Coordinate Regulation of Collagen and Alkaline Phosphatase Levels in Chick Embryo Chondrocytes*

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Chick embryo tibial chondrocytes release into their extracellular matrix several species of proteochondroitin sulfate and collagen as well as matrix vesicles which are rich in Ca²⁺ and alkaline phosphatase and that appear to play a role in the calcification of cartilage. To determine whether there is any parallel regulation of the production of these products, the rates of collagen synthesis by cultured chick embryo tibial chondrocytes were altered, and the resulting changes in proteochondroitin sulfate synthesis and alkaline phosphatase levels in the cells were measured. As the rate of collagen synthesis was increased by adding increasing amounts of ascorbic acid to the culture medium, there was a parallel increase in the level of alkaline phosphatase. Similarly, when the rate of collagen synthesis was inhibited by adding 3,4-dehydroproline to the culture medium, the levels of alkaline phosphatase fell. The alkaline phosphatase in the culture medium was associated with vesicles which appeared to be matrix vesicles. It was recovered quantitatively by filtration through membranes with a pore size of 0.1 μm and measured by solubilizing the alkaline phosphatase from the membrane with detergent and assaying with 4-methylumbelliferyl phosphate as the substrate. When the matrix vesicles from the culture medium were analyzed for collagen types, it was found that only Type X collagen was recovered in this fraction. The implications of the association of Type X collagen and the matrix vesicles, both of which are found primarily in growth plate cartilage in the zone of hypertrophied chondrocytes which is in the process of mineralization, are discussed.

Chondrocytes from the cartilage of embryonic bones secrete a matrix composed primarily of collagen and proteochondroitin sulfate which serves as a scaffold for the deposition of hydroxyapatite. The major collagen synthesized by chondrocytes is Type II collagen, but these cells also synthesize several minor collagens (1-3). The chondrocytes secrete a large, cartilage-specific proteochondroitin sulfate and two smaller proteochondroitin sulfate species (4). All of these products are synthesized and secreted by primary cultures of chick embryo chondrocytes, but the different types of collagen and proteochondroitin sulfate are not equally distributed in the cell

matrix (Ma²⁺) and culture medium (CM) pools (3, 5). In addition, the cultured chondrocytes release into the extracellular pools vesicles which are rich in alkaline phosphatase and Ca²⁺ and which have a unique phospholipid composition typical of matrix vesicles (6). These vesicles are believed to be the site of earliest hydroxyapatite deposition in the zone of hypertrophying chondrocytes in embryonic long bones (see Ref. 7 for a review). Since the matrix vesicles, the proteochondroitin sulfate, and the collagen represent the major products secreted by chondrocytes, it seemed possible that the formation of two or more of these products might be coordinately regulated. If this were the case, the specific perturbation of the synthesis or secretion of one of the major types of products might be expected to cause changes in the synthesis and/or secretion of one of the others. This report describes the effects of altered collagen metabolism on the formation of proteochondroitin sulfate and membrane-associated alkaline phosphatase in cultured chondrocytes. The results show that specific activation or inhibition of collagen synthesis results in a corresponding change in the synthesis and secretion of alkaline phosphatase.

EXPERIMENTAL PROCEDURES

Cell Culture—Chondrocytes were prepared from a mixture of zones 1, 2, and 3 of the distal end of the tibiotarsus of 12-day chick embryos and grown as previously described in 60-mm Falcon dishes (5, 8). Unless otherwise indicated, on day 7 and 9 the medium was replaced with fresh Dulbecco's modified Eagle's medium containing 2 g of glucose/liter and 10% heat-inactivated fetal calf serum and Na ascorbate. On day 9, fresh medium containing isotopic precursors was added to the cultures for measurement of proteochondroitin sulfate and collagen synthesis (see below), and the cultures were harvested and assayed 24 h later. The heat-inactivated serum was prepared by heating fetal calf serum to 60 °C for 60 min to inactivate serum alkaline phosphatase activity. Just prior to its addition to the culture medium, Na ascorbate was made up fresh at a concentration of 10 mg/ml in Tris-saline buffer and filter sterilized.

Matrix Vesicle Assay—Medium was removed from cell cultures and centrifuged at 500 × g for 5 min to pellet any detached whole cells. Matrix vesicles were then recovered from the supernatant by the centrifugation procedure used previously (6) or by a new filtration procedure. For the filtration assay, the medium was first incubated at 37 °C for 15 min in the presence of 0.02% trypsin (Sigma, Type III) and then passed through a Schleicher and Schuell nitrocellulose membrane filter (PH 79, 25-mm diameter, 0.1-μm pore size) which was prerinsed with 5 ml of Tris-saline (0.15 M NaCl in 3.5 mM Tris, pH 7.4). The culture medium was filtered under vacuum and the filter was rinsed with 5 ml of Tris-saline. The filter was then placed in a 12 × 75-mm test tube containing 0.5 ml of 2% Zwittergent 3-12 (Calbiochem-Behring) in Tris-saline. The filter was vortexed vigorously, and an aliquot of the solubilized vesicles was assayed for alkaline phosphatase activity (see below).

In order to prepare the matrix vesicle fraction from the pericellular matrix (Ma pool), the washed cells remaining on the dish after the

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† The abbreviations used are: Ma, pericellular matrix; IC, intracellular; CM, culture medium.
medium was removed and washed with saline for 10 min at 37 °C. The trypsinate was centrifuged in a clinical centrifuge to pellet free cells, and the supernatant containing the cells was retrieved and designated as the CM pool. One ml of 4 M guanidinium chloride in buffer A (8) was added to the cell layer, and the cells were washed with 0.5 ml of 4 M guanidinium chloride in 1-butanol, glacial acetic acid, and 10 μCi/ml H\textsuperscript{3}O\textsuperscript{14}, (carrier-free, New England Nuclear) for measurement of total \textsuperscript{3}H incorporation into glycosaminoglycans. After 24 h, the culture medium was removed and the cell layer was washed with 1 ml of Tris-saline. The medium and wash were combined and designated as the CM pool. One ml of 4 M guanidinium chloride in buffer A (8) was added to the cell layer, and the wash was combined with the cells.

**Chondroitin Sulfate Analysis—**Chondroitin sulfate synthesis was assayed by measuring the \textsuperscript{35}S\textsuperscript{O} incorporation into chondroitin sulfates as before (5, 8). On day 3 of culture, cells were incubated in 5 ml of fresh medium containing 10% heat-inactivated fetal calf serum and 10 μCi/ml H\textsuperscript{3}O\textsuperscript{14} (carrier-free, New England Nuclear) for measurement of total \textsuperscript{3}H incorporation into glycosaminoglycans. After 24 h, the culture medium was removed and the cell layer was washed with 0.5 ml of 4 M guanidinium chloride in buffer A for 4 h and centrifuged. The supernatants from the two extracts were combined and designated as the cell-associated pool. The incorporation of \textsuperscript{3}H was determined by the assay procedure described in Ref. 5.

**Collagen Analysis—** Cultures were incubated for 24 h with either 10 or 30 μCi/ml L-[2,3-\textsuperscript{3}H]proline (46 Ci/mmol, Amersham Corp.) in Dulbecco's modified Eagle's medium containing 2 g of glucose/liter, containing 100 μg/ml β-aminopropionitrile, 0–50 μg/ml of l-ascorbate, and 10% heat-inactivated fetal calf serum. The intracellular (IC) pool, and CM pools were isolated as previously described (3), and aliquots of the three pools were chromatographed on 1 × 22-inch strips of Whatman No. 3 paper overnight in 1-butanol, glacial acetic acid, 1 N ammonia (2:3:1.5) to move the free proline away from the origin which retains labeled polymer. The 1-inch origin segment was cut, hydrolyzed, and analyzed as described to determine the total [\textsuperscript{3}H]proline incorporated into proteins. The distribution of \textsuperscript{3}H in proteins-bound proline and hydroxyproline was determined by paper chromatographic analysis of an aliquot of the hydrolyzed polysaccharides in 0.1 M sodium perchloric acid, with the analysis being performed in 0.1 M sodium bicarbonate, pH 9.0, for 1 hr. After incubation for 30 min at 37 °C, 1.9 ml of 0.1 M sodium bicarbonate buffer containing 1% Tris (pH 7.4), and 10 mM EDTA was added to stop the reaction. The fluorescence of the 4-methylumbelliferone formed by action of the alkaline phosphatase was determined using 4-methylumbelliferone (Sigma) in the glycine/carbonate/EDTA buffer. The assay could detect 100 pmol of product.

**DNA Determination—**With the aid of a rubber policeman, cells were removed from two 60 dishes in a total of 3.0 ml of 0.03% Pronase (grade B, 45,000 proteolytic units/g, Calbiochem-Behring) in 0.02 M Tris-HCl, pH 8.0, 10 mM EDTA buffer and incubated at 37 °C for 1 h. Three volumes of 95% ethanol containing 1% K acetate were added, and the reaction mixture was passed through the filter. Panel b shows the recovery of alkaline phosphatase activity on 0.1-μm membrane filters. The symbols below the arrow indicate amounts of alkaline phosphatase recovered when a 2.5-ml sample was treated with 2% Zwittergent prior to filtration.

![Fig. 1. Retention of alkaline phosphatase activity on membrane filters. Panel a shows the amount of alkaline phosphatase retained on Schleicher and Schuell membrane filters of different porosity when 5 ml of tissue culture medium from cultured cells was passed through the filter. Panel b shows the recovery of alkaline phosphatase activity on 0.1-μm membrane filters. The symbols below the arrow indicate amounts of alkaline phosphatase recovered when a 2.5-ml sample was treated with 2% Zwittergent prior to filtration.](image)

**Table I**

<table>
<thead>
<tr>
<th>Isolation method</th>
<th>Alkaline phosphatase units/dish</th>
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</thead>
<tbody>
<tr>
<td>Centrifugation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>135</td>
</tr>
<tr>
<td>Filtration, 0.1-μm filter</td>
<td>356</td>
</tr>
</tbody>
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<sup>a</sup>147,000 × g, 90 min.
Correlation of Collagen and Alkaline Phosphatase Levels—Initial studies showed that the production of matrix vesicles could be correlated with the biosynthesis of collagen but not that of proteochondroitin sulfate. Fig. 2 demonstrates that the incubation of chondrocytes for 24 h with increasing concentrations of ascorbic acid caused a marked increase in the rate of collagen synthesis. The greatest change was observed in the matrix fraction, which showed an 80-fold increase in the appearance of polymer-bound hydroxyproline. Accompanying the increase in collagen synthesis, there was a large increase in the alkaline phosphatase activity in the cell fraction and in the vesicle fractions recovered from both the culture medium and the cell matrix. Ascorbic acid caused only a modest increase in $^{35}$S incorporation in proteoglycan. The increase of proteochondroitin sulfate synthesis rate that did occur was observed at lower ascorbic acid concentrations than required for the changes in collagen synthesis and alkaline phosphatase accumulation.

Fig. 3 shows a time course for the induction of alkaline phosphatase activity and the increase in collagen synthesis. In this experiment, medium containing ascorbic acid (50 μg/ml) was added at the time indicated and the cells were fed with fresh medium at 48-h intervals. Cells were harvested on day 10 and assayed for collagen synthesis and alkaline phosphatase levels. Fig. 3a shows that the total alkaline phosphatase activity/20 μg of DNA and the total collagen synthesis/
Fig. 5. Gel electrophoresis of [3H]proline-labeled collagen pools before (lanes 1–6) and after (lanes 7–12) pepsin treatment. Matrix pools are shown for control cells (lanes 1 and 7), ascorbate-treated cells (lanes 2 and 8), and cells treated with both ascorbate and dehydroproline (lanes 3 and 9). CM pools are shown for control cells (lanes 4 and 10), ascorbate-treated cells (lanes 5 and 11), and cells treated with both ascorbate and 3,4-dehydroproline (lanes 6 and 12). All lanes were loaded with 20,000 cpm of [3H]. Pepsin converts the precursors of a1(II) chains to a1(II) chains and Type X chains to a M, = 45,000 chains.

but the smaller pools both showed a lag period before reaching maximal levels (see "Discussion").

The parallel changes in collagen synthesis and alkaline phosphatase levels were further demonstrated by observing the changes in collagen metabolism and alkaline phosphatase activity when 3,4-dehydroproline was added to the cultures during the 24-h [3H]proline labeling period to inhibit collagen synthesis. Fig. 4 shows that, as the concentration of dehydroproline was increased, collagen synthesis decreased, with a parallel decrease in alkaline phosphatase levels in the cell. As above, it was the amount of collagen appearing in the Ma pool of collagen that was altered the most, falling to 15% of control levels. Treatment for 24 h with the proline analog caused a decrease in total cellular alkaline phosphatase to 77% of control levels, but relatively little effect on the sulfate incorporation into proteochondroitin sulfate.

Analysis of the Collagen Pools—Fig. 5 shows a comparison of the gel electrophoresis patterns of the [3H]proline-labeled collagen species from the Ma and CM pools of control cells (no ascorbic acid) and cells grown in the presence of ascorbate or ascorbate plus dehydroproline. Each lane was loaded with 20,000 cpm of [3H]. The electrophoresis profiles of the Ma and CM pools in lanes 1–6 are qualitatively similar, indicating that, in spite of the marked differences in the rates of synthesis of collagen under the different culture conditions, the collagen species that were formed were similar. The Type X collagen is present primarily in the CM pool as before (3), and the H and J collagens are present in the Ma pool. In the presence of ascorbic acid, the processing of the pro-a1(II) and the pc-a1(II) to the mature a1(II) form appears to proceed more efficiently than when ascorbic acid was omitted. Lanes 7–12 show the results of limited pepsin treatment of each of the collagen samples. The procollagen precursors are converted by pepsin to the a1(II) chains, which represent the major collagen synthesized in all cases. The Type X collagen is converted to a M, = 45,000 chain (3). The recovery of a2 chains after pepsin treatment appears to be increased in both the Ma and CM pools in the presence of ascorbic acid.

The correlation between the rate of collagen synthesis and alkaline phosphatase accumulation suggested the possibility that matrix vesicles might be released from the plasma membrane in association with collagen. To test this possibility, cells were labeled with [3H]proline and matrix vesicles were isolated by the centrifugation procedure, solubilized in detergent, and analyzed by gel electrophoresis. Fig. 6 compares the collagen recovered in the matrix vesicle fraction with the collagen species found in the CM supernatant from the matrix vesicle isolation and in the Ma pool. The matrix vesicle fraction in lane 1 shows the Type X collagen as the most prominent [3H]proline-labeled protein in the matrix vesicle fraction. Higher molecular weight proteins are also present, but relatively little a1(II) collagen chain is present. Typical profiles are observed for the CM pool in lane 2 and for the Ma pool in lane 3. After pepsin treatment of the samples (lanes 4–6), collagen precursors were converted completely to the mature a1(II) chains in the CM and Ma pools, and the Type X collagen species was converted to the previously observed M, = 45,000 chain. In the matrix vesicle fraction, the only collagen band present after pepsin treatment was the M, = 45,000 collagen chain. There was no a1(II) band present, indicating the non-collagenous nature of the higher molecular weight [3H]proline-labeled proteins in the matrix vesicle fraction (lane 1).

Discussion

Although proteochondroitin sulfate, collagen, and alkaline phosphatase, the three most prominent products secreted by chondrocytes, are readily assayed in chondrocyte cultures, the chondrocyte product whose metabolism is most readily controlled is collagen, which must be converted to a triple helical structure prior to its secretion from the cell (for reviews see

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*Type X collagen* (27) is the M, = 59,000 collagen that has been referred to as G collagen (2), 59K collagen (3), or SC (short chain) collagen (22) in previous literature.
The intracellular accumulation of underhydroxylated collagen results in the lowering of the rates of collagen synthesis, perhaps via a feedback mechanism (15). The experiments reported here show that the altered rates of collagen synthesis which result from varying the exposure of the chondrocytes to ascorbic acid, a required cofactor for prolyl and lysyl hydroxylase (13), are accompanied by parallel changes in the levels of the alkaline phosphatase synthesized by the cells. A comparison of the total alkaline phosphatase levels and the total collagen synthesis (Fig. 3a) shows that the rises of collagen and alkaline phosphatase with increasing time of exposure to ascorbic acid paralleled each other and that when collagen synthesis fell with longer exposure to ascorbic acid, alkaline phosphatase levels also fell. Similarly, when collagen synthesis was inhibited by the incorporation of the dehydroproline to prevent the hydroxylation of collagen (14), the levels of alkaline phosphatase in the cells fell in a corresponding manner. Identical results were obtained when hydroxylation was blocked by adding dipyriridine to the culture medium (results not shown).

Each of the three characteristic products secreted by chondrocytes in culture are distributed among the IC, the Ma, and the CM pools. Although all three of the collagen pools were altered when the rates of collagen synthesis changed, the most dramatic effects were observed for the Ma pool. This pool, which contains very little collagen when cells are cultured in the absence of ascorbic acid, is markedly increased in a dose-and time-dependent manner when ascorbic acid is included in the culture medium. Similarly, when collagen synthesis was inhibited by 3,4-dehydroproline, the major decrease in collagen occurred in the Ma pool. In contrast, the largest pool of alkaline phosphatase is the IC pool which contained approximately 90% of the total alkaline phosphatase, and, quantitatively, the largest increase in alkaline phosphatase took place in the IC pool. However, in terms of percentage increase in alkaline phosphatase, the two extracellular pools show the greatest increases. Thus, ascorbic acid causes a large increase in the extracellular pools of both collagen and alkaline phosphatase.

The quantitative retention of the alkaline phosphatase on the membrane filters in the assay procedure used in this work, and the solubilization of the alkaline phosphatase from the filter by detergent establishes that all of the alkaline phosphatase of the extracellular pools is membrane-associated. This is consistent with the earlier report that vesicle-bound alkaline phosphatase is released by cultured chondrocytes into the culture medium (6). In addition, the alkaline phosphatase secreted by the vesicles released into the medium contains high levels of Ca** and has a unique phospholipid composition (6). These properties are typical of matrix vesicles, which were first observed by Bonucci (11) and Anderson (10, 16) as the site of initial hydroxyapatite deposition during endochondral calcification in the extracellular matrix surrounding hypertrophying chondrocytes. Thus, the extracellular vesicles formed in culture appear to be matrix vesicles, and the measurements of alkaline phosphatase in the Ma and CM fractions were adopted here as a sensitive assay for these vesicles. It was shown previously that the number of matrix vesicles in the growth plate of long bones increased as the chondrocytes progressed from the proliferative zone to the hypertrophic zone (17) and that when chondrocytes from these different zones of the developing tibiotarsus of the chick embryo were grown in monolayers (6), the amount of matrix vesicle protein and the specific activity of the alkaline phosphatase in the vesicles increased in going from the zone of proliferative cells to the zone of hypertrophied cells. In the present study the chondrocytes were prepared from a mixture of the zones of proliferative, elongated, and hypertrophying chondrocytes from the tibiotarsi of 12-day chick embryos. Whether similar changes in the number of vesicles or the specific activity of the alkaline phosphatase took place with different culture conditions could not be determined using the filtration assay, but the changes of alkaline phosphatase levels with the changes in culture conditions do reflect changes in matrix vesicle activity.

When alkaline phosphatase was induced by ascorbic acid, the activity increased first in the cellular fraction, then in the Ma fraction, and finally in the CM fraction (Fig. 3c). This sequence appears to be a reflection of the progression of matrix vesicles into the extracellular matrix of the cells in which there is initially an increased synthesis of the plasma membrane alkaline phosphatase followed by a transient appearance of the matrix vesicles in the periplasmic matrix as they pinch off from the plasma membrane and then are released into the CM pool. Thus, a steady state level of matrix vesicles is found in the Ma pool, while the alkaline phosphatase in the CM pool continues to increase. Studies of scurvy patients and human and guinea pigs have shown lower serum levels of alkaline phosphatase during ascorbic acid deficiency, but an induction of the enzyme by ascorbic acid has not been demonstrated previously. The intestinal form of alkaline phosphatase has been shown to be inducible by a number of steroid hormones and parathyroid hormones, but little information is available on hormone responsiveness of the enzyme.

The finding that the Type X collagen was uniquely associated with the matrix vesicles and the CM is of note. Type X collagen was originally observed in cultures of sternal (2) and tibial (3) chick embryo chondrocytes and has been referred to as G collagen (2, 18), 59K collagen (3, 19), or SC (short chain) collagen (20–22). It is a collagen that is characterized by chains which are approximately half the length of interstitial collagens (M<sub>r</sub> = 59,000), that are rich in methionine and that form triple helices which are not cross-linked by disulfide bonds (2, 3, 18–22). In the chick embryo tibiotarsus, its synthesis was shown to occur only in chondrocytes from the zone of hypertrophic chondrocytes which were undergoing calcification (19). The observation that Type X collagen is localized in the zone of calcifying cartilage has been repeated more recently (23, 24), and Schmid and Linsenmayer (25) have shown that monoclonal antibodies directed against the Type X collagen bind specifically to tissues of skeletal origin which are undergoing calcification, including both endochondral and intramembranous calcifying tissues. These are tissues which are also rich in matrix vesicles. Although matrix vesicles have been considered to the site of initiation of hydroxyapatite deposition in bone formation, there has been some recent debate over whether the first deposits of hydroxyapatite are directly associated with the matrix vesicles (26–28). However, there seems little question that matrix vesicles play a role in the calcification event, and the confinement of Type X collagen to regions of cartilage which are undergoing calcification suggests that it too plays some role in calcification. The observations reported here that collagen synthesis and matrix vesicle formation by chondrocytes rise and fall together and that the matrix vesicle fraction that is isolated from the culture medium is associated with a single type of collagen, namely, Type X collagen, suggest the possibility that their roles in calcification may be coupled.
REFERENCES