Type X Collagen Synthesis during Endochondral Ossification in Fracture Repair*

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Collagen synthesis in normal connective tissue development and repair is integral to tissue stability. The appearance of a short chain collagen, designated Type X, was studied in experimental fractures created in the chicken humerus. Biosynthetic studies using [14C]proline incorporation coupled with histologic examination of the cartilaginous callus demonstrated that Type X collagen synthesis occurs during endochondral ossification in the fracture callus. Type X synthesis occurred in the areas of cartilaginous callus composed of hypertrophic and degenerative chondrocytes that were associated with increased vascularity and matrix mineralization. Synthesis of short chain collagen was not detected in either skeletal muscle or bone.

Two-dimensional peptide mapping of cyanogen bromide and proteolytic fragments derived from fracture callus short chain collagen confirmed the identity of this collagen as Type X. The synthesis of Type X collagen by fracture callus is further evidence supporting its close association with the process of endochondral ossification.

Several extracellular matrix macromolecules are synthesized at various developmental stages in normal chondrogenesis, limb growth, and maturation of the skeleton (1). Within cartilage, collagens comprise approximately 40–50% of its dry weight, the predominant form being Type II collagen. Several other collagens are present in cartilage in smaller amounts than Type II, these include 1α,2α,3α, high molecular weight and low molecular weight collagens, classified as Type I collagen and a short chain collagen, referred to as Type X (2). Alterations in the appearance of some of these collagens (3–8) occur during cell differentiation and may be involved with changes in cell phenotype (9). Type X collagen is synthesized in the cartilaginous tissues of the growth plate (3, 10, 11) and the calcifying region of the sternum (12) and appears to be "advertis- 

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regions of the callus composed of hypertrophic cells and matrix that is undergoing vascularization and mineralization.

MATERIALS AND METHODS

Animal Model—Closed midshaft humerus fractures created in skeletally mature White Leghorn chickens were nonrigidly immobilized with orthopaedic stockinette about the thorax and the triceps wing. Radiographs were obtained just prior to fracture, immediately post-fracture, and at death 10 days later (see Fig. 1) at which time the fracture callus was freshly dissected from the bone fragments. The callus was divided into three tissue subgroups according to color, consistency, and vascularity using the dissecting microscope (13). Skeletal muscle and cortical bone were obtained from the contralateral limb as controls.

Organ Cultures and Extractions—In all studies, representative portions of fracture callus were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for histologic examination. The remainder of the tissue was placed in Dulbecco's modified Eagle's medium containing 25 mm HEPES,1 and supplemented with ascorbic acid (50 μg/ml), β-aminopropionitrile (50 μg/ml), penicillin (10,000 units/ml), streptomycin (10,000 μg/ml), and then preincubated at 37 °C for 1 h. The medium was removed and replaced with the same tissue culture medium supplemented with 5 μCi/ml of [U-14C]proline (New England Nuclear). The tissue was incubated for 20 h in this medium and rinsed with 0.15 M NaCl, 0.05 M Tris(hydroxy- 

1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; FAGE, polya- 

acrylamide gel electrophoresis.
and seeded at a cell density of $7 \times 10^5$ per cm$^2$ in Dulbecco's modified Eagle's medium containing 25 mM HEPES supplemented with 10% fetal bovine serum. To prepare radioactively labeled collagens, cultured cells were incubated with 5 μCi/ml of [$^{14}$C]proline for 24 h, the culture medium was collected into protease inhibitors, and the proteins were precipitated by the addition of 10% trichloroacetic acid.

Gel Electrophoresis—Organ culture and cell culture medium proteins and the products from enzymatic digestion were analyzed by SDS-PAGE on 7.5% slab gels (16). Gels were stained with Coomassie Brilliant Blue R-250, destained in 10% methanol, 7% acetic acid, and processed with ENHANCE (New England Nuclear) for fluorography. Presensitized X-Omat AR x-ray film (Kodak) was exposed at $-70^\circ$ C (17).

Quantitative estimation of radioactivity within individual bands was performed by densitometric scanning (E-C Apparatus Corp.) and electronic integration (SP-4270, Spectra-Physics). Fracture callus was performed by densitometric scanning (E-C Apparatus Corp.) and equilibrated with SDS-PAGE sample buffer containing 100 mM dithiothreitol. After removing these lanes from the gel, each lane was washed three times with 70% formic acid and then placed in a solution of cyanogen bromide at 10 mg/ml in 70% formic acid for 2 h at room temperature while shaking. Following digestion, the lanes were rinsed with water and equilibrated with SDS-PAGE sample buffer containing 100 mM dithiothreitol. The lanes were then placed horizontally in contact with a separate second dimension 5% stacking, 12.5% separating gel and electrophoresed (18). A similar two-dimensional SDS-PAGE technique was used with Staphylococcus aureus V-8 protease (500 μg/ml) to compare peptide fragments from fracture callus collagens to those synthesized by calcifying chick sternal chondrocytes.

Molecular weights of collagen chains were estimated using cyanogen bromide peptides of Type I collagen extracted from rat tail tendon and of Type II collagen from rabbit structural cartilage. Peptides were prepared by digestion in 70% formic acid with an equal weight of cyanogen bromide for 4 h at 30 °C.

RESULTS

Fracture Callus Collagen Synthesis Related to Histologic Regions—Representative histologic sections made of the three tissue subgroups dissected from 10-day chicken fracture callus are shown in Fig. 2. Tissue sample (subgroup) 1 was grossly white, avascular, and, upon microscopic examination, consisted of fibrous tissue and fibrocartilage with areas of residual skeletal muscle. Sample 2 was chondroid, more transparent, and had few areas of vascular invasion. Many of the chondrocytes were undergoing hypertrophy and a few areas of calcified matrix were seen. Sample 3 was more vascular, consisted primarily of hypertrophic chondrocytes, and had large areas of calcified matrix and immature bone.

Metabolic labeling with [$^{14}$C]proline followed by SDS-PAGE demonstrated that Type I collagen was predominant in bone, muscle, and the first callus sample, whereas Type II collagen synthesis was increased in the hyaline cartilaginous areas of the callus represented by samples 2 and 3 (see Fig. 3). Synthesis of the 55-kDa form of a short chain collagen was initially observed in the second sample of the fracture callus and became the major form in association with hypertrophic chondrocytes and matrix mineralization in sample 3 (Fig. 3, lanes 2 and 3).

Densitometric scanning of several fluorograms was used to quantitate the relative densities of $\alpha$ chain and short chain collagen bands for the three callus subgroups (see Table I). The ratio of $\alpha_1:\alpha_2$ calculated for the first subgroup was 2.0, consistent with the synthesis of Type I collagen, the expected product of fibrous tissue and fibrocartilage. For subgroup 2, the ratio was 5.2, indicating a relative increase in the synthesis of $\alpha_1$ chains making up Type II collagen. This value decreases in subgroup 3 to 2.9, presumably due to synthesis of the Type I collagen of osteoid. The short chain collagen comprised approximately 15% of the total radioactivity incorporated into the collagens of the second subgroup, and increased to 31% in the third subgroup.

Comparison of Fracture Callus Collagens with Collagen from Cultured Chondrocytes—Fig. 4 demonstrates the electrophoretic patterns of radiolabeled proteins synthesized by fracture callus in organ culture (lanes 1 and 2) compared with proteins synthesized and secreted into the medium of cultured sternal chondrocytes derived from the region of "presumptive calcification" of 17-day-old chick sterna (lanes 3 and 4). Lanes 2 and 4 represent the products of limited pepsin digestion of fracture callus and cell culture proteins, respectively. The major collagens synthesized by fracture callus include Type I, as noted by the presence of $\alpha_1$ and $\alpha_2$ chains, and of Type II collagen, as indicated by the ratio of $\alpha_1$ to $\alpha_2$ being greater than 3.0.

![Fracture radiographs.](image-url)

**Fig. 1. Fracture radiographs.** Radiographs of right midshaft humerus in skele tally mature White Leghorn chicken. A, prefracture; B, immediately postfracture; and C, 10 days postfracture.
than 2:1. The non-disulfide-bonded short chain collagen was synthesized by this tissue with an estimated molecular mass of 55 kDa (by collagenous standards) and was converted to 40 kDa by limited pepsin proteolysis. Digestion of the [14C] proline-labeled fracture callus collagens with bacterial collagenase demonstrated the sensitivity of both 55 kDa and 40 kDa in addition to Types I and II (data not shown). The short chain collagen of chicken fracture callus migrates on SDS-PAGE with a 3–4-kDa smaller apparent molecular mass than the Type X collagen isolated from cell culture. Such small differences in molecular mass could reflect differences in post-translational modification, such as hydroxylation or glycosylation, that may exist between the cell culture and organ culture systems.

Cyanogen Bromide Digest Peptide Mapping—To further establish the identity of this fracture callus short chain collagen as Type X, cyanogen bromide digestion peptides of the 40-kDa form (pepsinized) were compared to the peptides from a similar digestion of (pepsinized) embryonic chick sternal chondrocyte Type X collagen. The peptides resulting from cyanogen bromide digestion of fracture callus and sternal chondrocyte collagens were analyzed by two-dimensional gel electrophoresis (Fig. 5). The digestion products demonstrate at least eight definite peptide fragments. The correspondence between the cyanogen bromide peptide maps for these two short chain collagens indicates that the fracture callus short chain collagen is a product of the Type X collagen gene.

Using the same two-dimensional SDS-PAGE technique, S. aureus V-8 protease was used to produce peptide fragments of the collagen chains. Peptide maps of the fracture callus short chain collagen and chondrocyte culture medium Type X were also similar using V-8 protease, confirming the results with cyanogen bromide cleavage (data not shown).

DISCUSSION

Type X collagen synthesis has been previously associated with endochondral bone formation in the developing chicken embryo tibiotarsus (10, 11), chondrocytes in culture derived from the “presumptive calcification” region of the sternum (12), and the hypertrophic region of the growth plate in several mammals (3, 6, 19). Our previous study further determined that synthesis of Type X collagen is limited within the growth plate to the zones of provisional calcification and “degeneration” (3). The fracture callus model establishes Type X collagen synthesis in another form of endochondral bone formation, the process of bone repair. The results show synthesis of Type X collagen is increased in the areas composed of hypertrophic chondrocytes associated with calcified cartilaginous matrix.

Consistent with the findings of Kettenjian et al. (13) and Lane et al. (14), our histologic studies substantiate the role of chondrocytes in fracture callus to be similar to the cells in the hypertrophic region of the growth plate. The primary difference is the organization within fracture callus of numerous centers of ossification rather than the uniform transition to a calcified matrix that is seen in the growth plate. Similar histologic changes also occur during the induction of new bone by subcutaneously implanted demineralized bone matrix (20). This involves a transition process in which chondroblasts are first seen at day 5 following implantation, then proliferate, and by day 9 are associated with calcifying matrix.

Biochemical studies of fracture callus regions have shown parallels between this tissue and zones of the growth plate with respect to concentration of enzymatic and metabolic intermediates (14, 15). Analysis of specific collagen types in fracture callus demonstrates the synthesis of Type X collagen during matrix calcification, similar to processes in the growth plate. Type X collagen is not synthesized by bone, skeletal muscle, or by the fibrocartilaginous component of fracture callus. This collagen appears to be a consistent marker of endochondral ossification.

Matrix vesicles have been identified within the extracellular environment of chondrocytes (21, 22). These membranous structures are thought to be derived from cytoplasmic processes of chondrocytes and have been implicated with a role in the calcification of the vertical sepa of growth plate.
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Fig. 3. Regional collagen synthesis by fracture callus. The three regions of fracture callus were cultured in the presence of [14C]proline, and labeled proteins were extracted and analyzed by SDS-PAGE both before (−pepsin) and after (+pepsin) limited digestion with pepsin. Similar organ cultures of skeletal muscle (M) and diaphyseal cortical bone (B) demonstrate predominantly Type I collagen synthesis (lanes 1–3). There is a relative increase in Type II collagen synthesis seen in samples 2 and 3 of fracture callus.

Short chain collagen, synthesized as a 55-kDa component, is initially seen in fracture callus sample 2 and to an even greater extent in sample 3 (consisting of hypertrophic chondrocytes and large areas of calcified matrix). Following pepsin digestion to remove non-triple helical sequences, short chain collagen is converted to a 40-kDa component.

Table 1

| Subgroup | Density | α1 | α2 | α1α2 | Short chain |
|----------|---------|----|----|------|-------------|
| 1        |         | 61.8| 31.5| 2.0  | 0           |
| 2        |         | 63.0| 12.2| 5.2  | 14.8        |
| 3        |         | 49.4| 16.8| 2.9  | 30.8        |

cartilage (22, 23). Accumulation of alkaline phosphatase and ionized calcium within the vesicles is presumed to be important to the nucleation of apatite crystals in the hypertrophic zone (22). Habuchi et al. (24) demonstrated that only Type X collagen was associated with the matrix vesicle fraction isolated from chondrocyte culture medium. This observation supports the contention that Type X could be involved in the process of cartilage mineralization.

A number of reports deal with possible biosynthetic precursors to Type X collagen and their proteolytic processing. Kiely et al. (5) have observed a time-dependent conversion of Type X "procollagen" (59 kDa) in chick tibiotarsal explants of the hypertrophic region to a 49-kDa polypeptide, and, more recently, the same group has identified precursor components to Type X (25). Remington et al. (19) reported the synthesis of a 180-kDa precursor to Type X collagen in rabbit growth plate, but did not observe processing beyond 60 kDa. We have been unable to detect either a larger precursor form of the Type X collagen chain in bovine growth plate organ culture nor have we been able to demonstrate processing of the initial form of the Type X collagen chain to a smaller species even after a 20-h chase in culture (3). Likewise, in the 24-h fracture callus metabolic labelings, no evidence for proteolytic conversion could be detected by analysis on SDS-PAGE.

Comparison of chick Type X collagen synthesized in cultures of chondrocytes from the "presumptive calcification" area of sterna to Type X synthesized in fracture callus revealed a 3–4-kDa difference between the two, the molecular mass of the fracture callus form being smaller. This may be due to differences in post-translational modification that may be inherent to unique characteristics of the two organs studied, the fracture callus versus the sternum. Alternatively, we cannot rule out the possibility of differences between protein synthesis in organ culture compared to conditions in cell culture that may contribute to changes in mobility. However, there were no obvious differences in the cyanogen bromide
and chondrocyte cultures. After collagen from culture medium of chick sternal chondrocytes has callus is converted to apparent molecular mass of fide-bonded 55-kDa short chain collagen synthesized by fracture slightly larger molecular mass, (Xwp) 55 kDa- 40 kDa- (X pep)

organ cultures chondrocytes from the region of "presumptive calcification" peptides. 

Fig. 4. Comparison of collagen synthesis in fracture callus and chondrocyte cultures. Collagens synthesized by fracture callus in the presence of [14C]proline, lanes 1 and 2, and by chondrocyte cultures, lanes 3 and 4, were analyzed by SDS-PAGE before (−) and after (+) digestion with pepsin either with (+) or without (−) the disulfide bond reducing agent dithiothreitol (DTT). The non-disulfide-bonded 55-kDa short chain collagen synthesized by fracture callus is converted to 40 kDa following pepsin digestion. Type X collagen from culture medium of chick sternal chondrocytes has a slightly larger molecular mass, 58 kDa, than that seen in fracture callus. This is true also for the pepsin-digested form (X_p) with an apparent molecular mass of 44 kDa.

Fig. 5. Two-dimensional mapping of cyanogen bromide peptides. Pepsin-digested collagens from fracture callus region 3 organ cultures (A) and culture medium of chick embryo sternal chondrocytes from the region of "presumptive calcification" (B) were run in the first dimension on SDS-PAGE using a 7.5% acrylamide gel, the lanes were excised and treated with cyanogen bromide, and the resultant peptide fragments were analyzed in the second dimension on a 12.5% acrylamide gel. The cyanogen bromide peptides from fracture callus short chain (40 kDa) and sternal chondrocyte Type X (X_p) show extensive similarities.

peptide maps that would explain the observed difference in molecular weight.

Further knowledge of the properties of Type X collagen will help establish its role in endochondral ossification. Interactions between this collagen and other noncollagenous proteins (26-28), proteoglycans, or growth factors (29) could play a part in endochondral ossification. The association of Type X collagen synthesis in cartilage matrix mineralization may be a molecular marker that would distinguish between successful fracture repair and nonunion. Identification of environmental factors that induce synthesis of Type X collagen by fracture callus may be important in the treatment of fractures.

The formation of a cartilaginous callus during fracture healing may be favored by the mechanical movement of bone (30). A recent report indicates that Type II collagen detected by immunolocalization appears only in areas of motion during fracture repair (31), another describes changes in collagen composition during the repair of small defects in cortical bone without endochondral ossification (32). In our present study, we used a nonrigidly immobilized fracture allowing a certain degree of motion. The appearance of Type X collagen synthesis in the areas showing hypertrophic chondrocytes and chondroid tissue with the concomitant synthesis of Type II may be dependent on the restricted movement that was permitted to occur in our system. Utilization of a model in which bone repair can be studied at a mechanically stable site could further elucidate the biomechanical conditions that stimulate the synthesis of specific collagen types and the ensuing cartilaginous tissue.

REFERENCES
1. von der Mark, K. (1980) Curr. Top. Dev. Biol. 14, 199-225
2. Mayne, R., Reese, C. A., Williams, C. C., and Mayne, P. M. (1983) in *Limb Development and Regeneration* (Kelley, R. O., Goe- trinicke, P. F., and MacCabe, J. A., eds) Vol. 110, Part B, pp. 125-135, Alan R. Liss, New York
3. Grant, W. T., Sussman, M. D., and Balian, G. (1985) *J. Biol. Chem.* 260, 3798-3803
4. Schmid, T. M., and Linsenmayer, T. F. (1985) *Dev. Biol.* 107, 373-381
5. Kielty, C. M., Kwan, A. P. L., Holmes, D. F., Schor, S. L., and Grant, M. E. (1985) *Biochem. J.* 27, 545-554
6. Sussman, M. D., Ogle, R. C., and Balian, G. (1984) *J. Orthop. Res.* 2, 134-142
7. Capasso, O., Tajana, G., and Cancedda, R. (1984) *Mol. Cell. Biol.* 4, 1163-1168
8. Gibson, G. J., Schor, S. L., and Grant, M. E. (1982) *J. Cell Biol.* 93, 767-774
9. Mayne, R., Elrod, B. W., Mayne, P. M., Sanderson, R. D., and Linsenmeyer, T. F. (1984) *Exp. Cell Res.* 151, 171-182
10. Schmid, T. M., and Conrad, H. E. (1982) *J. Biol. Chem.* 257, 12444-12450
11. Schmid, T. M., and Conrad, H. E. (1982) *J. Biol. Chem.* 257, 12451-12457
12. Gibson, G. J., Beaumont, B. W., and Flint, M. H. (1984) *J. Cell Biol.* 99, 208-216
13. Ketenjian, A. Y., and Arsenis, C. (1975) *Clin. Orthop. Relat. Res.* 107, 266-273
14. Lane, J. M., Boskey, A. L., Li, W. K. P., Eaton, B., and Posner, A. S. (1979) *Metab. Bone Dis. Rel. Res.* 1, 319-324
15. Ruhlman, R. E., and Bakowski, M. J. (1975) *Clin. Orthop. Relat. Res.* 107, 258-265
16. Laemmli, U. K. (1970) *Nature* 227, 680-685
17. Bonner, W. M., and Laskey, R. A. (1974) *Eur. J. Biochem.* 46, 83-88
18. Barsh, G. S., and Byers, P. H. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 5142-5146
19. Remington, M. C., Bashey, R. I., Brighton, C. T., and Jimenez, S. A. (1983) *Collagen Relat. Res.* 3, 271-278
20. Reddi, A. H. (1981) *Collagen Relat. Res.* 1, 209-226
21. Bonucci, J. (1967) *J. Ultrastruct. Res.* 20, 33-50
Type X Collagen in Fracture Repair

22. Anderson, H. C. (1969) J. Cell Biol. 41, 59–72
23. Ali, S. Y. (1983) in Cartilage (Hall, B. K., ed) Vol. 1, pp. 343–378, Academic Press, Orlando, FL
24. Habuchi, H., Conrad, H. E., and Glaser, J. H. (1985) J. Biol. Chem. 260, 13029–13034
25. Kwan, A. P. L., Sear, C. H. J., and Grant, M. E. (1986) FEBS Lett. 206, 267–272
26. Termine, J. D., Kleinman, H. K., Whitson, S. W., Conn, K. M., McGarvey, M. I., and Martin, G. R. (1981) Cell 26, 99–105
27. Termine, J. D., Beicourt, A. B., Conn, K. M., and Kleinman, H. K. (1981) J. Biol. Chem. 256, 10403–10408
28. Syftestad, G. T., and Caplan, A. I. (1984) Dev. Biol. 104, 348–356
29. Seyedin, S. M., Thompson, A. Y., Rosen, D. M., and Piez, K. A. (1985) J. Cell Biol. 97, 1950–1953
30. Goss, R. J. (1983) in Cartilage (Hall, B. K., ed) Vol. 3, pp. 267–307, Academic Press, Orlando, FL
31. Laze, J. M., Suda, M., von der Mark, K., and Timpl, R. (1986) J. Orthop. Res. 4, 319–329
32. Glimcher, M. J., Shapiro, F., Ellis, R. D., and Eyre, D. R. (1980) J. Bone Jt. Surg. Am. Vol. 62, 964–973