vesicles

The regulation of mineralization in cartilage and bone is not fully understood, but collagens, proteoglycans, and matrix molecules are clearly involved in this process. Bone mineralization is thought to occur in two phases, initially via MV for the formation of hydloxyapatite, and later through nucleation at collagen fibrils for the

proliferation of mineral crystal within the extracelluar matrix (47). MV are extracellular microstructures containing mineral and alkaline phosphatase. They are formed by chondrocytes by budding from the cytoplasmic membrane into the extracellular matrix (48). Thus, in hypertrophy cartilage, MV become entrapped in a matrix composed largely of collagens, mainly type X and II, and a proteoglycan network. These vesicles are abundant in the inter-territorial matrix in the upper hypertrophic zone and are proposed to be the initial nucleational foci for mineralization (47). MV in the growth plate are associated with proteoglycans and can bind to type X and II collagen (49).

Ca^{2+} influx into matrix vesicles

Although the nature of many constitutive vesicle proteins has been explored recently, the mechanism and regulation of MV-induced mineralization remain to be elucidated. The important proteins in MV are three EGTA soluble proteins that bind to Ca^{2+} in the presence of phosphatidylserine, a lipid notable in MV membranes. These MV proteins belong to the annexin family, a group of Ca^{2+}-dependent acid phospholipid-binding proteins and are shown to be identical to annexin V. There is evidence that the binding of type X collagen to MV may be mediated by annexin V (50). It has been reported that annexin V binds directly to type X collagen in a Ca^{2+}-independent manner (51). Annexin V exhibits distinctive Ca^{2+} ion channel properties, enabling the rapid uptake of Ca^{2+} by the vesicles. All together, the above properties suggest that annexin V acts as a Ca^{2+} channel protein in MV that might be modulated by binding of type X collagen to MV. Thus, attachment of MV to the collagen network in hypertrophic cartilage would facilitate the rapid influx of Ca^{2+} into MV. Therefore, the interaction between collagen X with MV activates the influx of Ca^{2+} into MV, suggesting a regulatory role of collagen X in mineralization (49).

Compartmentalizing cartilage matrix components

Type X collagen is important for the compartmentalization of matrix components to the hypertrophic zone of growth cartilage, providing the proper environment for mineralization and modeling (44). The hexagonal lattice structure proposed for type X collagen and its affinity for proteoglycans and collagen fibrils, would be consistent with a role in compartmentalization. Type X collagen may act as a pericellular network retaining or trapping the necessary types and amounts of matrix components, including the MV and proteoglycans, into a correct location of the hypertrophic zone of the cartilage, promoting initiation of normal mineralization. Thus, in the absence of collagen X there is wider spread distribution of matrix components normally restricted to, or more concentrated in, hypertrophic zone (44). As consequences, this improper distribution of matrix molecules alters the structure of newly formed bone as well as the physical properties of cartilage, resulting in compression and deformation of parts of the growth plate (44).

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