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Abnormal Collagen Metabolism in Cultured Cells in Osteogenesis Imperfecta*

(collagen types/fibroblasts)

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ABSTRACT Cells obtained from normal human skin synthesize predominantly type I collagen in culture. Cells obtained from the skin of an infant with a severe form of osteogenesis imperfecta were found to synthesize as much type III as type I collagen. Decreased synthesis of type I collagen could explain the tissue fragility observed in this case.

Osteogenesis imperfecta (OI) is a heritable disorder of connective tissue characterized by fragile bones, but also including opalescent teeth, thin skin, and blue sclera (1). Although the disease is genetically and clinically heterogeneous, two main clinical varieties are recognized according to the onset and severity of symptoms. Defects in the tissues of the congenital form of the disease may prove lethal at birth while patients with the tarda form exhibit milder symptoms, most notably increased fractures of bones. Histological studies of tissues from patients with OI point toward an abnormality in collagen (2–4). The irregular pattern of collagen fibrils normally present in the skeletal tissues and cornea of fetuses and newborns persists until a much older age in patients with OI.

Recent studies have established the existence of several chemically and genetically distinct collagens. Type I collagen has the chain composition $[\alpha 1(I)]_2\alpha 2$ and is the major collagen species in mature skin, bone, and tendon (5). Skin, aorta, and certain other tissues also contain various amounts of type III collagen, $[\alpha 1(III)]_3$ (6–8). Type III collagen may account for half or more of the collagen in fetal skin, but less is found in the skin of older individuals. Type I collagen is the only collagen known to occur in mature bone (9). We show here that cells cultured from the skin of certain patients with severe forms of OI synthesize a higher proportion of type III collagen than cells from control skin.

MATERIALS AND METHODS

Source and Labeling of Skin Fibroblast Cultures. Cell strains were grown from skin biopsies of individuals with various forms of osteogenesis imperfecta at the American Type Culture Collection (ATCC), Rockville, Md., in modified Dulbecco–Vogt medium by standard techniques, and stored in liquid nitrogen. OI strains and control strains both age-matched and from fetal skin were thawed, plated in 75-cm^3 Falcon plastic flasks, and passed at least once prior to use in biosynthetic studies. To obtain maximal labeling of collagenous protein, the growth media were replaced with 10 ml of the Dulbecco–Vogt medium lacking glycine, lysine, glutamate, and serum but supplemented with ascorbic acid (50 μg/ml) and β-aminopropionitrile (50 μg/ml) (10). Twenty microcuries each of [*U*-^{14}C]glycine, [*U*-^{14}C]proline, and [*U*-^{14}C]-lysine were added to each flask. Usually cells were exposed to label for 24 hr, except in the studies designed to measure the extractability and synthetic rates of collagen where labeling was carried out for 96 hr without β-aminopropionitrile.

Preparation of Labeled Proteins. At the termination of the labeling period, phenylmethylsulfonylfluoride (50 μg/ml) was added to the medium as well as to the cell layer suspension to suppress possible proteolytic alterations of the labeled proteins (11). The cell layer was suspended in 10 ml of 1 M NaCl, 0.05 M Tris·HCl (pH 7.5) at 4° for 1–3 days; insoluble material was recovered by centrifugation. The cell layers from cultures labeled without β-aminopropionitrile were extracted with 1 M NaCl as above, and the residues were re-extracted with 10 ml of 0.5 M acetic acid and subsequently with 5 ml of 2 M $CaCl_2$.

Analysis and Chromatography of Labeled Collagens. After dialysis of medium and cell layer fractions, we estimated the proportion of collagenous and noncollagenous protein by a specific enzymatic method, using bacterial collagenase (12). In some studies the ratio of [^{14}C]hydroxyproline to [^{14}C]-proline and [^{14}C]hydroxylysine to [^{14}C]lysine in proteins was determined in hydrolyzed samples with an automated amino-acid analyzer coupled to a liquid scintillation spectrometer equipped for flow counting.

The amounts of type I and type III procollagens present in the medium after the labeling period were estimated by separating these proteins by chromatography on DEAE–cellulose (13) and measuring the incorporated label.

In other experiments, medium and cell layer were combined, carrier collagen (20 mg) was added, and these samples were incubated with pepsin under conditions (10) designed to effect the limited cleavage of precursor forms to collagen and solubilize precipitated collagen. After 6 hr the pepsin was inactivated and insoluble material was removed by centrifugation. A portion of the supernatant fluid was reduced in 8 M urea at pH 8.6 with 0.02 M mercaptoethanol for 4 hr. Reduced and nonreduced samples were dialyzed against 0.03 M sodium acetate (pH 4.8) and chromatographed in 4 M urea at 45° on carboxymethyl(CM)–cellulose (14).

Preparation of Standards. Type I collagen was extracted with acid from rat and human skin and purified by a standard

Abbreviations: OI, osteogenesis imperfecta; CM–cellulose, carboxymethyl-cellulose.

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procedure (14). Type III collagen was solubilized by limited digestion of human skin with pepsin, as described by Epstein (7), Chung and Miller (8), and Trelstad (15). Type III collagen was separated from type I collagen by an initial precipitation with 1.5 M NaCl and purified further by chromatography on CM-cellulose (7, 8, 15). In some studies labeled components, as well as authentic $\alpha 1$(I), $\alpha 2$, and $\alpha 1$(III), were digested with CNBr after reduction and alkylation. Peptides produced by CNBr cleavage were partially resolved by chromatography on CM-cellulose (6) or by electrophoresis on 7.5% sodium dodecyl sulfate-acrylamide gels (16). The radioactivity in labeled extracts, column effluents, and gel fractions was assayed by standard techniques of scintillation spectrometry.

RESULTS

Rates of Collagen Synthesis. In our initial studies the proportions of collagenous to noncollagenous proteins synthesized by an OI cell strain (ATCC no. 1262, baby girl D), and an age-matched control skin (no. 1146) were estimated by two methods. In one study, cells were labeled with radioactive amino acids for 24 hr, and the radioactivity incorporated into peptide-bound hydroxyproline, proline, hydroxylysine, and lysine in dialyzed aliquots of media, into extracts of the cell layer, and into the insoluble residues, was determined with an amino-acid analyzer. In the second experiment (done without β-aminopropionitrile), we used collagenase digestion to measure the collagen content. Both studies showed that similar amounts of collagen were synthesized by the OI and control strains. In a labeling experiment without β-aminopropionitrile, 85% of the collagenous protein synthesized by both cell strains was located in the medium and only 15% in the cell layer 96 hr after the addition of labeled amino acids. No significant difference was noted in the extractability of collagen from the cell layer. The ratios of peptidyl [^{14}C]hydroxyproline to [^{14}C]proline and [^{14}C]hydroxylysine to [^{14}C]lysine were similar in these two strains and resembled those values previously published (10).

Characterization of Collagen Precursors Secreted by OI and Control Cells. Subsequent studies were directed toward characterizing the collagenous proteins made by the cell strains. It has been shown that most of the collagenous protein found in the medium is soluble precursors of type I and type III collagens, which can be separated by chromatography on DEAE-cellulose. By this method radioactive protein was eluted by the gradient in three regions labeled A, B, and C in Fig. 1. Peak A contains primarily type I collagen. Peak B contains precursors of type I collagen, while peak C contains precursors of type III collagen (17). The identification of the three fractions A, B, and C from OI strain no. 1262 was confirmed by digesting the proteins with CNBr and chromatographing the resulting peptides on CM-cellulose. Fractions A and B produced the profile of peptides expected from type I collagen, while fraction C produced the profile of peptides found in type III collagen. Type I procollagen was the most prominent constituent in control medium, whereas the proportions of type I and type III procollagen were markedly different in the OI strain no. 1262. When the same amount of radioactive protein was applied to the column, more collagenase-digestible material was recovered in the region identified as fraction C in Fig. 1 in the medium of strain no. 1262 than from the control strain no. 1146. The ratio of type I to type III

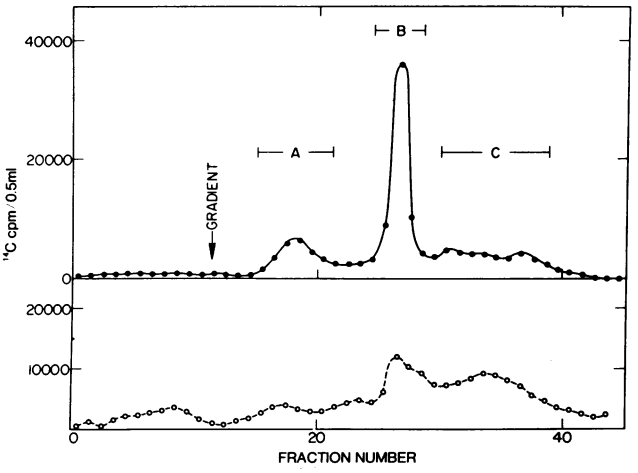
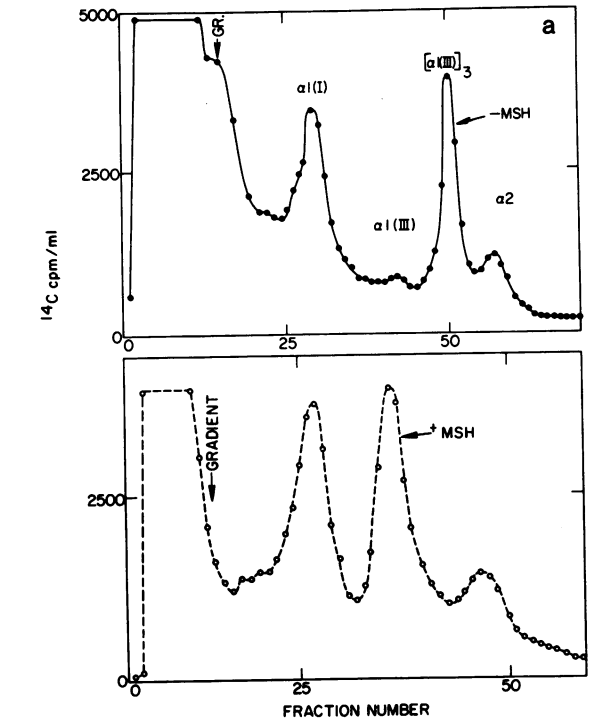


FIG. 1. DEAE-cellulose chromatography of the labeled proteins secreted by the fibroblasts into the medium. Top panel, control medium (ATCC no. 1146); bottom panel, OI medium (no. 1262).

precursors of collagen in the media of control cells averaged 3-4 to 1, whereas the ratio in the medium from OI strain no. 1262 was closer to 1. These results indicate that the OI strain no. 1262 secretes type I and III procollagens in altered proportion.

Characterization of Pepsin-Treated Collagens. In subsequent studies, we estimated the synthesis of type I and type III collagen in medium as well as in cell layer by measuring the amounts of $\alpha 1$(I) and $\alpha 1$(III). Here we took advantage of the fact that the denatured components of type III collagen are resolved from the α chains of type I collagen on CM-cellulose. In these experiments, medium and cell-associated proteins were combined and incubated with pepsin under conditions that cause only a limited digestion of native molecules (10, 18). This procedure converts precursor forms to collagen-like molecules. The components of this digest were denatured and resolved by chromatography on CM-cellulose. In addition to the material not bound to the column, four radioactive, collagenase-susceptible fractions were recovered (Fig. 2). Two peaks were identified as $\alpha 1$(I) and $\alpha 2$ on the basis of their chromatographic and electrophoretic properties (not shown) and by the profile of peptides produced from them by digestion with CNBr (not shown). The radioactive species eluting between $\alpha 1$(I) and $\alpha 2$ were identified as $\alpha 1$(III) and [$\alpha 1$-(III)]$_3$ by the following criteria. First, the peak labeled [$\alpha 1$-(III)]$_3$ was recovered in much smaller amounts when the sample was treated with β-mercaptoethanol prior to chromatography, and the size of the peak labeled $\alpha 1$(III) was increased. Similar chromatographic properties were noted with authentic [$\alpha 1$(III)]$_3$ and $\alpha 1$(III) obtained from fetal human skin after incubation with pepsin. Second, the material in the fraction labeled [$\alpha 1$(III)]$_3$ electrophoresed as an approximately 300,000 molecular weight component without reduction and with authentic $\alpha 1$(III) after reduction (Fig. 3). Third, the profiles of CNBr peptides produced by CNBr from the peaks labeled $\alpha 1$(III) and [$\alpha 1$(III)]$_3$ were identical and resembled that obtained from authentic [$\alpha 1$(III)]$_3$ (not shown).

Marked differences in the proportion of $\alpha 1$(I) to $\alpha 1$(III) chains were noted between the control strain (no. 1146) and OI strain (no. 1262). Radioactivity in $\alpha 1$(III) was 5-18% of



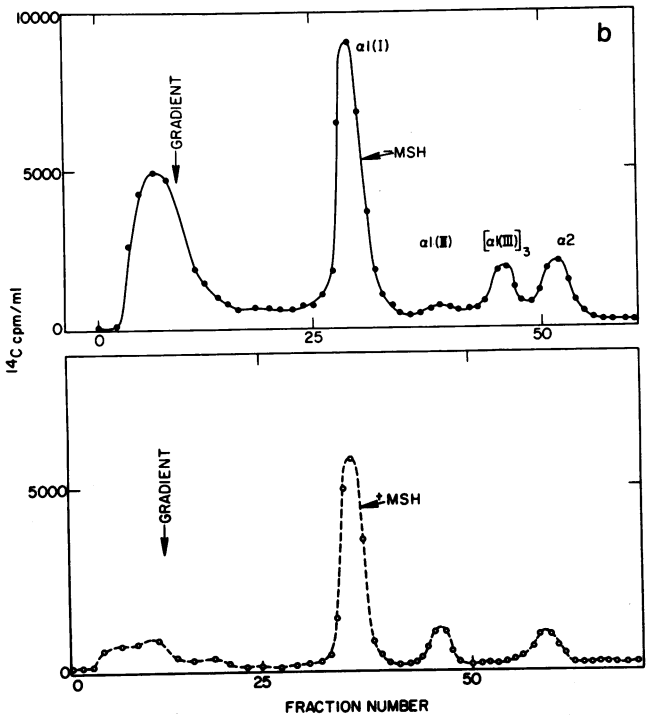


FIG. 2. CM-cellulose chromatography of the collagenous proteins obtained from fibroblast cultures after limited digestion with pepsin at 15°. (a) OI sample (strain no. 1262); (b) control sample (strain no. 1146). The sample labeled +MSH was reduced with mercaptoethanol before chromatography.

the label in α1(I) in controls but 38–46% in the OI strain. Similar results were obtained when only media proteins were chromatographed after pepsin treatment. However, proteins of the cell layer showed no alteration in the ratio of α1(III) and α1(I) from the controls.

Several other control strains have given identical results to those described for no. 1146. These strains included cells from the skin of a 20- to 22-week fetus (ATCC no. 1106) and a 17-week fetus (Zeber).

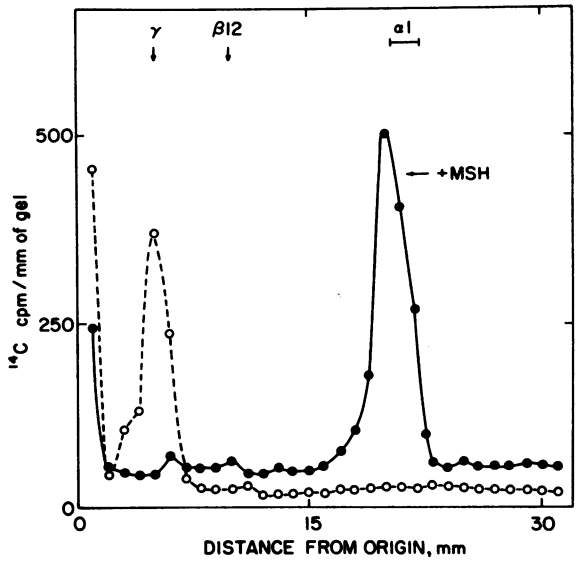


FIG. 3. Electrophoresis of fraction labeled [α1(III)]3 isolated by CM-cellulose chromatography in Fig. 2a. Aliquots were subjected to electrophoresis in sodium dodecyl sulfate with (●) or without (○) mercaptoethanol.

Six other OI strains tested had a normal profile of medium procollagens resolved by DEAE-cellulose chromatography. However, strains 1280 (B.P.), 1251 (E.R.), and 1248 (G.R., mother of E.R.) had a higher than normal amount of α1(III), a ratio of 0.25 and 0.40 of α1(III) to α1(I) as judged by the pattern of components resolved by CM-cellulose after pepsin treatment.

DISCUSSION

The studies reported here deal primarily with alterations observed in the synthesis of collagens secreted by cells obtained from a fetus that died at birth. Clinical and pathological studies, to be reported elsewhere (19), are consistent with the diagnosis of osteogenesis imperfecta congenita. A number of tissues, in addition to bone, were severely affected in this individual. During delivery of this child there was an avulsion of the right foot and head while the abdominal wall and anterior thorax were lacerated. The placenta was soft and appeared to lack supportive (fibrous) tissue. These observations are consistent with a generalized defect in collagen in those tissues known to consist primarily of type I collagen.

Recent studies (17, 20) have shown that cell strains from skin synthesize two different collagens, type I and type III. The precursor forms of these collagens are readily resolved on DEAE-cellulose and the denatured proteins on CM-cellulose. We used these two methods to estimate the ratio in which the two types of collagens are synthesized by cells in culture and have found that control cell strains synthesize predominantly type I collagenous protein (17). Cells from patients with osteogenesis imperfecta fell into three groups.

In the first group, type III collagen accounted for a greater proportion of the total accumulated in the cultures of OI strain no. 1262 as judged by the proportion of precursor forms in the medium and the proportion of α1(III) to α1(I) chains isolated after pepsin digestion of medium and cell layer.

In the second group, the proportion of type III collagen synthesized by strains 1280, 1251, and 1248 was elevated as judged by the CM-cellulose assay, but the profiles of proteins on DEAE-cellulose appeared normal. It should be noted that these procedures measure different parameters. Precursors of type I and III collagens present in the media are estimated after chromatography on DEAE-cellulose. The assay of α1(I) and α1(III) chains by CM-cellulose involves an initial treatment with pepsin to convert precursor forms to collagen and also to solubilize precipitated collagens. It is likely that there is some loss of α2 during this procedure, since the ratio of α1(I) to α2 is greater than 2. If the OI strains 1280, 1251, and 1248 produced type I collagen containing an amino-acid substitution weakening the helical structure of collagen, degradation of type I collagen could alter the apparent ratio of α1(I) to α1(III). Alternatively, differences in the extent of conversion of procollagens type I and III in these cell strains might explain the discrepancy. The third group is comprised of cell strains from several patients with osteogenesis imperfecta that showed no difference in the proportion of type I and III collagens by either test. Such diversity of findings in the cells from these patients is neither unexpected nor without precedence. Patients grouped under the diagnosis of osteogenesis imperfecta are genetically and clinically heterogeneous (1), and it would not be surprising that different molecular defects would give rise to similar clinical findings. This has been the case with patients with the Ehlers-Danlos syndrome (21–23).

While the alterations noted in OI strain no. 1262 could result from differences in the synthesis of either protein, we favor the interpretation that the synthesis of type I collagen is reduced since bone is largely made of type I collagen and is more affected than skin, which contains both types of collagen. Decreased synthesis of type I collagen in strain no. 1262 would lessen tissue contents and explain the generalized tissue fragility.

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