Defects in the Processing of Procollagen to Collagen Are Demonstrable in Cultured Fibroblasts from Patients with the Ehlers-Danlos and Osteogenesis Imperfecta Syndromes*

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This is a study of the processing of procollagen to collagen in cultures of skin and tendon fibroblasts. Processing was markedly increased by growing cells for 2-4 days postconfluence and then adding ascorbate to the medium for 2 days prior to labeling with [3H]proline. With this system, more than two-thirds of the pro-α chains of type I procollagen in the culture medium, and more than 90% of those in the cell layer, were rapidly processed to p-α, pN-α, or α chains. Purified, exogenous procollagen was also rapidly processed in cell-free culture medium. The results showed for the first time that exogenous procollagen can be processed in conditioned cell-free medium. The system was then used to compare the processing of procollagen in the medium of normal fibroblasts, cells from one bovine and four human variants of osteogenesis imperfecta, and those from eight human variants of the Ehlers-Danlos syndrome. The cells could be divided into three groups, based on their ability to process type I procollagen: 1) normal, 2) consistently slow, and 3) very slow. The cause of the decreased processing was shown to be associated with either a mutation causing a shortening of an α chain or decreased activity of procollagen N-proteinase in cell-free culture medium. Decreased processing of procollagen to collagen occurred with cultured fibroblasts from patients with different forms of both osteogenesis imperfecta and Ehlers-Danlos syndrome. Both of these disease syndromes are associated with abnormalities in the structure or metabolism of procollagen in fibrous connective tissues, bones, and teeth. The results show that defects in the structure, synthesis, or processing of procollagen are readily demonstrated with cultured fibroblasts.

The Ehlers-Danlos syndrome (EDS') and osteogenesis imperfecta (OI) are two major syndromes of heritable diseases with fragility or hyperextensibility of tissues whose tensile strength is primarily dependent upon fibrillar collagens (1-8). Mutations that change the structure or decrease the processing of type I procollagen have been found in a few variants of EDS, and mutations that change the structure of type I procollagen have been found in a few variants of OI (9-17). In most variants of EDS or OI, however, neither a specific genetic mutation nor decreased procollagen processing has been identified. Lichtenstein et al. (18) found that they were not able to extract procollagen proteinase activity from the cell layer or culture medium of fibroblasts from three unrelated cases of EDS, while Layman and Ross (19) were able to demonstrate procollagen peptidase activity in extracts of both the cell layer and medium of cultures of normal human fibroblasts. In a subsequent study, however, Layman (20) found that the procollagen proteinase activity was quite variable in extracts of the cell layer and culture medium of confluent cultures of fetal and adult human fibroblasts. This is consistent with numerous observations that only small and variable amounts of procollagen processing are found when normal fibroblasts are grown with the usual culture conditions (18-21). Also, because of the small amount of processing that occurs in most fibroblast cultures, conflicting observations have been reported as to whether type I procollagen is processed immediately before or after secretion, whether the processing occurs at a time and place that are distant from secretion, or whether the processing can occur in cell-free culture medium (17-20).

Sonohara et al. (22) recently showed that considerable amounts of procollagen processing occur in postconfluent cultures of embryonic guinea pig fibroblasts. We observed similar amounts of processing in the course of studies of a bovine variant with osteogenesis imperfecta. Processing was maximal when the fibroblasts were maintained at confluence for 5-10 days or if cultures that were 2-4 days postconfluent were fed for 2 days with medium containing ascorbate. Here, we used the modified culture system to compare the processing of endogenous 3H-procollagen and chromatographically purified, exogenous 14C-procollagen that was added to cell-free culture medium and to the postconfluent fibroblasts. The system was then used to compare the processing of procollagen to collagen by normal human fibroblasts and by fibroblasts from eight human variants of EDS and four human variants of OI.

MATERIALS AND METHODS

Source of Bovine Fibroblasts—Biopsies were obtained from gastrocnemius tendon of fetal and newborn Holstein calves with the Australian variant of bovine osteogenesis imperfecta (BOA-Aust). Normal Holstein cows were bred at Cornell University with semen from the Australian Holstein bull that was shown to transmit BOI as an autosomal dominant trait (23). The biopsies were obtained in a collaboration with Drs. Joyce A. M. Wootton, Laurence Denholm, Lennart Krook, and Charles Hall. Primary cultures of fibroblasts were established from biopsies of a normal twin 180-day-old fetus.
and from an affected twin with BOI. The affected twin was readily identified by the presence of thin fragile tendons, bones, and teeth. Primary cultures were also established from biopsies of normal and affected half-sibling newborn calves, but no differences were detected in the function of the fetal and newborn cells. Since the fetal fibroblasts were from affected and unaffected twins, these cells were selected for the studies of procollagen processing described here.

The cells were established from a tissue mince, grown in Dulbecco's modified Eagle's medium without pyruvate, containing 20% fetal bovine serum, 1% fungizone solution (GIBCO, 600-5295), and 1% penicillin/streptomycin solution (GIBCO, 600-5140). After 2 days of culture, the tissue mince was removed, medium without fungizone was added, and the attached fibroblasts were allowed to form large confluent colonies. These colonies were digested with 0.5% trypsin-EDTA solution (GIBCO, 610-5300), and 2.5 x 10⁶ cells were plated in 5 ml of medium in 25 cm² tissue culture flasks. The cultures were fed every other day and were passed at a density of 10⁵ cells/cm² within 2 days of reaching confluence. In later passages all antibiotics were omitted from the medium, and the concentration of fetal bovine serum was reduced to 10%. All of the studies described here were done with cells between passage 3 and 10. When these cells were to be labeled, they were grown for 2-4 days postconfluence and were fed for 2 days with the same medium containing 25 μg/ml sodium ascorbate.

Sources of Human Fibroblasts—The sources of normal and patient fibroblasts are shown in Table I. Fibroblasts from passage 3 through 15 were grown under standard conditions (9-12) in 25-cm² tissue culture flasks in Dulbecco's modified Eagle's medium containing 1% fetal bovine serum and no antibiotics. After reaching confluence, the cells were fed for 2 days with the same medium containing 25 μg/ml sodium ascorbate.

Continuous Labeling Experiments—Cells fed for 2 days with medium containing ascorbate were preincubated for 30 min with 1.5 ml of Dulbecco's medium containing 1% fetal bovine serum. The preincubation medium was replaced with the same medium containing 10-50 μCi/ml [2,3,4,5-3H]proline (Amersham Corp.), and the flasks were incubated for 4 h at 37 °C. To block the hydroxylation of proline and lysine in selected cultures, a,a'-dipyridyl was added to both the preincubation medium and labeling medium to a concentration of 0.3 mM.

Pulse-Chase Experiments—To study the kinetics of procollagen processing, five flasks each of unaffected and affected cells of BOI-Aust were preincubated for 30 min and pulsed for 1 h with 50 μCi/ml [3H]proline as described above. The labeled medium was removed, the cell layers were washed with Dulbecco's medium, and the flasks were incubated with 1.5 ml of Dulbecco's medium containing 1% fetal bovine serum without label for 0, 1, 2, 4, or 8 h.

Procesing of Exogenous 14C-Procollagen—To study the enzymatic processing of exogenous procollagen on the cell layer and in cell-free culture medium, a substrate of chromatographically purified 14C-labeled chicken procollagen was prepared as described previously (24-26). One mg of this purified 14C-procollagen, containing 5 x 10⁶ cpn of 14C, was incubated for 4 h at 37 °C in 5 ml of cell-free culture medium or in 1.5 ml of fresh culture medium that was added to the cell layer. The cell-free medium was culture medium that was removed from postconfluent cultures of bovine or human fibroblasts after 2 days and was centrifuged for 10 min at 1000 X g. When used in a clinical centrifuge to remove floating cells and other particulates. At the end of the incubation, cold protease inhibitor solution and ammonium sulfate were added to both the cell-free medium and medium from the cell layer, and the cell layer was homogenized and boiled in homogenizing buffer as described below.

Analysis of Labeled Medium and Cell Layer—As described previously (9-12), the medium proteins were recovered by adding a stock protease inhibitor solution to give final concentrations of 11 mM N-ethylmaleimide, 1 mM p-aminobenzamidine, 1 mM phenylmethylsulfonyl fluoride, and 30 mM EDTA adjusted to pH 7.4. Ammonium sulfate was added to 30% saturation (176 mg/ml), and the samples were centrifuged to remove cells and other particulates. The supernatants were acidified to pH 2.3 with 50 μl of 5 M HCl and were subjected to chromatography on a column of Dowex-50. The elution of two pools was studied. First, the labeled amino acids of the eluate were analyzed by liquid scintillation spectrometry, and the fractions that contained labeled amino acids were collected and concentrated by evaporation. Second, the labeled amino acids were subjected to high-performance liquid chromatography on a Whatman (Clifton, NJ) 10 x 250 mm precolumn and a Whatman 10 x 100 mm analytical column. The eluates were collected at 1 ml/min in 2 ml fractions, and the radioactivity of each fraction was determined with a liquid scintillation spectrometer.

| Clinical diagnosis | Cell line No. | Age | Sex | Source |
|-------------------|---------------|-----|-----|--------|
| Normal            | CRL-1106      | Fetus | M   | American Type Culture Collection |
|                   | IMR-8555      | Fetus | M   | Institute for Medical Research |
|                   | IMR-970       | 3 days | M   | Institute for Medical Research |
|                   | IMR-3348      | 10 yr | M   | Institute for Medical Research |
|                   | IMR-3349      | 10 yr | M   | Institute for Medical Research |
|                   | CRL-1290      | 15 yr | M   | American Type Culture Collection |
|                   | IMR-3652      | 24 yr | M   | Institute for Medical Research |
|                   | RMS-C1        | 61 yr | F   | J. Utto, Torrance, CA |
| Lethal OI (type II) | IMR-2962 | 0 days | F   | Institute for Medical Research |
| Lethal OI (type II) | RMS-2      | 0 days | M   | C. E. Anderson, Long Beach, CA |
| Lethal OI (type II) | RMS-18      | 2 days | M   | H. Chen, Shreveport, LA |
| Moderate OI (type III) | RMS-25 | 7 yr | F   | F. M. Pope, London |
| Mild OI (atypical) | RMS-44       | 24 yr | M   | S. Kaffe, NY |
| EDS (type I)      | RMS-52        | 2 yr | M   | B. G. Kousseff, Tampa |
| EDS (type VII)    | CRL-1193      | 3 yr | F   | American Type Culture Collection |
| EDS (type VII)    | CRL-1274      | 12 yr | F   | American Type Culture Collection |
| EDS (type VII)    | CRL-1150      | 16 yr | F   | American Type Culture Collection |
| EDS (type VII)    | CRL-1149      | 31 yr | F   | American Type Culture Collection |
| EDS (type VII)    | RMS-54        | 11 yr | F   | S. Farrell & R. Weksberg, Toronto |
| EDS (atypical)    | RMS-75        | 7 yr | M   | B. G. Kousseff, Tampa |
| EDS (atypical)    | RMS-74        | 8 yr | F   | B. G. Kousseff, Tampa |

*The types of OI are according to the classification of Sillence (3) and the types of EDS are as suggested by several investigators (1, 4, 6, 7, 13). The diagnosis of lethal OI (type II) was made for cell line (IMR-2962) by Dr. Thaddeus Kelly (10), for RMS-2 by Dr. Carol E. Anderson, and for RMS-18 by Dr. Harold Chen. The clinical manifestations in the patient with moderately severe OI (RMS-25) were described by Nicholls et al. (37). The patient with mild atypical OI (RMS-44) and affected members of his family were noted by Dr. Sandra Kaffe in patient RMS-54 by Drs. Sandra Farrell and Rosanna Weksberg. With the two half-siblings RMS-74 and RMS-75, Dr. Kousseff found that the primary clinical signs were easy bruisability and thin atrophic scars, with very little hyperextensibility of skin or joints. Therefore, he concluded that this was a clinically unclassified EDS variant with some characteristics of EDS type IV.
were stirred overnight at 4 °C and centrifuged at 20,000 × g, at 4 °C, for 1 h. The cell layer was immediately washed three times with cold (4 °C) phosphate-buffered saline and homogenized on ice in 500 μl of homogenizing buffer consisting of 270 mM NaCl, 22 mM N-ethylmaleimide (Sigma), 2 mM p-aminobenzamidine (Sigma), 2 mM phenylmethylsulfonyl fluoride (Sigma), 0.1% Triton X-100, and 80 mM EDTA in 270 mM Tris-HCl buffer adjusted to pH 7.4 at room temperature. The homogenate was immediately boiled 3 min in a 1:10 volume of 20% SDS and again for 3 min after adding 2-mercaptoethanol to a final concentration of 1–5%. The precipitate of the medium proteins was dissolved in 300 μl of the same homogenizing buffer and boiled 3 min with 2% SDS and again for 3 min after adding 2-mercaptoethanol to 1–5%. For electrophoresis, a 1/5 volume of 5× electrophoresis sample buffer was added, and the samples were dialyzed for 24 h at room temperature against 2 changes of 1× sample buffer consisting of 2% SDS, 10% glycerol, and 0.001% bromphenol blue in 0.01 M Tris-HCl buffer adjusted to pH 6.8 at room temperature.

Slab gels of 6% acrylamide or a 4–8% acrylamide gradient in SDS were used to separate the labeled peptides (27). In most gels urea was added to a concentration of 0.5 M, and in selected cases the same samples were rerun with either no urea or 2.0 M urea in the gel system. These comparisons showed that increasing amounts of urea caused the band of type III procollagen to be more focused and those of type V procollagen to be less focused, but urea did not change the density of the bands of type I procollagen. The differences in concentration of the peptides in the gels changed the apparent ratio of the different types of procollagen chains, but there was no difference in the separation of the pro-α1(I) and pro-α1(III) chains in the gels with different concentrations of urea. The gels were dehydrated with dimethyl sulfoxide, infiltrated with 2,5-diphenyloxazole, dried, and fluorograms were prepared on Kodak XAR film (28). The films were exposed for periods from 2 to 14 days, and multiple exposures of each gel were scanned at 600 nm with a Gilford Response gel scanner to quantitate the ratios of absorbance in each band containing peptides derived from type I procollagen. Since this instrument did not reliably integrate separate peaks, the traces of the peaks from the fluorograms were cut out and weighed to confirm the quantitation of ratios of the areas of the peaks representing procollagen, partially processed procollagen, and collagen chains.

RESULTS

Processing of Type I Procollagen in Culture Medium on the Cell Layer and in Cell-free Culture Medium from Bovine Fibroblasts—In the initial experiments with fetal bovine tendon fibroblasts, we found that procollagen processing was maximized when the cells were grown for 2 days beyond confluence and then fed for 2 days with medium containing 25 μg/ml ascorbate (Fig. 1A, 1st and 2nd lanes). More than

![Fig. 1. SDS-polyacrylamide gel electrophoresis showing the processing of endogenous procollagen (panel A) and exogenous procollagen (panel B) in culture medium from tendon fibroblasts from an unaffected (U) bovine fetus and its affected (A) twin with BOI-Aust. In both panels, 1st lane is to the left, 1st and 3rd lanes are from cells from the unaffected twin, and 2nd and 4th lanes are from the affected twin. In panel A, 1st and 2nd lanes, the cultures were grown for 4 days beyond confluence, were then fed for 2 days with medium containing 25 μg/ml ascorbate, and were labeled for 4 h with medium containing [3H]proline (60 μCi/ml). In panel B, 3rd and 4th lanes, the cells were grown for 10 days beyond confluence without added ascorbate and were labeled for 4 h with medium containing [3H]proline (50 μCi/ml). The 3H-procollagens in the medium were precipitated, reduced, by boiling with mercaptoethanol, and were separated on a gradient gel of 4–8% acrylamide. As shown the gradient gel gave excellent separation of the collagenous chains, but the band above the pro-α1(I) chain is unidentified. In panel B, 1st and 2nd lanes, cell-free culture medium was removed from cultures of bovine tendon fibroblasts that were grown for 4 days beyond confluence and then fed for 2 more days with medium containing added ascorbate (25 μg/ml). This conditioned medium was removed from the cultures after the 2 days and was centrifuged to remove all cells and other particulates. Chromatographically purified 14C-procollagen (50 μg, 5 × 10^6 dpm), synthesized by embryonic chick tendon fibroblasts (25), was then incubated for 4 h in the cell-free supernatant of the medium from the bovine fibroblasts. In panel B, 3rd and 4th lanes, 14C-labeled chicken procollagen (50 μg, 5 × 10^6 dpm) was added to 1.5 ml of fresh medium on the ascorbate-stimulated bovine tendon fibroblast cell layer for a 4-h incubation. After incubation, the 14C-collagens and procollagens were precipitated, reduced, and separated on a gel of 6% acrylamide. As shown there was no difference in the processing of either endogenous or exogenous procollagens by the cultures of unaffected and affected bovine fibroblasts.](image-url)
half of the newly synthesized type I procollagen was processed to pC-collagen, pN-collagen, or collagen in the medium of fibroblasts that were labeled for 4 h with [3H]proline. For example, densitometry of the 2nd lane showed that the distribution of $^3$H in pro-α1(I) or processed pro-α1(I) chains was 24% in pro-α1(I), 30% in pC-α1(I), about 8% in pN-α1(I), and 26% in α1(I) chains. For this calculation, the proportion of pN-α1(I), which co-migrates with pro-α2(I), was estimated as twice the value of the pN-α2(I) chain. Therefore, about 65% of the newly synthesized type I procollagen was partially or completely processed. For convenience, the ratio of pC-α1(I) to the sum of pro-α1(I) + PC-α1(I) chains was used as a minimal estimate of processing. By this measure about 47% of the procollagen in the 2nd lane of Fig. 1 was processed to PC-collagen. This value is a minimal estimate, since pC-α1(I) chains are also being processed to α1(I) chains.

Similar degrees of procollagen processing were seen in the medium of confluent cultures that were grown for 7–10 days beyond confluence in medium without ascorbate (Figs. 1A, 3rd and 4th lanes). The results also showed that during 4 h of labeling there was no difference in the extent of processing of $^3$H-procollagen in cultures of affected and unaffected fibroblasts from BOI-Aust (Fig. 1).

In the same series of experiments, exogenous $^{14}$C-procollagen was chromatographically purified and was used to determine 1) whether the processing of procollagen occurred in cell-free culture medium and 2) whether the extent of processing of exogenous procollagen was comparable to the processing of endogenous procollagen that occurred when the enzymes and substrates were synthesized and secreted by the same cells. As shown in Fig. 1, there was little or no difference in the extent of processing of endogenous $^3$H-procollagen that was synthesized by the bovine fibroblasts and exogenous $^{14}$C-procollagen.
labeled chicken procollagen that was added to fresh culture medium on postconfluent bovine fibroblasts. Essentially the same degree of processing of the exogenous $^{13}$C-procollagen occurred in cell-free medium that was removed from postconfluent bovine fibroblasts after 2 days of culture and was centrifuged to remove floating cells and other particulates (Fig. 1B, 1st and 2nd lanes). Densitometry of the fluorograms shown in Fig. 1B (3rd lane) indicated that 40% of the $^{13}$C was in pro-α(1), 40% in pC-α(1), 10% in pN-α(1), and 10% in α1(I) chains. These values were essentially the same as values obtained for processing of endogenous procollagen in the medium in 4 h (Fig. 1A). Most (84%) of the exogenous procollagen remained in the medium, and only 16% of the recovered $^{13}$C was in the cell layer. As also indicated in Fig. 1B, there was no difference in the processing of the exogenous procollagen in the culture medium of unaffected and affected fibroblasts of BOI-Aust.

In further experiments, bovine fibroblasts were pulse-labeled for 1 h with $^{3}$H-proline, and the label was chased for 0, 1, 2, 4, or 8 h by replacing the labeled medium with fresh medium without label (Fig. 2). Densitometry of the fluorograms indicated that more than 40% of the pro-α(I) chains in the culture medium were processed during the first h of chase and more than 80% were processed by 8 h. Again, there was no difference in the rate of procollagen processing in the affected and unaffected bovine fibroblasts (Fig. 2).

**Processing of Type I Procollagen in the Cell Layer—**Bovine fibroblasts were labeled with $^{3}$H-proline for 4 h, and the labeled proteins in the cell layer and medium were compared. After 4 h the cell layer contained about two-thirds of the nondialyzable $^{3}$H, but only 15% of this $^{3}$H was in labeled procollagen or collagen chains. The procollagen in the cell layer, however, was more extensively and rapidly processed. About 80% of the labeled collagen in the cell layer was recovered as α1(I) and α2(I) chains. Electron microscopy showed that thin (~20 nm) fibrils accumulated between the layers of lamellipodia of the postconfluent fibroblasts (not shown). This rapid and complete processing of procollagen to collagen in the cell layer was even more evident in fluorograms from a pulse-chase experiment. As indicated in Fig. 3A, most of the procollagen in the cell layer was processed to α chains after 2 h of chase. Similar amounts of processing occurred in the cell layer in a pulse-chase experiment with postconfluent human fibroblasts (Fig. 3B).

**Processing of Type I Procollagen in the Culture Medium of Human Fibroblasts**—The same culture conditions used to increase the processing of procollagen by bovine fibroblasts were used to study human fibroblasts. Processing of type I procollagen was readily demonstrated in the culture medium of human fibroblasts (Figs. 4 and 5). The ratio of pC-α(1) to the sum of pro-α(1) + pC-α(1) plateaued at about 35% during the second h of chase (Fig. 6A), while the amounts of α1(I) and α2(I) chains continued to increase throughout the chase in the cultures of normal human fibroblasts (Figs. 4 and 5). In contrast, in the culture medium of fibroblasts from a patient with a mild variant of OI (RMS-44) the ratio remained less than 10% (Figs. 4 and 6B). Previous studies showed that fibroblasts from this patient synthesized equal amounts of normal and shorter pro-α2(I) chains, and procollagen molecules containing these shorter chains were resistant to cleavage in vitro by partially purified procollagen N-proteinase (10). Therefore, fibroblasts from a series of variants of OI and EDS were examined for evidence of defects in the processing of type I procollagen.

As indicated in Fig. 6A, control fibroblasts consistently showed a 30-50% ratio of pC-α1(I) to the sum of pC-α1(I) +

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**FIG. 5.** SDS-polyacrylamide gel electrophoresis showing greatly reduced processing of $^{3}$H-procollagen in a pulse-chase experiment with postconfluent cultures of skin fibroblasts from a new patient with EDS type VII (RMS-54) and a new patient with an atypical form of EDS (RMS-74). The controls were postconfluent cultures of normal human skin fibroblasts (IMR-1106 and IMR-3348). The procedures were the same as those used in the experiment described in Fig. 3. Densitometric scans of the fluorograms showed that in the cultures of both patients fibroblasts less than 10% of the $^{3}$H-procollagen chains were processed to altered procollagen or collagen chains. Panel A, IMR-54 and RMS-54; panel B, IMR-3348 and RMS-74.
pro-$\alpha_1(I)$ chains. As shown in Fig. 6B and in Table II, patient fibroblasts could be assigned to three groups on the basis of procollagen processing: 1) normal, 2) consistently slow, and 3) very slow. The slow group was defined as variants whose ratio was more than 2 S.D. below the mean of the control values at four out of five time points, and the very slow group were those with values less than 10%.

The variants showing normal procollagen processing included two variants of EDS type VII (CRL-1150 and CRL-1193), two variants of severe OI type II (RMS-2 and RMS-18), and one variant each of moderately severe OI type III (RMS-25) and EDS type I (RMS-52) (Fig. 6B and Table I). The variants of EDS type VII (CRL-1150 and CRL-1193) were previously reported to have a defect in the procollagen processing (13), but the procollagen in the medium of these fibroblasts consistently had a normal 30-50% ratio of pC-$\alpha_1(I)$ to pro-$\alpha_1(I)$ + pC-$\alpha_1(I)$ in the studies carried out here.

The variants with consistently slow processing included one variant of severe OI type II (IMR-2962), two variants of EDS type VII (CRL-1183 and CRL-1149), and a clinically unclassified, atypical EDS (RMS-75).

The variants with very slow processing of procollagen to collagen included one mild variant of OI (RMS-44), two of EDS type VII (CRL-1274 and RMS-54), and one atypical EDS (RMS-74) (Fig. 6B and Table I). As noted above, RMS-44 was previously shown to have a structural defect in the N-terminal region of the $\alpha_2(I)$ chain that made the type I procollagen resistant to cleavage by partially purified procollagen N-proteinase (11). One variant of EDS (CRL-1274) was also previously shown to have decreased procollagen processing (13). However, defects in processing had not been identified previously in either new variant of EDS (RMS-54 and RMS-74).

Identification of a Structural Defect in Type I Procollagen and a Deficiency of Procollagen N-Proteinase—Previous studies indicated that decreased processing of procollagen can be caused either by mutations that change the structure of a pro-$\alpha$ chain or mutations that decrease the activity of procollagen N-proteinase. Therefore, the cell lines from the three new EDS variants (RMS-54, RMS-74, and RMS-75) showing decreased processing were examined further. As shown in Fig. 7, the pro-$\alpha_2(I)$ chains synthesized by cells from RMS-54 migrated slightly further than normal pro-$\alpha_2(I)$ chains in polyacrylamide gels. The increased migration was observed when the procollagen chains were hydroxylated, as well as when the cells were labeled in the presence of the iron chelator and hydroxylation inhibitor $\alpha,\alpha'$-dipyridyl. The results suggest, therefore, that the slow processing in this variant was explained by a mutation that led to the synthesis of a shortened pro-$\alpha_2(I)$ chain. Of special interest was the observation that no normal length pro-$\alpha_2(I)$ chains were seen in these cultures (Fig. 7). In contrast, the pro-$\alpha$ chains from the variants RMS-74 and RMS-75 had a normal migration on polyacrylamide gels (data not shown).

Additional experiments were done to determine whether there was a decreased activity of procollagen N-proteinase in the cell lines from RMS-74 and RMS-75 who were half-siblings. Chromatographically purified $^{14}$C-procollagen was added to cell-free medium that was removed from postconfluent cultures of these cells and control fibroblasts. The medium was centrifuged and chromatographically purified $^{14}$C-procollagen was added to the supernatant for a 4-h incubation at 37 °C (Fig. 8). Densitometry of the fluorograms indicated that the extent of processing of exogenous pro-$\alpha_1(I)$ chains to pC-$\alpha_1(I)$ chains was 23% in the cell-free medium of control fibroblasts, 16% in the medium from RMS-75, and 11% in the medium from RMS-74. Therefore, both of these variants appear to have mutations that decrease the activity of procollagen N-proteinase.

DISCUSSION

The conditions used here to increase procollagen processing were the relatively simple manipulations of allowing the cells
to grow past confluence and supplementing the medium with sodium ascorbate for 2 days prior to labeling. The reason why these manipulations increased processing is not clear, but it may be related to the multilayering of fibroblasts and to the previous reported effects of ascorbate-stimulating procollagen synthesis and secretion (29–31).

The results confirm previous observations that procollagen N-proteinase activity is present in culture medium (18–20) and showed for the first time that exogenous procollagen can be processed in cell-free medium from cultured fibroblasts. The exogenous procollagen was processed at about the same rate and to about the same extent as endogenously synthesized procollagen. Therefore, it is clear that processing can occur after secretion of procollagen. Also, it is clear that processing does not require the fibroblast cell surface or the surface of growing collagen fibrils. These surfaces or processes may play a role in procollagen processing in vivo, but neither the fibroblast cell surface nor growing collagen fibrils are essential requirements for procollagen processing.

There was more extensive and more rapid processing of procollagen in the cell layer than in the culture medium. Unique pools of tissue fluids may be created between the layers of discoid cells as they multilayer, and these pools may represent an environment that favors procollagen processing and collagen fibril formation. Electron microscopy shows that thin collagen fibrils accumulate between the layers of cell processes in postconfluent cultures, and recent studies indicate that the processing of procollagen and assembly of collagen into fibrils may occur in specialized compartments formed by the plasma membranes of fibroblasts (32, 33). The simplest explanation, however, for the more rapid and extensive processing of procollagen in the cell layer is that the substrate and enzyme are in high concentration here, whereas they are too dilute for rapid and complete processing after secretion into the relatively large volume of medium used to culture fibroblasts. Also, it is possible that there is more inhibition of processing of the C-terminal propeptides by the serum in the culture medium than in the cell layer (34).

Because of the extensive processing of procollagen to pC-collagen in the postconfluent cultures, the system described here makes it possible to identify readily two general categories of mutations: those that decrease the activity of procollagen N-proteinase, and those that alter the structure of type I procollagen so as to make it resistant to procollagen N-proteinase. Procollagen N-proteinase is one of a small class of proteinases that require a substrate in a native conforma-

**TABLE II**

Grouping patient fibroblasts by the rate of processing of procollagen to collagen in the culture medium

| Procollagen processing groupa | Clinical diagnosisa | Identifying no. | Mutationb |
|-----------------------------|-------------------|----------------|----------|
| Normal                      | Lethal OI (type II) | RMS-2          | Unknown  |
|                             | Moderate OI (type III) | RMS-25       | Pro-a2* (12) |
|                             | Lethal OI (type II) | RMS-18         | Unknown  |
|                             | EDS (type I)       | RMS-52         | Unknown  |
|                             | EDS (type VII)     | CRL-1190       | Unknown  |
|                             | EDS (type VII)     | CRL-1193       | Unknown  |
| Consistently slow           | Lethal OI (type II) | IMR-2962       | Pro-a2* (10) |
|                             | EDS (type VII)     | CRL-1183       | PRO-a2* (9)  |
|                             | EDS (type VII)     | CRL-1149       | Probably N-proteinase (13) |
|                             | EDS (atypical)     | RMS-75         | Probably N-proteinase |
| Very slow                   | Mild OI (atypical) | RMS-44         | Pro-a2* (11) |
|                             | EDS (type VII)     | CRL-1274       | Probably N-proteinase (13) |
|                             | EDS (type VII)     | RMS-54         | Pro-a2* |
|                             | EDS (atypical)     | RMS-74         | Probably N-proteinase |

a Values for the ratio of pC-a1(I) to pC-a1(I) + pro-a1(I) chains were used to define the three processing groups as follows: Normal group, values between 30 and 50%; Consistently slow group, values more than 2 S.D. below the normal mean but more than 10% at four out of five time points; Very slow group, values less than 10%.

b Numbers in parentheses are references on previously reported variants. Pro-a2*, pro-a2(I) chains with a structural alteration in the C-propeptide; pro-a2*, shortened pro-a2(I) chains; pro-a2*, pro-a2(I) chains with an unknown structural alteration.

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**Fig. 7.** SDS-polyacrylamide gel electrophoresis showing an increase in the electrophoretic mobility of the a2(I) chains synthesized by cultures of skin fibroblasts of a patient with EDS type VII (RMS-54). Also see Fig. 5A. Confluent cultures of the patients cells (EDS) and control skin fibroblasts (C) were preincubated 30 min and labeled for 4 h with [3H]proline (20 µCi/ml) in the presence (+) and absence (−) of α,α′-dipyridyl (0.3 mM). The increased electrophoretic mobility of both the unhydroxylated and hydroxylated a2(I) chains (1st and 5th lanes) suggests that there is a shortening of the a2(I) chain synthesized by the patients fibroblasts.

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+  
-  

C EDS C C EDS C  

Pro a1 (I)  
pC a1 (I)  
Pro a2 (I)  
pC a2 (I)
detect mutations that decrease the activity of procollagen N-proteinase.

Previous reports suggested that there is a consistent correlation between the clinical syndrome of EDS type VII and a decrease in the processing of the N-propeptide of type I procollagen. However, two variants that were previously classified clinically as EDS type VII (CRL-1193 and CRL-1150, Ref. 13) were found here to show normal processing. In addition, the results demonstrated that processing can be deficient in patients who do not meet the clinical criteria of EDS type VII. One variant with consistently slow processing (IMR-2962) died in utero with the typical manifestations of the lethal, broad-boned form of OI. Two half-siblings with decreased procollagen processing (RMS-74 and RMS-75) had easy bruising and subcutaneous bleeding, thin atrophic scars, and only slight hyperextensibility of skin or joints. These manifestations are inconsistent with EDS type VII. Therefore, it is apparent that mutations decreasing the processing of type I procollagen can produce more than one clinical syndrome and that the clinical features of EDS type VII do not necessarily indicate that there is a defect in procollagen processing. One consequence of these observations is that postconfluent cultures of fibroblasts may be useful in the characterization of a broad spectrum of heritable diseases of connective tissues.

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