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EFFECTS OF CHICK EMBRYO EXTRACT FRACTIONS ON COLLAGEN AND GLYCOSAMINOGLYCAN METABOLISM BY CHICK CHONDROBLASTS *

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Summary

Chick embryo extract was fractionated by ion exchange columns to yield a basic protein (mol. wt. 72 000) which, at concentrations as low as 10^{-7} M, caused morphological dedifferentiation of cultured chick chondroblasts. Effects of a partially pure embryo extract fraction I on the metabolism of collagenous proteins, noncollagenous proteins and glycosaminoglycans by chondroblasts was studied. This material caused a decline in collagen synthetic rates during a 7-day growth period but did not affect noncollagenous protein and glycosaminoglycan synthetic rates. 'Pulse-chase' experiments demonstrated that turnover rates of cell layer collagen were low in culture (half-life 4 months), whereas the half-life of noncollagenous proteins was 55 h and glycosaminoglycans 75 h. Fraction I had no effect on turnover of any of these molecules. This material caused a concentration-dependent reduction of [^{3}H]hydroxyproline accumulation by chondroblasts during a 24-h period (70% maximal reduction), only a 10% reduction on noncollagenous proteins and no effect on glycosaminoglycans. Collagenase digestion experiments showed the effect was not due to inhibition of prolyl hydroxylation. Similar inhibition of collagen synthesis by this fraction occurred in chick embryo tendon fibroblasts, but not in human fibroblasts, suggesting species specificity. The pure fraction from embryo extract exerted a biphasic effect on chondroblast collagen synthesis. Synthesis was inhibited at lower concentrations, but synthetic rates were

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similar to those of controls at higher concentrations.

We suggest that the role of the active embryo extract fraction in vivo may be to regulate the type and amount of collagen synthesized by connective tissue cells.

Introduction

In early studies it was noted that embryonic chick cartilage cells (chondroblasts) 'dedifferentiated' in culture. These cells lost their characteristic extracellular metachromatic matrix, became motile, assumed a spindle shaped fibroblastic morphology [1], and the ultrastructure is considerably different from that of the definitive chondroblasts [2]. Coon [3] showed that the chondroblast morphology could be maintained, however, if chick embryo extract was excluded from the medium. Similar activity is present in chick serum [4] and 5-bromo-2′-deoxyuridine (BrdUrd) also caused chondroblasts to become dedifferentiated [5]. It was suggested the phenomenon was caused by growth of contaminating fibroblasts [6], but when chondroblasts were cloned, the progeny cells became fibroblastic [7]. Thus it was established that the dedifferentiated chondroblast descended from the chondroblast.

Studies have demonstrated that dedifferentiated chondroblasts synthesize hyaluronic acid [8,9], which is typically a fibroblast product [10]. During a 5-day growth period in embryo extract or BrdUrd, the morphological dedifferentiation was accompanied by a shift in the types of collagen and glycosaminoglycans synthesized [9]. Control cultures synthesized exclusively cartilage type II collagen and chondroitin sulfate, whereas the dedifferentiated chondroblasts synthesized type I collagen, chondroitin sulfate and hyaluronic acid. The switch in collagen gene expression for cells grown in embryo extract and BrdUrd has been verified by analysis of cyanogen bromide peptide profiles [11,12]. A similar shift in collagen gene expression was induced with a chick lysosomal enzyme preparation [13] and spontaneously dedifferentiated chondroblasts also switch collagen type [14]. Thus, when cultured with embryo extract, there is an extensive 'remodeling' of the chondroblast to that of a dedifferentiated 'fibroblastic' progeny. In this study we have isolated a protein from this complex extract which causes the morphological changes, and have examined aspects of chondroblast metabolism of collagenous proteins, noncollagenous proteins and glycosaminoglycans during dedifferentiation.

Materials and Methods

Cell culture techniques

Pure populations of chondroblasts from 11-day chick embryo cartilagenous vertebrae were prepared by the 'floater' technique as described [9]. The floaters were plated in Falcon tissue culture dishes, and all cultures were incubated for a 24-h period before the start of any experiment. Chick fibroblasts were prepared from tendons of 14-day embryos, and human fibroblasts were obtained from explant outgrowths of human dermis. The culture

medium was Ham's F-10 supplemented with 10% fetal calf serum, 1% bovine serum albumin, twice the normal concentrations of amino acids and pyruvate [3] and a mixture containing antibiotics and antimycotics (Grand Island Biological Co.). Cultures were maintained in a humid atmosphere containing 5% CO_2/95% air at 37°C.

Collagen and glycosaminoglycan analysis

Cell cultures were incubated with 3 ml of proline-free F-10 medium to which was added 50 μg/ml freshly-prepared ascorbic acid and 185 kBq per dish [^{3}H]-proline (New England Nuclear, NET-285). At the end of the incubations cells and medium were analyzed separately for total radioactivity, [^{3}H]proline and [^{3}H]hydroxyproline [15,16]. Some of the data are expressed as

$$\text{'\%Hyp'} = \frac{(\text{dpm Hyp})}{(\text{total dpm})} \times 100.$$

This value represents an estimate of the amount of collagen synthesized during the pulse in relation to all other proline-containing proteins. Since collagen contains approximately 110 residues of proline and 100 residues of hydroxyproline, the total proline-derived radioactivity in collagen is 2.1 times the hydroxyproline. Subtraction of this value from the total radioactivity gives an estimate of the noncollagenous protein. Content of newly-synthesized collagen using a collagenase assay was performed as described by Peterkofsky and Diegelmann [19] using clostridial collagenase (CLSPA, Worthington Biochemical Corp.).

To determine glycosaminoglycan synthesis, cultures were labeled with 37 kBq/ml [^{3}H]glucosamine (New England Nuclear, NET-190). The cells and medium were separated and digested with pronase [8] (Sigma Chemical Co. P-5130, 300 μg/ml in 0.2 M Tris-HCl, pH 8) at 55°C for 24 h. The digests were chilled, treated with 10% trichloroacetic acid to remove the protein and the soluble glycosaminoglycans from the supernatants dialyzed against distilled water and counted.

Preparation of embryo extract

Chick embryos (10—11 days) were rinsed with Hank's balanced salt solution, added to an equal volume of 0.1 M potassium phosphate buffer (pH 8.0) and forced through the orifice of a 50 ml plastic syringe. The material was then subjected to freeze/thaw 3 times (dry ice in acetone followed by 37°C water bath) and centrifuged 15 min in the clinical centrifuge. The supernatant was centrifuged at 28 000 × *g* for 1 h at 4°C in the Beckman L-2 ultracentrifuge. This supernatant is the starting material for fractionation, and the concentration of the derived fractions is based on this material, which is referred to as 1*X*. For example, a fraction from 100 ml whole embryo extract reconstituted in 10 ml balanced salt solution would have a concentration of 10*X*. Addition of 1 part of this stock solution to 9 parts culture medium gives a 1*X* solution, which is equivalent to 100% whole embryo extract. The ability for a given fraction to cause morphological dedifferentiation was assessed visually. Chondroblasts were plated ($7.5 \cdot 10^4$ cells/60 mm dish) and 24 h later the fractions were added. Morphological changes were usually seen after 24 h, but cultures were

observed for at least 5 days. Since the morphological switch is essentially an all-or-none phenomenon, no attempt was made to quantitate the change.

DEAE-cellulose column chromatography

Embryo extract (150 ml, 8—10 mg/ml protein) was dialyzed against 0.1 M potassium phosphate buffer (pH 8.0) and pumped onto a DEAE-cellulose column (Whatman DE 52, 2.5 cm × 40 cm, flow rate 1 ml/min). After the void volume had eluted, 4 major peaks eluted with a 1-liter linear gradient consisting of increasing NaCl (0 to 1 M) and decreasing pH (8.0 to 6.5). All the morphological dedifferentiation activity (tested at 0.1*X* and 0.5*X*) and approximately 30% of the total protein was recovered in the void volume peak, which is referred to as fraction I.

CM-cellulose column chromatography

Fraction I from the DEAE-cellulose column was lyophilized, dialyzed into 0.1 M potassium phosphate buffer containing 0.5 M NaCl (pH 4.5) and pumped onto a 2.5 cm × 40 cm column of carboxymethyl cellulose (Whatman CM 52, flow rate 1 ml/min). After the void volume had eluted the column was eluted with 0.2 M $H_3PO_4^+$ 1 M NaCl (Fig. 1). Fraction II was collected, lyophilized, dialyzed into starting buffer and rechromatographed twice on the same column to yield a single protein on SDS-polyacrylamide gels [20]. This pure protein is referred to as fraction II.

Results

Effects of embryo extract fractions on chondroblast morphology

Fraction II from CM-cellulose column chromatography (Fig. 1) migrated as a single component on SDS-polyacrylamide disc gels with a molecular weight of about 72 000 (Fig. 2) and represented approximately 0.2% of the total protein of whole embryo extract. The effects of this material on chondroblast morphology were determined. Control cells were polygonal and grew in clones, (Fig. 3A), whereas cells grown 3 days in 0.5*X* fraction II were elongated and spindle shaped (Fig. 3B) and were similar to those grown in whole embryo extract or fraction I. Since fraction II caused morphological changes as low as 0.1*X* (17 μg/ml protein), it was calculated that the material was effective at concentrations as low as $2 \cdot 10^{-7}$ M. The protein nature of fraction II was shown since activity was destroyed by boiling or by treatment with pepsin. The isoelectric point (focusing on polyacrylamide gels) was about 8.4 which is consistent with the ion exchange elution properties and amino acid content. Preliminary analysis indicated that about 22% of the amino acid residues consisted of lysine, histidine and arginine.

Effects of fraction I on the synthesis and partitioning of collagen and glycosaminoglycans

During an 8-day growth period, chondroblasts converted radioactive proline to hydroxyproline. The % Hyp values for the controls were relatively constant between 15 and 18%, whereas in cultures grown and pulsed with fraction I, this value was lower during the initial 24 h and steadily decreased during the first

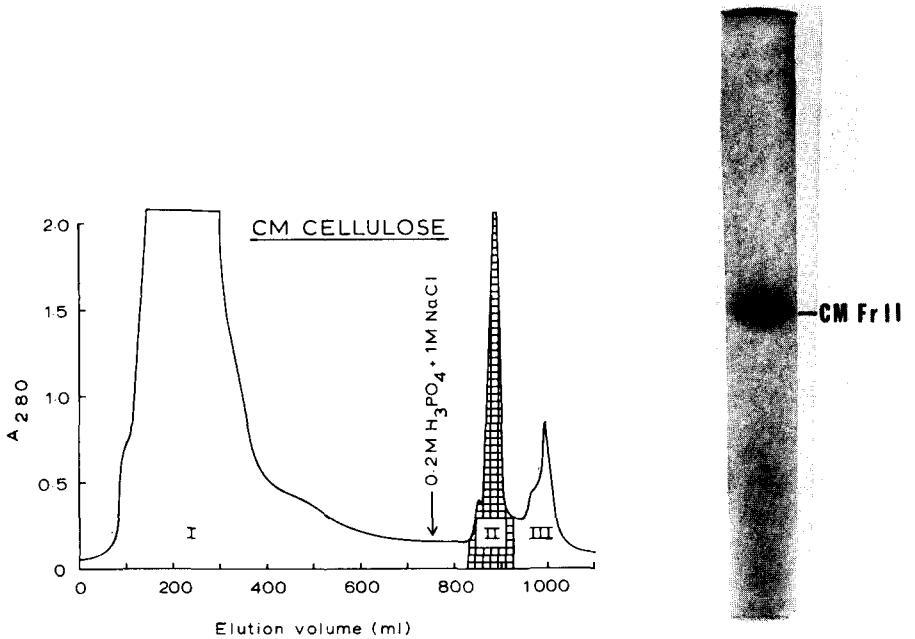

Fig. 1. Carboxymethyl cellulose column chromatograph profile of the void volume peak (fraction I) from a DEAE cellulose column. All the morphological dedifferentiation activity was recovered in the shaded peak fraction II.

Fig. 2. Sodium dodecyl sulfate-polyacrylamide disc gel of fraction II. Fraction II from the CMC Column of Fig. 1 was rechromatographed twice, collected and subjected to electrophoresis. Although the sample was treated with 2-mercaptoethanol, the same pattern was obtained without reduction.

4 days (Fig. 4, upper panel). These data suggested that collagen synthesis was differentially reduced with respect to noncollagen proteins as morphological dedifferentiation proceeded. Fraction I had little effect on the growth rates of these cells. At the end of this experiment control cultures averaged $1.07 \cdot 10^6$ cells/dish for a 7.5-fold increase whereas fraction I treated cultures increased 7.8-fold. The partitioning of the newly-synthesized collagen between the cell layer and medium in this experiment was determined (Fig. 4, lower panel). The soluble medium collagens consist of large molecular weight procollagen species which are cleaved into smaller gamma components and assembled into fibers (see Ref. 21 for a review of procollagen). In the controls, during these 24-h pulses the proportion of the total labeled collagen which was recovered in the medium averaged about 10%. By contrast, after the first 24 h in fraction I up to 30% of the collagen was recovered in the medium.

In a parallel experiment to that shown in Fig. 4, chondroblasts were pulsed on consecutive days with [^{3}H]glucosamine to assess the effects of fraction I on glycosaminoglycan synthesis. Total synthesis rates increased during the 8 days (Fig. 5, upper panel) and cultures grown and pulsed in fraction I did not differ significantly from the controls. From the partitioning (Fig. 5, lower panel), it is apparent that as the cultures became more confluent a larger percentage of the newly-synthesized glycosaminoglycans were recovered in the cell layer,

Fig. 3. Effects of fraction II on chondroblast morphology. Chondroblasts were plated for a 40-h period in control medium, at which time fraction II (0.5X = 86 μg/ml protein) was added to half the cultures. Cultures were fixed in 10% phosphate-buffered formalin, stained by hematoxylin and eosin and photographed 3 days later (X320). A, control; B, fraction II-treated.

presumably as insoluble matrix components. As seen for collagen, the dedifferentiating chondroblasts were less capable of utilizing newly-synthesized glycosaminoglycans to form insoluble matrix.

Effects of fraction I on the turnover of collagen, noncollagen and glycosaminoglycans

'Pulse-chase' experiments were conducted to determine what happens to existing chondroblast macromolecules during morphological dedifferentiation. Cultures were 'pulsed' with [^{3}H]proline or [^{3}H]glucosamine for 40 h in control medium, and then 'chased' 5 days with nonlabeled medium with or without fraction I. The noncollagenous proteins were determined on the same cultures as the collagenous proteins. The results from this experiment (Fig. 6) are expressed in terms of the percentage of the original dpm of the particular class of macromolecule remaining in the cell layer. Noncollagenous proteins were lost during the 5-day chase period, and the half-life was estimated to be about 55 h. By contrast, loss of collagen from the cell layers was very low and a semilog plot of the best-fitting line through the points suggested a half life of about 130 days. Fraction I had no effect on turnover of either collagenous or noncollagenous proteins. The glycosaminoglycans displayed a rate of turnover estimated to be about 75 h, and fraction I did not affect this rate.

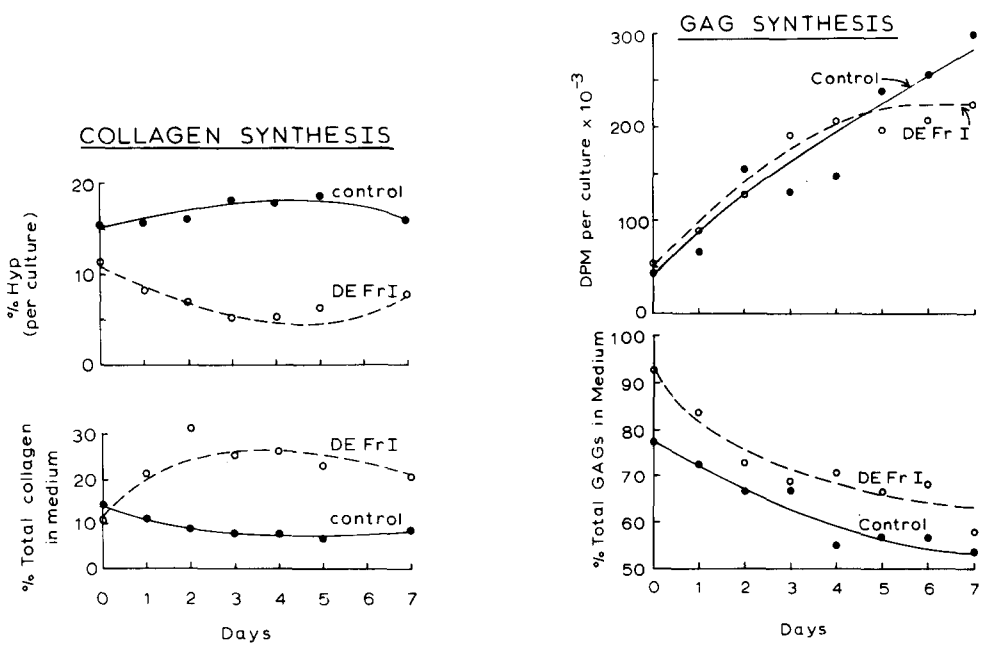

Fig. 4. Effects of fraction I on synthesis and partitioning of collagen by chondroblasts. Cultures were plated at $1.5 \cdot 10^5$ cells/dish and pulsed on consecutive days for 24-h periods with [^{3}H]proline (with or without fraction I). Cells and medium were analyzed separately for content of labeled proline and hydroxyproline. All cultures were fed daily with control medium or medium containing 0.2X fraction I (0.45 mg/ml protein). Upper panel, %, Hyp; lower panel, % of the total hydroxyproline recovered in the medium.

Fig. 5. Effects of fraction I on synthesis and partitioning of glycosaminoglycans. Chondroblast cultures were set up as described in the legend to Fig. 4, pulsed for 24-h periods with [^{3}]glucosamine and cells and medium analyzed separately for content of labeled glycosaminoglycans. Upper panel, total glycosaminoglycans synthesized; lower panel, % of total glycosaminoglycans recovered in the medium.

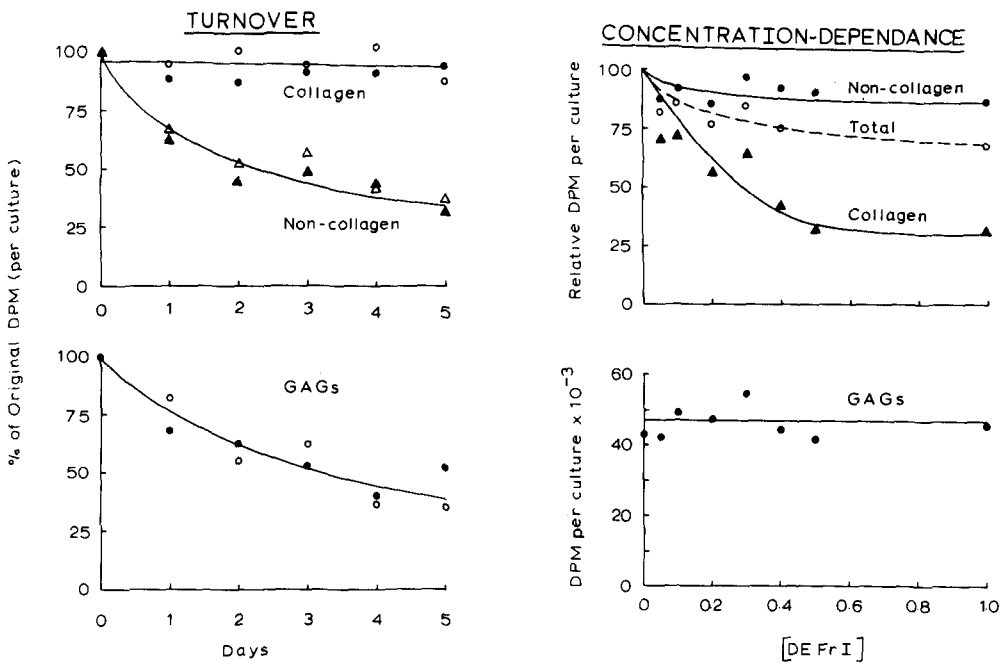

Fig. 6. Effects of fraction I on turnover of collagen, noncollagenous proteins and glycosaminoglycans. Replicate cultures were labeled 40 h with [^{3}H]proline or [^{3}H]glucosamine, and rinsed and incubated 30 min in medium without isotope. One-half of the cultures were then fed with fresh control medium (closed circles and triangles) and one-half in medium containing 0.2*X* fraction I (0.45 mg/ml protein, open circles and triangles). All cultures were fed daily in these respective media, triplicate cultures were removed at daily intervals and the cell layers examined for content of labeled collagenous proteins and noncollagenous proteins (upper panel) or glycosaminoglycans (lower panel).

Fig. 7. Effects of increasing concentrations of fraction I on synthesis of collagenous proteins, noncollagenous proteins and glycosaminoglycans. Triplicate cultures were incubated 24 h with [^{3}H]proline or [^{3}H]glucosamine in the presence of increasing concentrations of fraction I and assayed for synthesis of the three groups of macromolecules. Total protein synthesis, collagen synthesis and noncollagen protein synthesis was determined on the same cultures and the values were normalized to the untreated controls. Upper panel, relative dpm/culture of the proteins; lower panel, total glycosaminoglycans/culture. Fraction I concentration in '*X*' units, where 1*X* = 2.4 mg/ml protein.

Concentration effects of fraction I on collagen, noncollagen and glycosaminoglycan synthesis

The data presented in Figs. 4 and 5 demonstrated that fraction I caused inhibition of collagen synthesis but had no effect on glycosaminoglycan synthesis. An experiment was performed to determine if this effect was concentration-dependent. To directly compare the results for the proteins, the data (Fig. 7, upper panel) are expressed in terms of relative dpm of each class, and have been normalized to the control. At the highest concentration tested (1*X*), total protein synthesis was inhibited about 25% and the largest part of this inhibition was due to a 70% inhibition of collagenous proteins. Noncollagenous proteins were only slight affected (10%) and glycosaminoglycan synthesis was unaffected (Fig. 7, lower panel).

TABLE I

COMPARISON OF THE [^{3}H]HYDROXYPROLINE CONTENT AND COLLAGENASE SENSITIVITY OF LABELED PROTEINS SYNTHESIZED BY CONTROL OR FRACTION I-TREATED CELLS

Replicate log-phase cultures of chick chondroblasts, chick fibroblasts or human fibroblasts were incubated 24 h in F-10 medium with or without 0.5*X* concentration of fraction I (1.2 mg/ml). All media contained 50 μg/ml ascorbic acid and 55.5 kBq/ml [^{3}H]proline. At the end of the incubation one half of the cultures were analyzed for labeled proline and hydroxyproline and one half were assayed for digestibility with collagenase. Each value represents the mean ± S.D. from 9 cultures (3 separate experiments with 3 cultures per experiment).

Culture	Total dpm per culture	% Hyp	% Digested by collagenase
Chick chondroblast			
Control	265 520 ± 18 908	15.0 ± 1.4	10.9 ± 0.8
0.5*X* Fraction I	235 831 ± 44 027	9.9 ± 1.9	5.5 ± 1.7
Chick fibroblast			
Control	1 972 351 ± 136 941	5.7 ± 0.4	13.3 ± 3.7
0.5*X* Fraction I	1 757 611 ± 471 030	2.9 ± 1.1	7.9 ± 1.7
Human fibroblast			
Control	93 366 ± 5 514	5.0 ± 0.8	10.3 ± 3.7
0.5*X* Fraction I	98 207 ± 9 480	5.1 ± 1.2	11.7 ± 3.8

Collagen synthesis versus prolyl hydroxylation

Since newly-synthesized collagen was assayed by the extent of conversion of proline to hydroxyproline, decreased hydroxylation in the presence of fraction I would appear as inhibition of collagen synthesis. To test for effects on hydroxylation, the extent of hydroxylation of radioactive, peptide-bound proline was compared with the extent to which the total newly-synthesized proteins were digested with collagenase. This experiment (Table I) demonstrated that the reduction of [^{3}H]hydroxyproline in chondroblasts treated with fraction I was paralleled by a reduction in the amount of colla-

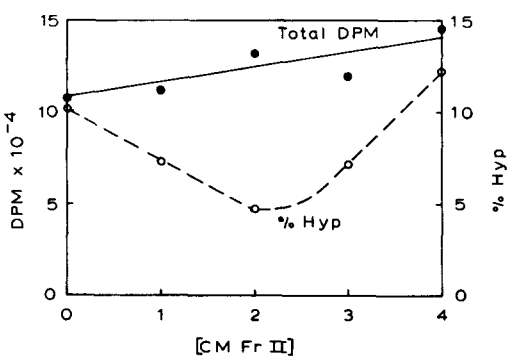

Fig. 8. Effects of increasing concentrations of fraction II on collagen and total protein synthesis by chondroblasts. Triplicate cultures were incubated 24 h with [^{3}H]proline in the presence of increasing concentration of fraction II and assayed for content of radioactive proline and hydroxyproline. The total dpm/culture is the sum of the proline and hydroxyproline. Fraction II concentration is expressed in 'X' units, where 1*X* = 172 μg/ml protein.

genase-sensitive radioactivity. This argues that fraction I caused decreased synthesis of collagen by these cells. Fraction I caused a similar inhibition of collagen synthesis by chick tendon fibroblasts, but had no effect on human fibroblasts. Thus, fraction I exhibited species but not tissue specificity.

Effects of fraction II on chondroblast collagen synthesis

Pure fraction II was tested for effects on chondroblast collagen and noncollagen protein synthesis, and the results (Fig. 8) demonstrated that the material caused a reduction in collagen synthesis at concentrations up to $2X$, but synthesis returned to control levels at $4X$. Unlike fraction I, this material appeared to cause a concentration-dependent increase in total protein synthesis.

Discussion

Embryo extract causes replicating chick chondroblasts in culture to undergo numerous morphological changes and the synthetic program for collagen and glycosaminoglycans changes from that of a chondroblast to that of a fibroblast. In this study we have examined this dedifferentiating population of cells for their changing capcity to synthesize, partition and retain these two major classes of macromolecules. We have found that embryo extract fraction I (DEAE-cellulose column chromatography) caused a concentration-dependent reduction in collagen synthesis, with a maximal inhibition of about 70%, whereas noncollagen proteins were only slightly affected (10%) and glycosaminoglycan synthesis unaffected. This differential inhibition of collagen synthesis appeared to be species but not tissue specific, since similar inhibition occurred in chick, but not in human fibroblasts. The turnover of macromolecules was measured in control and fraction I-treated chondroblasts, and the half-lives, which were similar in both group of cells, were estimated to be greater than 4 months for collagenous proteins, 55 h for noncollagenous proteins, and 75 h for glycosaminoglycans. Thus, even though the species of collagen and glycosaminoglycans synthesized changes during dedifferentiation [9], the existing cartilage species were not differentially lost.

Although the impure fraction I and the pure fraction II both significantly suppressed collagen synthesis by control and dedifferentiated chondroblasts, it has not yet been determined if types I and II collagens were inhibited to the same extent. We know that both collagen types are synthesized by the dedifferentiated chondroblast and type I predominates [9,12]. When dedifferentiation occurs spontaneously or is induced by BrdUrd or low concentrations of embryo extract, total collagen synthetic rates remain unchanged while type II synthesis is reduced and type I is induced. Consequently, it is likely that the net synthesis of a given collagen species will reflect both the direct inhibitory action of the embryo extract active fraction and the changed synthetic program of the dedifferentiated cell. The pure embryo extract fraction II caused reduction in the % hypro values at concentrations up to $2X$, but at $4X$ the values returned to those of the controls. We have no explanation for this biphasic response, and since concentrations of the impure fraction I greater than $2X$ were found to be cytotoxic, it could not be determined whether this

material caused a similar response. It is also of interest that the pure fraction II appeared to cause a concentration-dependent increase in noncollagen protein synthesis whereas fraction I was slightly inhibitory. Reduced synthesis in the presence of fraction I could be caused by toxic agents which would be absent in fraction II. Alternatively, either fraction may have modified the rates of labeled proline uptake or changed the intracellular proline pool sizes, thus creating an apparent change in total synthetic rate.

The protein we have isolated from embryo extract (fraction II) which causes chondroblasts to dedifferentiate has a molecular weight of about 72 000 and is a basic protein based on ion exchange elution characteristics, isoelectric point and content of lysine, arginine and histidine. This material may be the active component of the 'H' or heavy fraction of embryo extract which was studied by Coon and Cahn [22]. Using graded Sephadex columns, they showed embryo extract contained a fraction which prevented the phenotypic expression of chick chondroblast and pigmented retinal cells in culture. This H fraction was heat-labile, insensitive to hyaluronidase digestion, and the molecular weight was between 50 000 and 100 000.

What, if any, is the physiological significance of the embryo extract active fraction? One function could be to regulate the amount of collagen a cell synthesizes. Since the active fraction is probably present at all times, its ability to suppress collagen synthesis by a given cell may depend upon the acquisition by that cell of specific receptor sites for interaction. Such sites could arise in cells at specific times in development or even during specific portions of the cell cycle (G1, S, G2 or M). This latter possibility could explain why embryo extract does not induce chondroblast dedifferentiation in pellets or intact cartilaginous vertebrae [9,23] since cell replication is known to be minimal in both these culture conditions. Schiltz [24] presented evidence that chick embryo chondroblasts consist of 2 subpopulations of cells which have different generation times and synthesize collagen at different phases of the cell cycle. It will be interesting to determine if both these populations are responsive to embryo extract. Another function of the active fraction could be to regulate the type of collagen synthesized by cells in the chondrogenic lineage, perhaps to prevent the synthesis of type II collagen in cartilage precursor cells. It is known that type I collagen is synthesized by cartilage precursor cells of somite mesoderm [25,26]. Once chondrogenesis has occurred however, the definitive chondroblast in the embryo may no longer be responsive to the active fraction, and unless conditions were altered which would reintroduce responsiveness, the cells would continue to synthesize cartilage-type collagen and the organ would grow. Such alterations could be programmed in the embryo or could arise as a result of injury (which would induce dedifferentiative changes in vivo) or from the induction of cell replication during culture.

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