Reduced secretion of structurally abnormal type I procollagen in a form of osteogenesis imperfecta

(collagen/genetic heterogeneity/peptide mapping)

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ABSTRACT  Osteogenesis imperfecta is a clinically and genetically heterogeneous group of inherited connective tissue disorders in which bone fragility is the predominant feature. Cultured dermal fibroblasts from one patient with the lethal perinatal form of osteogenesis imperfecta secrete type I procollagen at a rate half that of normal cells. Short-term labeling experiments and treatment with α,α' dipyridyl (which prevents post-translational prolyl and lysyl hydroxylation) demonstrated that these cells produce two distinct proα1(I) chains, which are synthesized at the same rate. Analysis of cyanogen bromide peptides indicated that the two chains differ in their primary structures. Thus, structural abnormalities in type I procollagen prevent this molecule from being secreted normally, resulting in an anomalously low ratio of type I procollagen to other extracellular matrix molecules. While the lethal perinatal form of osteogenesis imperfecta may be heterogeneous, we propose that the underlying pathogenesis of at least one form is decreased secretion of type I procollagen.

Osteogenesis imperfecta (OI) is a heterogeneous group of heritable disorders in which osseous fragility is the common feature (1). The inheritance pattern, the frequency of bone fractures, and the involvement of tissues other than bone have provided the basis for a recent classification that distinguishes four groups of OI (2). Additional genetic and biochemical heterogeneity may exist within some or all of these groups (3, 4). Abnormalities in the metabolism of collagen (5–10), glycosaminoglycans (11–13), and proteoglycans (14) have all been proposed to account for the clinical findings in OI, but the primary biochemical defect in each form remains unknown.

The most severe form of OI is type II, also known as the congenital crumpled bone or lethal perinatal variety (2). Affected infants have major abnormalities in bone formation and extremely friable connective tissue (15). The distribution of clinical findings corresponds to the distribution of type I collagen in tissues (16), and decreased production of type I collagen has been observed in cells from a patient with lethal perinatal OI (6, 17).

The pathway of collagen biosynthesis is complex, and several alterations could lead to decreased production of type I collagen (18, 19). In reexamining cultured cells derived from a well-studied patient (6, 14, 15, 17, 20) with lethal perinatal OI, we have found that the type I procollagen synthesized by these cells is not secreted at a normal rate, and we suggest that failure of secretion is secondary to a structural mutation in one or both proα1(I) chains.

MATERIALS AND METHODS

Cell Culture. OI cells (CRL 1262) frozen at subcultivation 9 were obtained from the American Type Culture Collection.

Rockville, MD. The cells are derived from the skin of a stillborn 28-week fetus with the typical features of lethal perinatal OI (15). Cells were grown in Dulbecco–Vogt modified Eagle’s medium that contained 10% newborn calf serum (GIBCO), penicillin at 100 units/ml, and streptomycin at 100 μg/ml. Control cells originated from a newborn donor and were frozen at subcultivation 3 and stored in liquid N₂. All results were confirmed in separate experiments with control cells of fetal (18 week) and adult origin. Cells were subcultured weekly with 0.25% trypsin (1:3 split); all studies of OI and control cells were performed on subcultivations 5–15.

Labeling and Electrophoresis. Cells were plated at a density of 2.5 × 10⁵ per 35-mm culture dish, and after 24 hr the medium was replaced with 0.5 ml of serum-free modified Eagle’s medium that contained ascorbic acid at 50 μg/ml and L-[2,3-³H] proline (25 Ci/mmol, New England Nuclear) at 25 μCi/ml (1 Ci = 3.7 × 10¹⁰ becquerels). Proline was used at 200 μCi/ml in peptide mapping experiments. The cell number remained constant throughout the labeling period. After the indicated labeling time, the medium was harvested, centrifuged to remove detached cells (Beckman Microfuge for 2 min), and brought to a final concentration of 25 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, and 10 mM N-ethylmaleimide. The cell layer was rinsed twice with cold phosphate-buffered saline that contained EDTA, phenylmethylsulfonyl fluoride, and N-ethylmaleimide at the concentrations indicated above, then scraped into 1.5 ml of the same solution and homogenized with 20 strokes in a Teflon/glass homogenizer. In experiments designed to show that the procollagen found in the OI cell layer was intracellular, the cells were harvested by incubation with 0.25% trypsin for 5 min at 25°C, diluted into 10 ml of modified Eagle’s medium that contained 20% fetal calf serum, and centrifuged at 500 × g for 10 min, and the cell pellet was homogenized as described above. To prepare the samples for electrophoresis, medium or cell homogenates were dialyzed against 1 mM ammonium bicarbonate/0.1 mM phenylmethylsulfonyl fluoride/0.5 mM N-ethylmaleimide and lyophilized. Dry samples were dissolved in 50 μl of electrophoresis sample buffer and denatured by immersion in boiling water for 5 min. Vertical slab sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed by the method of Laemmli (21), using 5% gels under reducing conditions unless otherwise indicated. Gels were processed for fluorography with dimethyl sulfoxide and 2,5-di-phenylxazozone (22), or EN³HANCE (New England Nuclear).

Peptide Mapping. The identity of collagenous proteins in gels was determined by examination of CNBr peptides derived from digestion of proteins in gel strips. To avoid decreased recovery as a consequence of acid or alcohol fixation (23), strips corresponding to individual lanes measuring 5 × 0.5 cm were cut out immediately after electrophoresis. The strips were

Abbreviation: OI, osteogenesis imperfecta.

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equilibrated with 70% (wt/wt) formic acid by three successive 30-min washes in a 15-fold volume excess, and then incubated in 5 ml of 70% formic acid that contained CNBr (50 mg/ml) for 6 hr at 25°C. At the end of the digestion, each strip was equilibrated with 100 mM Tris-HCl, pH 6.8/30% (vol/vol) glycerol/bromophenol blue and placed horizontally over a 12.5% separating gel with a 5% stacking gel. This second-dimension gel was prepared at a width 0.5 mm greater than the first-dimension gel to allow easy placement of the first-dimension gel strips. A 100 mM Tris-HCl buffer, pH 6.8, containing 5% (wt/vol) sodium dodecyl sulfate, 20% (vol/vol) glycerol, and bromophenol blue was then layered over the gel slices (two gel slices per second-dimension gel) to a height of 1 cm, and the CNBr-derived peptides were separated by electrophoresis at a current of 20 mA. Identical first-dimension lanes were processed for fluorography without CNBr digestion.

**Other Procedures.** Indirect immunofluorescence was performed as described (24), using a rabbit antibody specific for type I procollagen. Antibody specificity was established by sodium dodecyl sulfate/polyacrylamide gel electrophoresis of immunoprecipitates from radiolabeled medium of normal fibroblasts.

Cell layer or medium procollagen was quantitated by scanning densitometry of fluorograms (25). Studies with control cells indicated that, under the conditions of labeling used in these experiments, more than 90% of the secreted collagen was found in the medium compartment and not associated with the cells. Values for total procollagen were determined by scanning the area of the fluorogram from proa2(III) to a2. Values for type III procollagen were then determined from the density of proa2(III) because there was no conversion to smaller forms detectable by peptide mapping studies. Values for type I procollagen were determined by subtracting the contribution of type III procollagen from total procollagen, a procedure that gave results similar to adding individual values for type I procollagen components.

![Fig. 1](image1.png)

**Fig. 1.** Phase-contrast microscopy (A and B) and indirect immunofluorescence with antibody to type I procollagen (C and D) of OI and control fibroblasts. Cells were plated at a density of 5.2 × 10⁴ per cm² in 35-mm dishes (A and B) or on plastic cover slips (C and D) and allowed to attach overnight in growth medium. The medium was then supplemented with ascorbic acid at 50 μg/ml and after 24 hr the cells on coverslips were fixed and stained with antibody to type I procollagen (C and D). (All ×800.)

**RESULTS**

The OI and control cells appeared different by phase microscopy; the OI cells contained numerous cytoplasmic vacuoles not seen in control cells (Fig. 1 A and B). When examined by indirect immunofluorescence using antibodies to type I procollagen, staining of the OI cells was more intense than that of control cells (Fig. 1 C and D), which suggested that the type I procollagen synthesized by the OI cells accumulated intracellularly.

To examine type I procollagen production, we labeled cultures with ['H]proline for 16 hr and analyzed the cell layer and medium proteins by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 2). After 16 hr, the majority of procollagen in control cultures was found in the medium. In contrast, more than half of the procollagen in the OI cultures remained in the cell layer (Fig. 2, lanes e–h). The procollagen in the OI cell layer remained in the cell fraction when the cells were removed from the dish with trypsin, and thus was presumably intracellular (data not shown). In order to determine whether type I or type III procollagen was specifically retained by the OI cells, it was necessary to identify each band on the fluorogram. Although bands corresponding to the constituent proα chains of types I and III procollagen could be identified by electrophoretic mobility in control cultures (Fig. 2, lanes g and h), similar unambiguous identification was not possible for the OI cultures. Neither the mobility nor the relative intensity of the bands migrating in the region of proα1 in the OI cells or medium was characteristic of either proα1(I) or proα1(III) in control cells or

![Fig. 2](image2.png)
medium. Furthermore, the bands in the region of proα2 from OI cells and medium (Fig. 2, lanes f and g) migrated slightly slower than proα2 from control cells and medium (Fig. 2, lanes e and h).

In order to identify each band from the OI cultures, we developed a peptide mapping procedure using CNBr digestion of proteins within gels. Strips from a 5% gel (such as the one shown in Fig. 2, lanes e–h) were cut out while the gel was still wet and were incubated in CNBr. The resulting peptides were separated by electrophoresis in a 12.5% gel as described in Materials and Methods (Fig. 3). Identical first-dimension strips were processed for fluorography without prior digestion and are illustrated in the upper portion of Fig. 3. Under these conditions, virtually all of the radioactivity was recovered from the 5% gel. Cleavage with CNBr was incomplete, but it was not increased by prior treatment with reducing agents or by increasing the time and temperature of incubation or the concentration of CNBr used in the cleavage step. The pattern of partial cleavage products was highly reproducible.

Peptide maps of the proteins in the OI cells and control medium (Fig. 3A) demonstrated that the two bands that migrated in the region of proα1 chains from the OI cells were different forms of proα1(I) (right side, Fig. 3A), which we designated proα1(I)α and proα1(I)β, corresponding to their “slow” and “fast” electrophoretic mobility, respectively. Proα1(I)α was distinguished by the presence of a peptide (arrowhead, Fig. 3A) that was never seen in control proα1(I) (medium or cells, see Fig. 3B) or in proα1(I), from OI cells. (Identical results were obtained in five separate experiments.) Less dramatic differences in the pattern of the partial cleavage peptides were apparent between proα1(I)α and normal proα1(I). These findings indicated that proα1(I)α and possibly proα1(I)β differed from normal proα1(I) in some aspect of primary structure.

Peptide maps of the proteins in OI medium and control cells (Fig. 3B) demonstrated that the two bands that migrated in the region of proα1 chains from the OI medium represented proα1(III) and proα1(I). The proα1(III)α-derived peptide was present in the OI medium (arrowhead, Fig. 3B) but was much less intense than in the OI cell layer. This suggested that proα1(III) and a small amount of proα1(I)γ contributed to the band labeled proα1(I) in the OI medium (Upper Left, Fig. 3B). In contrast, the major proα1(I) chain in the OI cell layer was proα1(I)α (Fig. 3A). The pattern of CNBr peptides from proα2 was the same in OI and control cultures, so the slight differences in electrophoretic mobility between OI and control whole proα2 chains (Fig. 2, lanes e–h) could not be explained by alterations in primary structure, but it might be due to differences in posttranslational hydroxylation or glycosylation.

Once the bands from the OI cultures were specifically identified, we determined the distribution of procollagens between the cells and the medium by scanning gel densitometry (Table 1). The distribution of type I procollagen was markedly altered in the OI cultures; 52% of the type I procollagen in the OI cultures was retained in the cell layer compared to only 9% in the control. Because virtually all the type III procollagen in the control and OI cultures was in the medium, these results demonstrated a specific abnormality in the secretion of type I procollagen.

The ratio of type III to type I procollagen produced (cells and medium together) and the ratio of type III to type I procollagen secreted (medium alone) were also determined by scanning gel densitometry (Table 1). Type III constituted 11% of the procollagen produced by the OI cultures compared to 7% in controls. Because the OI cells specifically retained type I procol-

**Fig. 3.** Peptide mapping of proα chains from control medium, OI cells, OI medium, and control cells. Cultures were labeled for 16 hr and CNBr peptide maps were prepared. Duplicate samples subjected only to electrophoresis in the first dimension were processed for fluorography and are presented in the upper portion of each figure. Horizontal and vertical arrows indicate the directions of electrophoresis in the first and second dimension, respectively. The arrowhead (right side in A and left side in B) indicates the peptide characteristic of proα1(I)α produced by the OI cells (see text). Each sample represents the contents of a single 35-mm culture dish; first-dimension fluorograms were exposed for 2 days; the second-dimension fluorograms were exposed for 16 days.
The peptide mapping studies suggested that procollagen I and procollagen III, seen in the OI cells (Fig. 3A, right side) differed in primary structure. To exclude the possibility that they were related as precursor and product, we labeled the cells in the presence of α,α’-dipyridyl, which prevents prolyl and lysyl hydroxylation. Under these conditions the OI cells still pro-

collagen, type III constituted 23% of the procollagen secreted by these cells compared to 7% in controls.

To examine the kinetics of procollagen secretion, we pulse-labeled the cells and then quantitated the material secreted into the medium by scanning gel densitometry (Fig. 4). It was not possible to measure type III and type I procollagen separately because not enough radioactivity was incorporated during the pulse (4 hr) for definitive peptide mapping of the procollagen chains in the OI medium. Therefore, secretion as presented in Fig. 4 includes the contribution of type III procollagen. Nonetheless, the rate of secretion of procollagen by the OI cells was approximately half that of control cells.

The peptide mapping studies suggested that procollagen I and procollagen III, seen in the OI cells (Fig. 3A, right side) differed in primary structure. To exclude the possibility that they were related as precursor and product, we labeled the cells in the presence of α,α’-dipyridyl, which prevents prolyl and lysyl hydroxylation. Under these conditions the OI cells still pro-

The table presents data on the cell and medium distributions of types I and III procollagen after 16 hr labeling.

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Type I, cells/ type I, media</th>
<th>Type II, total/ type I, total</th>
<th>Type III, media/ type I, media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.096 (9%)</td>
<td>0.073 (7%)</td>
<td>0.075 (7%)</td>
</tr>
<tr>
<td>OI</td>
<td>0.083 (8%)</td>
<td>0.069 (6%)</td>
<td>0.080 (7%)</td>
</tr>
</tbody>
</table>

OI and control cultures were labeled for 16 hr, and a fluorogram was prepared as in Fig. 2. Types I and II procollagen in the cell layers and media were quantitated by scanning densitometry. The data include results from two separate experiments expressed as ratios. The numbers in parentheses are percentages calculated as [numerator/(numerator + denominator)] × 100.

**DISCUSSION**

These dermal fibroblasts from an infant with the lethal perinatal form of OI synthesize two distinct species of procollagen I chains, whereas control cells synthesize only one. Several lines of evidence indicate that the two procollagen I chains synthesized by the OI cells are structurally and, therefore, genetically distinct. Short-term continuous labeling shows that the two chains are synthesized at equal rates. Labeling in the presence of α,α’-dipyridyl indicates that the two chains are apparent even in the absence of the usual posttranslational hydroxylation reactions.

**FIG. 5.** (A) Collagenous proteins in OI and control (C) cells synthesized in the presence or absence of α,α’-dipyridyl. Cultures were preincubated for 30 min in serum-free medium containing either ascorbic acid at 50 μg/ml (lanes a–d) or 0.3 mM α,α’-dipyridyl (lanes e and f), then changed to labeling medium containing ascorbic acid or α,α’-dipyridyl. Cell layers were harvested after 2 hr (lanes a and b) or 4 hr (lanes c–d) and subjected to electrophoresis. The protein marked by an arrowhead (lane e) may represent unhydroxylated procollagen III. Each lane represents the contents of a single 3.5-mm dish; the fluorogram was exposed for 4 days. (B) Short-term labeling of OI cells. After a 30-min preincubation in serum-free medium containing ascorbic acid at 50 μg/ml, OI cultures were labeled for 15 min (lane a), 30 min (lane b), 60 min (lane c), or 120 min (lane d). Cell layers were harvested at the indicated times and the constituent proteins were separated by electrophoresis. Each lane represents the contents of a single 3.5-mm dish; the fluorogram was exposed for 4 days (not shown) and 16 days. When quantitated by scanning gel densitometry (the 4-day fluorogram was used for lanes c and d), the ratio of procollagen III to procollagen I equaled 1.0 throughout the labeling period.

Produced two procollagen chains, which migrated in the region of control procollagen I (Fig. 5A, lane e) and presumably corresponded to unhydroxylated procollagen I and procollagen II.

Additional evidence mitigating against a precursor–product relationship for procollagen I, and procollagen II, was obtained by labeling cells continuously for 15, 30, 60, and 120 min (Fig. 5B). Both procollagen I, and procollagen II, were detectable in equal amounts after 15 min of labeling and remained in the same proportion throughout the labeling period. In conjunction with the peptide mapping studies, these results demonstrated that procollagen I, and procollagen II, were two separate gene products synthesized at equal rates.
Finally, CNBr peptide maps of the separated chains show that proα1(I) produces a strikingly abnormal pattern of CNBr peptides. The pattern of CNBr peptides from proα1(I), is also different from normal, but we are uncertain whether this represents an abnormality in primary structure or differential cleavage by CNBr. Although proα1(I) and proα1(I) are synthesized at equal rates, after 16 hr, the major proα1(I) chain in the OI medium is proα1(I), but the major proα1(I) chain in the OI cells is proα1(I). Because over half of the type I procollagen synthesized by the OI cells is not secreted, this suggests that type I procollagen molecules that contain one or more proα1(I) chains do not satisfy structural criteria required for secretion.

To date, the major structural requirements for procollagen secretion that have been recognized are presence of an amino-terminal “signal sequence” (26, 27) and triple-helical conformation (28, 29). If one of the OI proα1(I) chains lacked a normal signal sequence or was unable to form a triple helix, the abnormal chain or trimer containing the abnormal chain would be degraded intracellularly (30, 31). However, the level of intracellular cleavage by CNBr. Although this represents an abnormality in primary structure or differential metabolism (28, 29). If one of the molecules that contain these chains do not satisfy structural requirements for secretion—e.g., selective interaction with membrane-associated carriers involved in secretion (32, 33).

This form of OI is genetically heterogeneous (34, 35); in some families there is clearly autosomal recessive inheritance, but the occurrence of the disorder in most infants (including the one studied here) is sporadic, so that a new dominant mutation cannot be excluded. Our findings do not distinguish between dominant and recessive inheritance, because it has not been possible to study cells from the parents, and because the number of functional proα1(I) loci is unknown. However, the two OI proα1(I) chains are synthesized at the same rate, suggesting that the genes that code for the two chains are present in the same number of copies. This is consistent with a single functional proα1(I) locus in bone and skin fibroblasts, so that in these cells one allele is represented by proα1(I), the other by proα1(I). If pro α1(I) represents a neutral polymorphism that does not interfere with either function or secretion, then this infant may have had the disorder as a result of a single mutation that produced proα1(I).

These OI cells (CRL 12562) have been studied by several other investigators who described a reduced ratio of type I to type III collagen production (6), decreased total collagen production, and normal levels of intracellular degradation (17), increased sulfate uptake (14), and increased activity of hyaluronate synthetase (20). Our results demonstrate that the primary genetic defect in these cells is a structural abnormality in type I procollagen that prevents the molecule from being secreted normally and suggests that the other observations are secondary phenomena. The mechanism by which proteolytic metabolism is altered is unclear, but our results show that the decrease in total collagen production is due largely to decreased type I collagen production. Because collagen degradation was not increased (17), the reduced ratio of type I to type III collagen produced by these cells (6) must result from a decreased concentration or decreased translational efficiency of type I procollagen mRNA.

We have recently found that cells from four phenotypically similar patients also are unable to secrete type I procollagen normally (unpublished data). Thus, different structural mutations in proα chains that prevent secretion of type I procollagen may underlie the pathogenesis of lethal perinatal OI.