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Changes in Synthesis of Types-I and -III Collagen during Matrix-Induced Endochondral Bone Differentiation in Rat

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The changes in rates of hydroxyproline formation and biosynthesis of types-I and -III collagen during bone-matrix-induced sequential differentiation of cartilage, bone and bone marrow in rat were investigated. Biosynthesis of types-I and -III collagen at different stages of this sequence was studied by labelling *in vivo* and *in vitro* with [2,3-^{3}H]proline. Pepsin-solubilized collagens were separated by sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis. The results revealed that maximal amounts of type-III collagen were synthesized on day 3 during mesenchymal-cell proliferation. Thereafter, there was a gradual decline in type-III collagen synthesis. On days 9–20 during bone formation predominantly type-I collagen was synthesized. Similar results were obtained by the use of labelling techniques both *in vivo* and *in vitro*.

The growth of the long bones occurs via proliferation of mesenchymal cells, differentiation and hypertrophy of chondrocytes and calcification of cartilage matrix, before replacement of the calcified cartilage by bone formation in the metaphyses. In view of the complicated spatial and temporal changes that occur in this process of endochondral bone development, biochemical analyses of changes in collagens during different stages of cellular differentiation are technically difficult. However, these difficulties can be circumvented by the matrix-induced endochondral bone-forming system (Reddi & Huggins, 1972; Reddi & Huggins, 1975; Reddi & Anderson, 1976). This method consists of subcutaneous implantation of demineralized diaphyseal bone matrix into allogenic rats and results in the induction of new endochondral bone formation. On day 3 responding fibroblasts (mesenchymal cells) appear in the vicinity of matrix, interact with the matrix, proliferate and emerge as chondroblasts on days 5 and 6. The implant consists of differentiated cartilage on day 7. These chondrocytes undergo hypertrophy, and on day 9 calcification of the matrix is evident. Bone formation begins on day 10 after vascular invasion. The newly formed bone is remodelled and the resultant ossicle permits bone-marrow differentiation by day 21. The response to bone matrix is specific and is not elicited by tendon matrix (Reddi, 1976).

We have utilized immunofluorescent techniques previously and demonstrated the intricate transitions in collagen types I, II and III during experimentally induced sequential differentiation of cartilage, bone and bone marrow (Reddi *et al.*, 1977). Type-III collagen was localized on day 3 around invading and proliferating mesenchymal cells. On days 4–6 smaller amounts of type I were detected. Type-II collagen was seen in differentiating cartilage matrix on day 7. With the advent of vascular invasion and osteogenesis, type-I collagen was detected in the new bone matrix. Type-III collagen was also localized in developing haematopoietic bone marrow. Although immunohistochemical studies are informative and provide the cellular and tissue localization, they do not give rates of synthesis. The present study describes the quantitative changes in hydroxyproline formation and the kinetics of synthesis of types-I and -III collagen both *in vivo* and *in vitro* during various phases of matrix-induced endochondral bone differentiation.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Materials and Methods

Preparation and implantation of matrix

Dehydrated diaphyseal shafts of rat femur and tibia were pulverized in a CRC Micro Mill (Technilab Instruments, Vineland, NJ, U.S.A.) and sieved to a discrete particle size of 74–420 μm. The powders

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were demineralized with 0.5 M-HCl, extracted sequentially with water, ethanol and ether, and prepared as described (Reddi & Huggins, 1972). Demineralized bone matrix (20–25 mg) was implanted subcutaneously in bilateral sites in the thoracic region in male rats of Long–Evans strain (age 28–44 days) under ether anaesthesia such that on the day of labelling the button-like plaques were 3, 7, 9, 12, 14 and 20 days old. These days correspond to various stages of endochondral bone development: day 3, proliferation of mesenchymal cells; day 7, chondrogenesis and cartilage formation; day 9, calcification of cartilage; day 12, bone differentiation and mineralization; day 14, bone formation and remodelling; day 20, bone-marrow differentiation. Each group consisted of six rats, four for labelling *in vivo* and two for incorporation *in vitro*.

Labelling with [^{3}H]proline in vivo

The 24 rats used for labelling weighed 170 ± 11 g (mean ± S.D.) and were 47–48 days old. They were injected intraperitoneally with 75 mg of β-aminopropionitrile fumarate in 1.5 ml of phosphate-buffered saline (0.02 M-sodium phosphate buffer, pH 7.4, containing 0.15 M-NaCl) 10 min before similar administration of [2,3-^{3}H]proline (5 μCi/g body wt.; specific radioactivity 25 Ci/mmol; New England Nuclear). The rats were killed 6 h later and the two plaques were dissected out, weighed and homogenized separately, with the aid of a Polytron homogenizer (Brinkmann Instruments), in ice-cold 0.5 M-acetic acid. Homogenization was performed at the maximum setting for three 10 s periods with 5 s pause, and the homogenates were dialysed extensively against 0.5 M-acetic acid in the cold. Pieces of liver and calvarium (parietal and frontal bones) were processed similarly and served as controls. To assess variations of labelling, blood was collected by cardiac puncture in heparinized syringes. Radioactivity was determined in the blood plasma and found to be $1.20 \times 10^6 \pm 0.14 \times 10^6$ d.p.m./100 μl. Of this radioactivity 30–40% remained in the supernatant after 5% (w/v) trichloroacetic acid precipitation.

Labelling in vitro

The two plaques from each animal were dissected free of adherent tissue, pooled, weighed and minced in small cubes of approx. 1 mm^3. The tissues were preincubated in the Dulbecco–Vogt modification of Eagle's medium (Gibco, Grand Island, NY, U.S.A.) (1 ml of medium/100 mg of tissue) supplemented with ascorbic acid (100 μg/ml), β-aminopropionitrile fumarate (100 μg/ml), penicillin (50 i.u./ml) and streptomycin (50 μg/ml), but lacking glutamine and serum, in humidified air/CO_2 (19:1) on a rocking platform. After a 30 min preincubation, the medium was removed and replaced by fresh medium supplemented in addition with 100 μCi of [2,3-^{3}H]proline/ml (25 Ci/mmol). At the end of 6 h, labelling was terminated by the addition of acetic acid to yield a final concentration of 0.5 M; the samples were homogenized, and the homogenates were dialysed and processed as described for the plaques labelled *in vivo*.

Determination of hydroxyproline and relative rates of collagen synthesis

Samples of the dialysed homogenates were hydrolysed in 6 M-HCl at 110°C for 24 h and the hydroxyproline was determined by the chemical method of Juva & Prockop (1966) as modified by Bradley *et al.* (1974). The recovery of [^{3}H]hydroxyproline was determined by addition of [U-^{14}C]-hydroxyproline (New England Nuclear) as a standard and the values were corrected. These values were compared with that obtained by ion-exchange chromatography on Dowex AG 50W (X8; 200–400) mesh; Bio-Rad) in 25 cm × 1 cm columns as described (Cutroneo *et al.*, 1972). The two methods yielded similar values, since the ratios of hydroxyproline formed to the total radioactivity incorporated, estimated on the same sample by the chemical and chromatographic methods, were 0.150 and 0.134 respectively. This finding ruled out any potential loss of ^{3}H during the oxidation of [2,3-^{3}H]hydroxyproline by the chemical method of Juva & Prockop (1966). The amount of newly synthesized collagen per total protein was calculated by the formula of Green & Goldberg (1964).

Radioactivity was routinely determined with 10 ml of Hydromix (Yorktown Research, Yorktown, PA, U.S.A.) in a Beckman liquid-scintillation counter, and results are expressed in d.p.m. DNA was determined in samples of the homogenates by the diphenylamine procedure (Burton, 1956).

Purification and quantification of labelled collagens

To extract and purify newly synthesized collagen, limited proteolysis was performed on portions of the homogenates with 1% (w/w) pepsin (3 × crystallized; Calbiochem) in 0.5 M-acetic acid, pH 2.0 (adjusted with HCl) at 15°C for 6 h with constant stirring. Pepsin was inactivated by increasing the pH of the reaction mixture to 8.0 with Tris (50 mM) and diluted NaOH. A mixture of proteinase inhibitors [20 mM-EDTA / 1 mM-*p*-hydroxymercuribenzoate/ 0.01 mM-phenylmethanesulphonyl fluoride (Sigma), final concentrations] was added to the samples, which were then stirred for 16 h. The collagenous proteins were precipitated with 20% (w/v) NaCl and collected by centrifugation at 27000 ***g*** for 60 min. The pellet was resuspended and dialysed against 0.5 M-acetic acid. The insoluble material was removed by centrifugation at 27000 ***g*** for 60 min and

portions of the supernatant were freeze-dried and used for electrophoresis.

Quantification of collagen types was performed by SDS/polyacrylamide-slab-gel electrophoresis and differential salt precipitation. Freeze-dried portions containing equal amounts of radioactivity were resuspended in the sample buffer, heated (100°C for 2min), and 25μl portions were electrophoresed on SDS/polyacrylamide slab gels (5% acrylamide/0.13% bisacrylamide for the separating gel; the stacking gel was 3% acrylamide). The buffer system was that of Laemmli (1970) with minor modifications; 0.5M-urea was added to all solutions and the running buffer was 0.2M-glycine/0.025M-Tris. Electrophoresis was performed at a constant current of 50mA/plate (140mm x 95mm x 1.5mm), and the assembly was cooled by circulating tap water. To assess the amount of reducible $\alpha 1(III)_3$ chains, appropriate samples were reduced after 45min by the addition of 2.5μl of 14M-β-mercaptoethanol (Sykes *et al.*, 1977), and electrophoresis was continued for another 45–60min until the dye front migrated to the end of the slab gel. The gels were stained (Fairbanks *et al.*, 1971) and fluorograms prepared as described (Bonner & Laskey, 1974; Laskey & Mills, 1975). The relative amounts of specific proteins were quantified by densitometry at 540nm. Further, differential salt precipitation was performed on pepsin-solubilized salt-precipitable (20%, w/v, NaCl) collagen. The collagenous proteins were dissolved in 1.0M-NaCl in 0.05M-Tris/HCl, pH7.4, and 1mg each of rat type I (Bornstein & Piez, 1966) and rat type II (Smith *et al.*, 1975) collagen were added as 'carriers'. The samples were centrifuged at 27000*g* for 1h and the supernatants then dialysed against 2.5M-NaCl/0.05M-Tris/HCl, pH7.4, for 48h. The material was centrifuged at 27000*g* for 1h and the radioactivity was determined in the precipitates and supernatants.

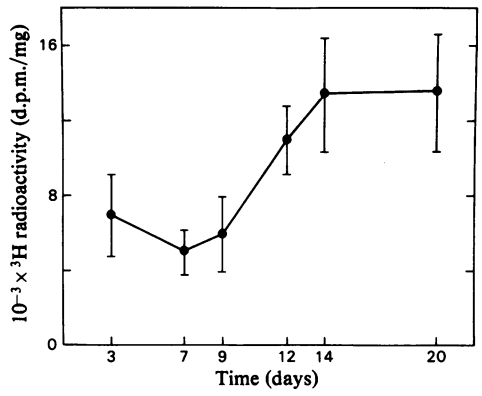

Fig. 1. *Incorporation in vivo of [^{3}H]proline into plaques at various stages of endochondral bone differentiation*
The rats were injected with 5μCi of [2,3-^{3}H]-proline/g body wt. 6h before being killed. The plaques were homogenized in 0.5M-acetic acid and dialysed as described in the Materials and Methods section. The results are expressed as radioactivity incorporated/mg wet wt. (d.p.m./mg). Each point represents the mean ± S.E.M. for 16 determinations from four rats.

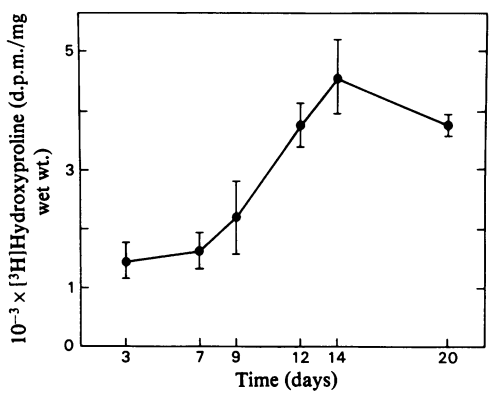

Fig. 2. *Formation in vivo of protein-bound [^{3}H]hydroxyproline in plaques at different stages of endochondral bone differentiation*
The plaques were labelled for 6h as described. Each point represents the mean ± S.E.M. for 16 determinations from four rats.

Results

Proline incorporation and hydroxyproline formation

The protein-bound non-dialysable hydroxyproline was determined as an index of collagen synthesis during discrete stages of matrix-induced endochondral bone formation. The experiments to be described were performed on two different occasions and identical results were obtained. The results presented are from the second experiment. Total incorporated radioactivity at different stages of bone development *in vivo* is depicted in Fig. 1. Maximal amounts of radioactivity were detected during bone formation on day 14. The production of hydroxyproline (d.p.m./mg of tissue) is shown in Fig. 2 and again the peak values are seen on day 14. Similar trends were seen when the results were expressed as d.p.m./mg of DNA. However, in this case there was a precipitous decline in the hydroxyproline/mg of DNA on day 20, possibly owing to increasing DNA content as a result of the appearance of haematopoietic bone marrow in the ossicle.

Relative amounts of collagen synthesized

The proportion of hydroxyproline per total radioactivity is presented in Fig. 3. There is a moderate increase in hydroxyproline on day 7 during cartilage differentiation. With the onset of calcification of the hypertrophied cartilage matrix there is a small but reproducible decline in collagen synthesis. During maximal bone formation the hydroxyproline values attained a much higher peak on day 14. The percentage of hydroxyproline in the newly induced bone (about 40%) is similar to that observed in the calvarium from these rats (about 45%). The hydroxyproline values were used to calculate the percentage of newly synthesized collagen per total protein synthesized (Green & Goldberg, 1964). It was observed that at the peak of bone differentiation newly synthesized collagen comprised 23.5% and is similar to the value found in the calvarium (Raisz *et al.*, 1976). Calvarium and liver from these rats were examined for collagen synthesis as examples of two tissues with marked differences in proportion of collagen synthesized. It is noteworthy that the collagen synthesized in the liver determined concurrently accounted for only 0.7% of total protein and was similar to the value reported by Dunn *et al.* (1977). In another experiment the capacity of plaques to synthesize collagen *in vitro* obtained at different stages were compared and found to be similar to the pattern *in vivo*; however, the proportion of hydroxyproline per total radioactivity was 30% lower.

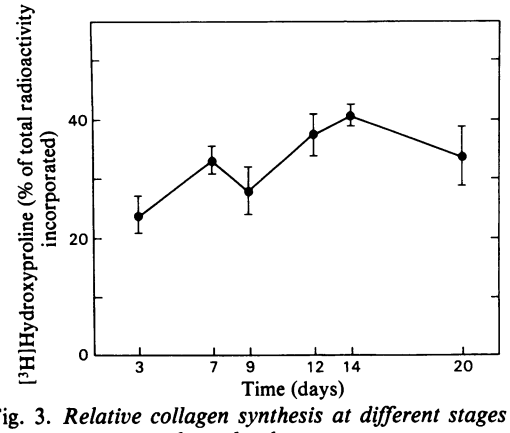

Fig. 3. *Relative collagen synthesis at different stages of bone development*

Results are expressed as [^{3}H]hydroxyproline (% of total radioactivity incorporated). Each point represents the mean ± S.E.M. for 16 determinations from four animals.

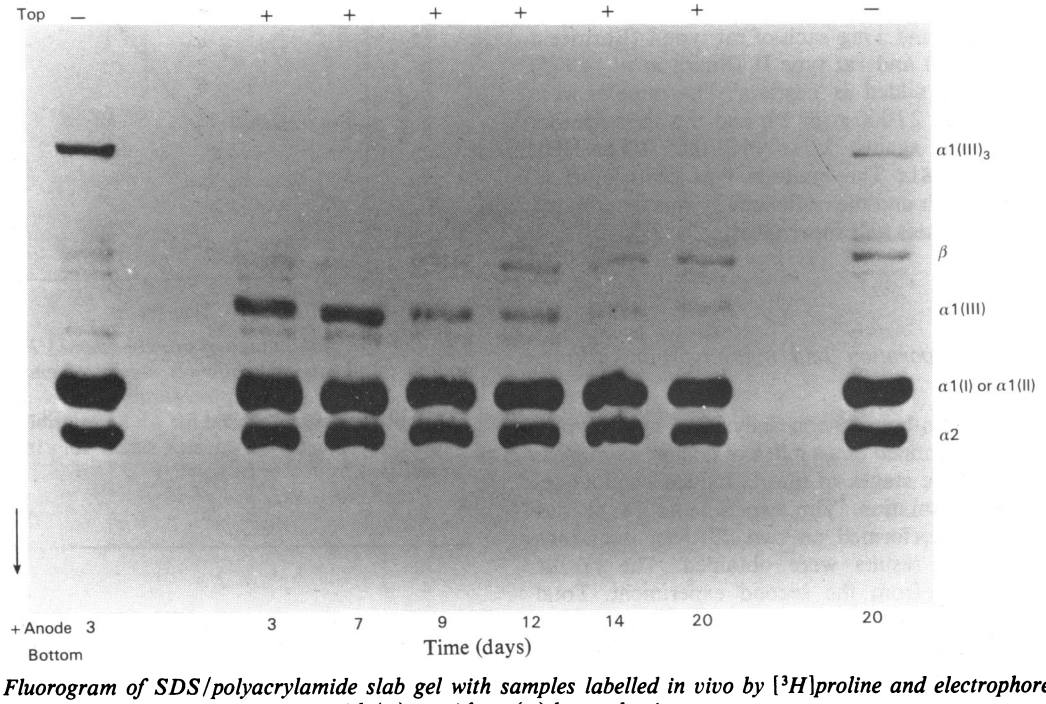

Fig. 4. *Fluorogram of SDS/polyacrylamide slab gel with samples labelled in vivo by [^{3}H]proline and electrophoresed with (+) or without (−) late reduction*

Each sample contained 12500d.p.m. The fluorogram was developed for 12 days. Note the gradual decline in reducible type-III collagen from day 3 to day 12.

Types of collagen synthesized

Previous immunohistochemical study revealed the occurrence of type-III collagen localization around proliferating mesenchymal cells as an early response to implantation of demineralized bone matrix. We quantified the relative proportions of types-I and -III synthesized at different phases of endochondral bone development. Pepsin-solubilized and purified radioactive collagen was subjected to SDS/polyacrylamide-slab-gel electrophoresis to separate the α 1(I) plus α 1(II) and α 2 chains from reducible α 1(III) components. The latter was distinguished by delayed-reduction with mercaptoethanol (Sykes *et al.*, 1977). The dried slab gels were used to develop fluorograms and the radioactive bands were quantified by densitometry. The results of the pulse-labelling of the plaques labelled *in vivo* revealed that maximal amounts of type-III collagen were synthesized on days 3–7 (Fig. 4). Thereafter there was a general decline, and minimal type-III collagen synthesis was observed on days 14 and 20. The $\alpha 1/\alpha 2$ chain ratios of the samples in the same gel were determined after exposure to X-ray film for shorter duration (2 days) to improve resolution for densitometry. The ratio was 3.2 on day 3, increased to 3.8 on day 7 and decreased to 2.8 and 2.3 on days 12 and 20 respectively. This suggested that on day 7 an appreciable amount of collagen was composed of α 1 chains. This finding was further confirmed by differential-salt-precipitation experiments. Dialysis of the samples with added types-I and -II carrier collagen against 2.5 M-NaCl in 0.05 M-Tris/HCl, pH 7.4, revealed that on day 7 the fraction of radioactivity soluble in 2.5 M-NaCl was higher (23%) than on days 3 and 12 (10.5 and 8.3% respectively). These observations substantiate the immunofluorescent localization of predominantly type-II collagen in day-7 plaques (Reddi *et al.*, 1977). Finally, under the present experimental conditions very little of the 'β' components was detected in the gels. Similar patterns of types of collagen were obtained with implants labelled *in vitro*.

Discussion

The bone-matrix-induced endochondral bone differentiation is an experimental model that recapitulates in the post-foetal rat the various phases of bone formation in epiphyseal growth plate (Reddi & Anderson, 1976). In brief, on implantation of demineralized bone matrix, a transient chemotaxis for polymorphonuclear leucocytes ensues. On day 3, fibroblast-like mesenchymal cells migrate to the vicinity of the matrix and undergo cell proliferation (Rath & Reddi, 1979). On day 7, the subcutaneous button-like implant contains newly differentiated hyaline cartilage interspersed among the particles of the implaned bone matrix in the central region while the periphery consists of perichondrium-like spindle-shaped cells. The chondrocytes undergo hypertrophy on day 9 and the cartilage begins to calcify. Bone formation begins on day 10 with the advent of vascular invasion. The newly formed bone is remodelled and the ossicle is the site of haematopoietic bone-marrow differentiation. The present study examined the relative rates of collagen synthesis in this system. There were two peaks of collagen synthesis: one on day 7 during chondrogenesis which decreased slightly during calcification of cartilage matrix on day 9; the second peak was larger and was seen on day 14, reflecting bone formation and remodelling and thereafter there was a decline. The proportion of collagen synthesized relative to total protein was similar to that in the calvarium.

The appearance and quantification of type-III collagen revealed that it is a major type of collagen synthesized on day 3. Lesser quantities of type III are observed in the plaques after day 7. On day 7 during maximal cartilage formation, type-II collagen synthesis was appreciable. These observations corroborate the previous immunohistochemical localization (Reddi *et al.*, 1977). However the previously documented localization of type-III collagen on day 17 could well be due to accumulated type-III collagen, as very little newly synthesized type-III collagen was detected on day 14 and later in the present study. Furthermore, this could be due to slow turnover of type-III collagen, as estimated in certain visceral organs (Klein & Chandrarajan, 1977).

The detection of type-III collagen synthesis during mesenchymal-cell proliferation in response to implanted matrix deserves additional comments. This phenomenon is similar to the occurrence of type-III collagen in the guinea-pig dermal wound scar (Barnes *et al.*, 1976). The matrix-induced endochondral bone formation described in the present study recapitulates the embryonic development of long bones. It is noteworthy that type-III collagen synthesis by mesenchymal cells is an early event before chondrogenesis. Previous investigations on the chick limb bud indicated that mesenchymal cells destined to form cartilage synthesized largely type-I collagen (Linsenmeyer *et al.*, 1973; von der Mark & von der Mark, 1977) both *in vivo* and *in vitro*. It is not clear whether these differences in transitions in collagen types reflect differences between mammalian and non-mammalian vertebrate skeletal morphogenesis.

The type-III collagen synthesis during early stages of mesenchymal proliferation is later replaced by predominantly type-I collagen during bone formation and remodelling in the present study. The switch in collagen synthesis to predominantly type I may not be operative in disease states such as osteogenesis imperfecta. Type-III collagen persisted in the

bones of one patient with osteogenesis imperfecta (Müller *et al.*, 1977).

Since the results obtained *in vivo* are similar to those observed in plaques incubated *in vitro*, it is likely that this would be an asset for future studies. For instance, it is known that the amount and proportion of types-I and -III collagen synthesized by fibroblasts in tissue culture is modulated by a variety of factors, such as cell density (Abe *et al.*, 1979), epidermal growth factor (Steinmann *et al.*, .1979) and prostaglandins (Baum *et al.*, 1978; Steinmann *et al.*, 1979). The availability of the present experimental model may be useful for further studies on factors modulating synthesis of types-I and -III collagen, such as hormones and other physiological and pharmacological agents *in vivo*.

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