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Research Article

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Enhanced Biosynthesis of Human Skin Collagenase in Fibroblast Cultures from Recessive Dystrophic Epidermolysis Bullosa

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ABSTRACT Using a sensitive, specific immunoprecipitation method, the biosynthesis of human skin collagenase was studied in fibroblast cultures from patients with recessive dystrophic epidermolysis bullosa. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of solubilized immunoprecipitates showed two ^{3}H-labeled procollagenase species that comigrated with those harvested from control cultures. Recessive dystrophic epidermolysis bullosa cultures accumulated increased amounts of collagenase. Both the initial rate of accumulation of intracellular enzyme and the rate of secretion were enhanced, suggesting that excessive accumulation is related to increased synthesis. Because the turnover of labeled collagenase was unaltered, the accumulation could not be attributed to diminished enzyme degradation. No preferential incorporation of [^{3}H]leucine into recessive dystrophic epidermolysis bullosa collagenase occurred. Furthermore, the mutant cultures displayed no alteration in total protein synthesis, the intracellular leucine pool, or the growth kinetics of the cells. Cells from a patient with dominant epidermolysis bullosa did not show enhanced accumulation of collagenase. The levels of collagenase synthesized in vitro correlated with those observed previously in vivo in recessive dystrophic epidermolysis bullosa patients, suggesting that this biochemical trait is pathogenetically significant in the disorder.

INTRODUCTION

The group of diseases collectively termed epidermolysis bullosa (EB)[1] is comprised of four major clinically and genetically distinct heritable disorders of the skin in which blistering is provoked by minor cutaneous trauma (1). In one of the most severe varieties, recessive dystrophic epidermolysis bullosa (RDEB), electron microscopic studies have disclosed marked collagen degeneration and phagocytosis of collagen fibrils by macrophages in the area of blistering (2), suggesting that mechanisms involved in collagen breakdown might be important in the pathogenesis of blister formation. Support for such a hypothesis was obtained when increased collagenase activity was observed in short term organ cultures of blistered skin from RDEB patients (3, 4). This postulate was further substantiated by the demonstration of increased tissue levels of immunoreactive collagenase in both the blistered and nonblistered skin of RDEB patients (5).

If a primary defect in the regulation of synthesis and/or degradation of collagenase were, indeed, basic to the etiology of RDEB, it is reasonable to expect that it might be expressed in fibroblast cultures. Recently, we have shown increased accumulation of collagenase in the culture medium of RDEB skin fibroblast cultures maintained for 24–48 h (6). In the cells of some patients, increased accumulation was associated with the production of an aberrant form of collagenase (7). These traits appeared to be genetically unique for RDEB cells because cultures from patients with other varieties of EB accumulated normal amounts of collagenase (6). Nevertheless, the cellular mechanisms by which increased collagenase accumulation occurred (i.e., increased synthesis vs. decreased degradation) remain unknown.

In the present investigation we have used fibroblast cultures derived from two prototypic patients with RDEB to examine the biosynthesis of collagenase using a highly sensitive immunoprecipitation method (8). Our major aims were twofold (*a*) to compare the in vitro kinetics of synthesis, secretion, and degradation of

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[1] *Abbreviations used in this paper*: EB, epidermolysis bullosa; RDEB, recessive dystrophic epidermolysis bullosa; SDS, sodium dodecyl sulfate.

collagenase by RDEB cells with those of control cell lines in an attempt to define the mechanism for increased collagenase accumulation, and (*b*) to characterize the qualitative nature of newly synthesized collagenase in RDEB cultures as a further measure of the degree of structural aberration.

METHODS

Fibroblast cultures. RDEB fibroblast cultures were established from two patients with typical findings of the disease after obtaining informed consent. All biopsies were taken from normal-appearing posterior trunk skin. Control cultures were initiated from trunk skin of healthy volunteers. As a further control, fibroblast cultures were established from a patient with the dominant simplex form of EB. All patients were examined by one of us (E.A.B.) and classified based on clinical, genetic, and histologic findings as described previously (1, 5). The cell lines employed in this study are detailed in Table I.

Cells were subcultivated in disposable plastic culture dishes or flasks (Corning Glass Works, Science Products Div., Corning, N. Y.) in Dulbecco's modified Eagle's medium-high glucose plus glutamine with 30 mM *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (pH 7.6), 20% fetal calf serum, and 200 U of penicillin, and 200 μg of streptomycin/ml at 37°C in a 95% air:5% CO_2 atmosphere. In these experiments, fibroblasts were grown to early confluence (i.e., stationary), a cell density at which collagenase accumulation is greatest (9). Serum-containing medium was then removed; the cells were washed three to five times with Hank's balanced salt solution and maintained in serum-free labeling medium as described below.

Labeling procedures. Replicate fibroblast cultures in early confluence were incubated in leucine-free Dulbecco's modified Eagle's medium-high glucose plus glutamine-containing antibiotics, and further modified to contain 30 mM Hepes buffer, vitamins, and a 40-μM concentration of each of the following amino acids: L-aspartic acid, L-proline, L-asparagine, L-glutamic acid, and L-alanine (8). [^{3}H]Leucine (net-460, 70–100 Ci/mmol; New England Nuclear, Boston, Mass.) was added in most experiments to an approximate concentration of 50 μCi/ml. Using cultures incubated at 37°C in an air-CO_2 atmosphere with 0.1–0.2 ml of the labeling medium per square centimeter of growth area, no significant change of culture pH was encountered during the duration of the experiments (10). After the desired incubation period, the medium was harvested, bovine serum albumin added to a final concentration of 0.5–1 mg/ml, and the entire mixture dialyzed at 4°C against several changes of 0.01 M Tris-HCl (pH 7.5) containing 1 mM $CaCl_2$ before immunoprecipitation (8). The cell layer was washed three times with Hank's balanced salt solution at 4°C, harvested by scraping, lysed by sonication, and assayed for total protein and incorporation of [^{3}H]leucine into protein.

TABLE I
Description of Human Skin Fibroblast Lines

Cell line	Sex	Age
		yr
Controls		
WUC 1124	F	40
WUN 77248	F	28
WUN 76130	F	20
WUN 77251	F	36
WUG 3348	M	3
Recessive dystrophic EB		
WUE 7572	F	34
WUE 76114	M	4
Dominant EB simplex		
WUE 7567	M	4

In experiments to determine the intracellular content of labeled enzyme protein during biosynthesis, the cultures were labeled for the desired length of time with [^{3}H]leucine, after which a 1,000-fold excess of nonradioactive leucine was added to the medium. A portion of the culture medium was harvested at the beginning of the chase period and at 2 h thereafter to measure the intracellular labeled enzyme protein that was chased into the culture medium. As shown previously (8), the intracellular enzyme could be chased into the medium within 2 h, after which no additional accumulation of labeled enzyme protein was seen for a period of up to 6 h. Thus, the amount of intracellular enzyme was taken as that amount precipitated from the medium over the amount present at the beginning of the chase period.

In some experiments, to assess the qualitative nature of the intracellular labeled enzyme protein, the medium was decanted from the cultures and the cells were washed five times with buffer (three times on the plate and two additional times with suspension followed by low-speed centrifugation). The cells were then suspended in 0.01 M Tris-HCl (pH 7.5) with 0.1 mM $CaCl_2$, 0.5% Triton X-100, and 0.3 mM phenlymethylsulfonylfluoride, and 5 mM *N*-ethylmaleimide (8). After sonication, the lysate was centrifuged at 13,000 *g* for 15 min, after which the supernate was subjected to ultracentrifugation at 105,000 *g* for 1 h. This 105,000-*g* supernate was then applied to a 0.9 × 4-cm column of carboxymethylcellulose equilibrated with 0.01 M Tris-HCl (pH 7.5) containing 0.1 mM $CaCl_2$ as described by Stricklin et al. (11). After application to the column, sample buffer was used to elute unbound protein until the radioactivity returned to baseline. The bound proteins were eluted in a single stepwise fashion with 0.3 M NaCl. Recovery in this system was consistently 90–100% (8). Bovine serum albumin and $CaCl_2$ were then added to final concentrations of 0.5 mg/ml and 1 mM, respectively, for precipitation.

The fate of intracellular labeled enzyme protein was further investigated in pulse-chase experiments in which replicate cultures of labeled cells were extensively washed as noted above. One set of cultures was processed immediately using lysis and ultracentrifugation, followed by ion exchange chromatography on carboxymethylcellulose. The proteins eluted from carboxymethylcellulose were precipitated with antiserum to human skin collagenase; that amount was a measure of the total enzyme protein present at zero time. In the second set of cultures, fresh unlabeled culture medium was added and then harvested after a 2-h chase period. The amount of immunoreactive collagenase in the chase medium represented the intracellular enzyme that had been secreted. Thus, the difference between the total amount at zero time and the amount secreted into the medium was taken as that labeled enzyme putatively lost through intracellular degradation.

Isolation of labeled collagenase by immunoprecipitation. For direct precipitation of labeled enzyme protein, dialyzed labeled cell culture medium was initially centrifuged at 10,000 *g* for 10 min at 4°C in siliconized glass centrifuge tubes to remove insoluble debris. Labeled human skin collagenase was then precipitated from the medium in a total

volume of 300 μl in disposable plastic microfuge tubes (Beckman Instruments, Inc., Fullerton, Calif.) using specific antiserum to the enzyme. A typical reaction mixture contained 50–200 μl of the ^{3}H-labeled culture medium, 3–10 μl of rabbit anti-human skin collagenase serum, and a sufficient quantity of electrophoretically pure (11) carrier human skin collagenase (usually 1.0 μg) to give precipitation in the zone of equivalence (8). After incubation for 3 h at 37°C and 18 h at 4°C with intermittent agitation, the resultant precipitates were harvested by centrifugation and washed four times with 0.01 M Tris-HCl(pH 7.5)–0.15 M NaCl buffer. The precipitates were dissolved in 250 μl of 0.1 M NaOH, added to 8 ml of scintillant solution, and counted in a liquid scintillation spectrometer with correction for quenching. As shown previously (8), 89–91% of the labeled collagenase was precipitated under these conditions.

As a control for the background radioactivity caused by nonspecific precipitation and trapping of labeled proteins in the immunoprecipitate, the same volume of labeled medium was incubated with 3–10 μl of antiserum to ovalbumin and a sufficient amount of ovalbumin shown by previous titration to give equivalence point precipitation in this system (8). The amounts of protein immunologically precipitated in the ovalbumin-antiovalbumin and collagenase-anticollagenase systems were equal. The difference between radioactivity precipitated by specific anti-human skin collagenase serum and antiovalbumin serum was taken as a measure of the labeled collagenase.

Total protein synthesis. Total protein synthesis was determined at varying time points by quantitating the [^{3}H]leucine incorporated into 10% trichloroacetic acid-insoluble material. For these studies, an equal volume of 20% trichloroacetic acid was added to 100 μl of labeled medium containing bovine serum albumin. The precipitates were harvested after 1 h at 0°C, washed three times with 10% trichloroacetic acid, and dissolved in 0.1 M NaOH for addition to scintillant solution and counting with quench correction. Cells were washed three times with Hank's balanced salt solution at 4°C, suspended in 0.01 M Tris-HCl (pH 7.5) with 0.15 M NaCl, and lysed by sonication. Trichloroacetic acid precipitation was then carried out as described above.

Analytical sodium dodecyl sulfate polyacrylamide gel electrophoresis. To assess the specificity of the precipitations, the washed immunoprecipitates were dissolved in 25–100 μl of sample buffer containing 0.065 M Tris-HCl (pH 6.8), 8 M urea, 3% sodium dodecyl sulfate (SDS), and 2% β-mercaptoethanol for gel electrophoresis. The precipitates were either allowed to dissolve in the sample buffer for 18 h at 25°C or were dissolved in a boiling water bath for 5 min before electrophoresis. Immediately before electrophoresis, one drop of glycerol containing bromphenol blue was added and the samples were applied to a discontinuous SDS slab gel (1 mm thick) made according to King and Laemmli (12) with 10% (wt/vol) acrylamide and 0.27% (wt/vol) *N,N'*-methylenebisacrylamide in the separating gel. Electrophoresis was carried out at 70 mA/mm slab thickness until the dye front approached the bottom of the gel. After the gel run, the positions of the top of the gel and the dye front were noted and the different gel slabs were either stained with Coomassie brilliant blue or processed for fluorography. For fluorography, the slab gels were equilibrated with dimethylsulfoxide, immersed in 20% 2,5-diphenyloxazole in dimethylsulfoxide for 3 h, rinsed in distilled water for 20 h, and dried under vacuum. The dried gels were exposed to Kodak XR-5 X-Omat R film (Eastman Kodak Co., Rochester, N. Y.) at −70° (13). Densitometric scans of the fluorographs were done with a Zeiss PM6 spectrophotometer.

Purification of human skin collagenase. Human skin procollagenase to be used for carrier protein or comparison of electrophoretic mobility with labeled enzyme was purified to homogeneity from the culture medium of normal human skin fibroblasts as described by Stricklin et al. (11). The enzyme was assayed after proteolytic activation as previously described (14). For each enzyme preparation, a range of trypsin concentrations (0.1–2.0 μg trypsin/50-μl enzyme sample) was employed to ensure that maximal collagenase activity was measured. After preincubation with trypsin for 10 min at 25°C, at least a fivefold molar excess of soybean trypsin inhibitor was added to inhibit further trypsin activity. Each mixture was then assayed for collagenase activity at 37°C in 0.05 M Tris-HCl (pH 7.5) in the presence of 10 mM $CaCl_2$ using native, reconstituted [^{14}C]glycine-labeled collagen fibrils containing ~4,000 cpm per substrate gel (15).

Preparation of antiserum to human skin collagenase. Human skin procollagenase was purified to homogeneity and used as the immunogen to prepare functionally monospecific antiserum to the enzyme as described in detail previously (11). The antiserum gave a single immunoprecipitin band when reacted in Ouchterlony analysis (16) with either the crude culture medium or with the electrophoretically pure human skin collagenase. Furthermore, a gamma globulin preparation of the antiserum produced >90% inhibition of the collagenase activity after reacting 2 h at 37°C compared with <5% inhibition by a nonimmune gamma globulin preparation (16). The amount of antiserum and pure carrier human skin collagenase required to achieve quantitative precipitation of the labeled enzyme has been determined previously (8) and was the same for control and RDEB collagenase preparations.

Radioimmunoassay of collagenase. In some experiments, the amount of labeled collagenase directly precipitated from the culture medium was compared with that quantitated by radioimmunoassay. In this case, immunoreactive collagenase was measured by a slight modification of the double antibody radioimmunoassay previously reported (17). The procollagenase used as the unlabeled standard and for iodination in the radioimmunoassay was purified to homogeneity (11). Standard radioimmunoassay curves were derived by incubating duplicate disposable plastic microfuge tubes (Beckman Instruments, Inc.) that contained a 1:2,500 dilution of the gamma globulin fraction of antiserum to human skin collagenase, ^{125}I-human skin collagenase (~20,000 cpm/tube), and 0–100 ng of electrophoretically homogeneous human skin collagenase in a total volume of 250 μl. After incubation for 24 h at 4°C, goat antirabbit IgG was added in excess and the resulting precipitates were isolated, washed, and counted in a single-channel gamma scintillation spectrometer. Unknowns were assayed for enzyme protein in an identical fashion using 100-μl portions of serial doubling dilutions of the enzyme preparations. Controls in which nonimmune rabbit gamma globulin was substituted for specific antiserum were routinely included.

Other assays. Protein was determined using a bovine serum albumin standard (18). The growth kinetics of control and RDEB fibroblasts were determined by seeding cultures from a single large pool at low density. Replicate cultures were fed serum-containing medium every 2 d for the duration of the experiment. Duplicate cultures were harvested daily for cell counts.

To measure the specific activity of the intracellular leucine pool during the incubation with [^{3}H]leucine, portions of cell lysates were precipitated with an equal volume of trichloroacetic acid, and the [^{3}H]leucine and the total leucine contents of the supernate were determined using an automated amino acid analyzer (Beckman Instruments, Inc.).

Statistical analysis of the data was performed by Dr. R. Wette of the Division of Biostatistics, Washington University. After ascertaining that variances within groups were homo-

geneous by Bartlett's test, the t test was used to compare the significance of the differences in the least squares estimates of the rates of biosynthesis between control and RDEB cultures (19).

RESULTS

Initially we investigated the specificity of the [^{3}H]-leucine-labeled material precipitated with anticollagenase serum from the culture medium of control and RDEB fibroblasts. The high degree of specificity of the immunoprecipitation of collagenase protein was shown by subjecting the precipitates to SDS gel electrophoresis followed by scintillation fluorography (Fig. 1). Fig. 1A shows the characteristic doublet pattern given by direct precipitation of the labeled procollagenase species from crude culture medium of a control cell line (8). For comparison, the paired arrows depict the position of the two control electrophoretically pure human skin procollagenase species obtained by quantitative purification of the control enzyme (11). Fig. 1B and C depict the SDS gel electrophoretic patterns of the labeled procollagenases precipitated from the crude culture medium of RDEB patients WUE 7572 and WUE 76114, respectively, which comigrated with those of the control preparation. In addition to the procollagenase doublet (arrows), both the control and RDEB preparations displayed a small amount of material migrating as a doublet corresponding to the position of the activated enzyme species (11). Fig. 1D shows the fluorograph of the labeled material precipitated in the ovalbumin-antiovalbumin system used as a control. Compared with the precipitates found with anti-human skin collagenase, the antiserum to ovalbumin gave no distinguishable bands, indicating that the radioactivity precipitated in this system was not the result of nonspecific trapping (8).

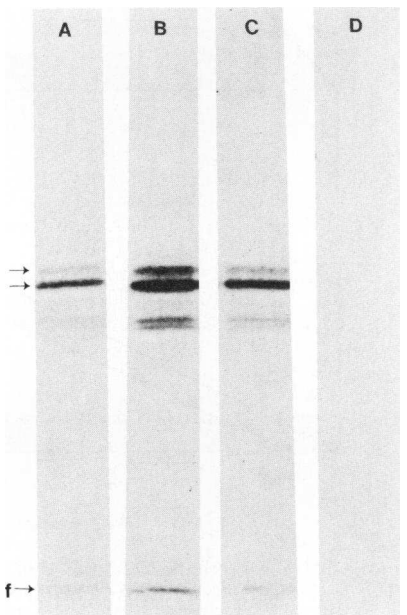

FIGURE 1 Fluorographs of the SDS gel electrophoresis patterns after immunoprecipitation from fibroblast culture medium. A. Anti-human skin collagenase precipitate from control culture medium. B. Anti-human skin collagenase precipitate from WUE 7572 medium. C. Anti-human skin collagenase precipitate from WUE 76114 medium. D. Ovalbumin-antiovalbumin precipitation of control culture medium. The paired arrows mark the electrophoretic mobilities of the two purified unlabeled human skin procollagenase standards; f, buffer front.

Because our previous studies suggested the existence of a structurally altered form of collagenase in certain patients with RDEB (7), we examined the electrophoretic mobilities of the immunoprecipitated RDEB procollagenase species. The apparent molecular weights of the RDEB procollagenases were 55,000 and 52,000, corresponding to those previously derived from quantitative amounts of the pure control proenzyme species using analytic ultracentrifugation (16). Further evidence for the high degree of similarity between the control and RDEB collagenases was shown by mixing material immunoprecipitated from control and RDEB cell lines before solubilization and SDS gel electrophoresis. In each case, the control and RDEB preparations comigrated, indicating little likelihood of any major alteration in molecular weight in the RDEB collagenases.

To assess the qualitative nature of the intracellular enzyme protein in RDEB cultures, we took advantage of the ion exchange behavior of the enzyme to achieve partial purification before precipitation (11). Fig. 2 shows a densitometric scan of the immunoreactive material found in RDEB cells. The radioactive doublet comigrated with the extracellular purified control collagenase standard, indicating that in RDEB, as in the control cell lines (8), some of the intracellular immunoreactive material exists in a form similar to or identical with the extracellular proenzyme species.

The rates of synthesis and secretion of collagenase were determined by quantitating both intracellular and extracellular labeled collagenase. The data in Figs. 3–5 are representative of nine such experiments in which replicate flasks were labeled with [^{3}H]leucine for variable lengths of time. A portion of the medium was harvested at each time point for determination of the extracellular enzyme content. Intracellular labeled enzyme protein was measured after addition of a 1,000-fold excess of cold leucine and subsequent chasing of the intracellular labeled material into the medium. As shown in Fig. 3, the intracellular labeled collagenase content of the two RDEB cell lines increased at a more rapid rate than that of the control line and reached a constant level ~1 h after the initiation of labeling (Fig. 3A and B). The amount of intracellular labeled enzyme protein was about twofold greater in the RDEB

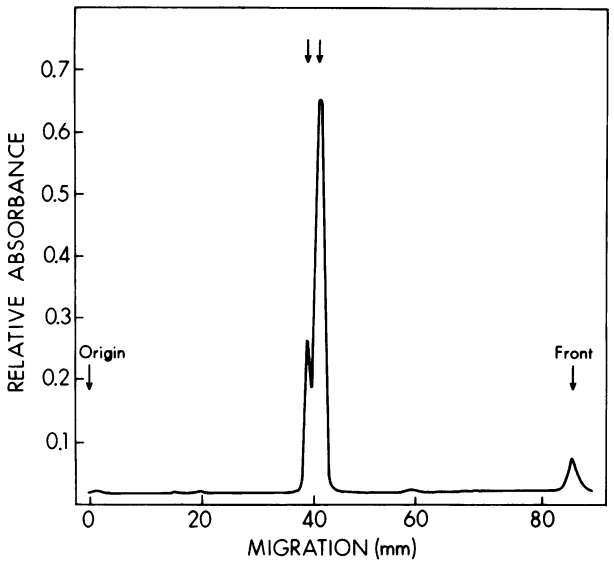

FIGURE 2 Densitometric scan of a fluorograph of the SDS gel electrophoretic pattern of immunoprecipitated intracellular material. A partially purified cell lysate (see text for details) was precipitated with anti-human skin collagenase before solubilization of the precipitates and electrophoresis. The paired arrows mark the electrophoretic mobilities of the two unlabeled purified extracellular control procollagenase standards.

cell lines (Fig. 3A and B) than in the control line (Fig. 3C), and remained at this level for the duration of the labeling period. Secretion of extracellular newly synthesized collagenase was easily seen at 1 h, the earliest time point examined in this experiment, and remained linear once the intracellular pool of labeled enzyme reached a constant level. This finding suggests that the RDEB cells are similar to control cells in that there is little intracellular storage of collagenase. Indeed, at the end of 6 h, in the RDEB cell lines, 89% (WUE 76114) and 81% (WUE 7572) of the labeled enzyme was found in the culture medium (i.e., extracellular) compared with 82% in the control cell line. Furthermore, these data suggest that secretion is largely reflective of *de novo* synthesis. Comparison of the slopes of the curves either for secretion alone or for total ^{3}H-labeled collagenase present in the medium and the cells showed that the RDEB cell lines WUE 76114 (Fig. 3A) and WUE 7572 (Fig. 3B) synthesized collagenase at rates respectively 2.6 and 2.4 times greater than the control cell line ($P < 0.005$).

Because of differences in the amount of [^{3}H]leucine used, the absolute values for radioactivity incorporated into collagenase protein cannot be compared between experiments. However, the time required for synthesis and secretion of collagenase by RDEB cells was examined in greater detail in separate experiments by labeling the fibroblast cultures for shorter intervals.

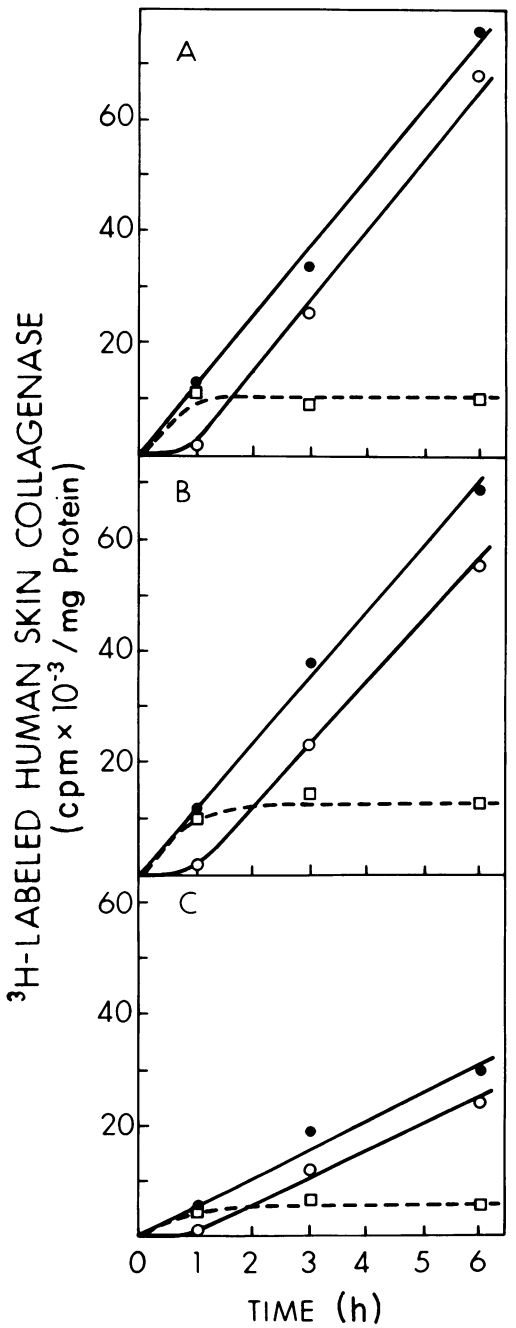

FIGURE 3 Synthesis and secretion of ^{3}H-labeled human skin collagenase. For each cell line, three identical confluent 10-cm^2 culture flasks were labeled with ~50 μCi/ml [^{3}H]-leucine for 1, 3, and 6 h. At the end of each respective labeling period, a portion of the medium was harvested to determine the ^{3}H-labeled collagenase content (extracellular). An excess of unlabeled leucine was then added and the intracellular enzyme was chased into the medium. A. WUE 76114; B. WUE 7572; C. control. ●, total (medium plus cells) ^{3}H-labeled collagenase; ○, extracellular ^{3}H-labeled collagenase; □, intracellular ^{3}H-labeled collagenase. The cell protein content was 0.45–0.48 mg/flask for WUE 76114, 0.45–0.5 mg/flask for WUE 7572, and 0.42–0.49 mg/flask for the control.

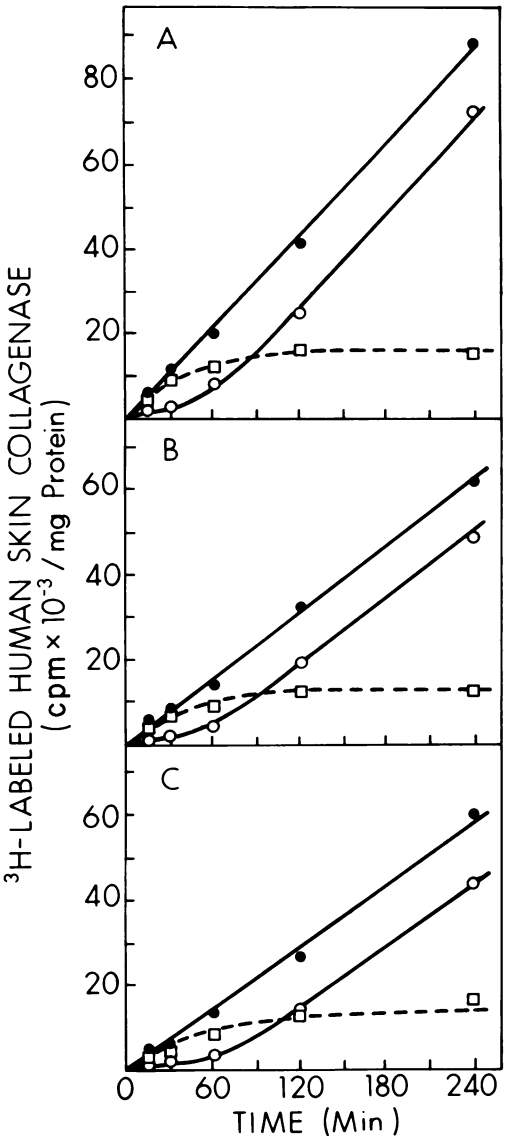

FIGURE 4 Short term synthesis and secretion of ^{3}H-labeled collagenase. Replicate culture flasks were labeled and processed as noted in Fig. 3. A. WUE 76114; B. normal control; C. EB simplex control, WUE 7567. ●, total ^{3}H-labeled collagenase; ○, extracellular ^{3}H-labeled collagenase; □, intracellular ^{3}H-labeled collagenase. The cell protein content was 0.4–0.46 mg/flask for WUE 76114, 0.41–0.49 mg/flask for the control, and 0.39–0.48 mg/flask for WUE 7567.

Fig. 4 depicts the short term kinetics of synthesis and secretion for WUE 76114 (Fig. 4A) compared with control cells (Fig. 4B) and, as a further control, compared with cells derived from a patient of the same sex and age with the dominant simplex form of EB (Fig. 4C). The slopes for total ^{3}H-labeled collagenase confirmed that overall synthesis in the RDEB cells was ~1.4 times greater than in the control fibroblast lines ($P < 0.02$). In six separate experiments employing WUE 76114, the rates of collagenase biosynthesis were 1.4 ($P < 0.02$)–4.4 ($P < 0.005$) times those of control cell strains with a mean increase of 2.7±0.5 (mean±SE) times. Short term incorporation of [^{3}H]leucine into collagenase was also examined in RDEB line WUE 7572 (Fig. 5). At the earliest time examined after the initiation of labeling, both intracellular and extracellular enzyme protein (Fig. 5A) were two to three times greater than in the control line (Fig. 5B). Here, too, the overall rate of synthesis of collagenase (medium plus cells) by the RDEB cell line was increased 2.7-fold ($P < 0.005$). The rates of collagenase biosynthesis in eight separate experiments for WUE 7572 ranged from 1.9 ($P < 0.001$)–9.2 ($P < 0.001$) times those of the control cultures with a mean increase in rate of 3.7±0.9 (mean±SE) times that of the controls.

The fate of intracellular ^{3}H-labeled collagenase was further examined in separate experiments by comparing that amount present in the intracellular pool

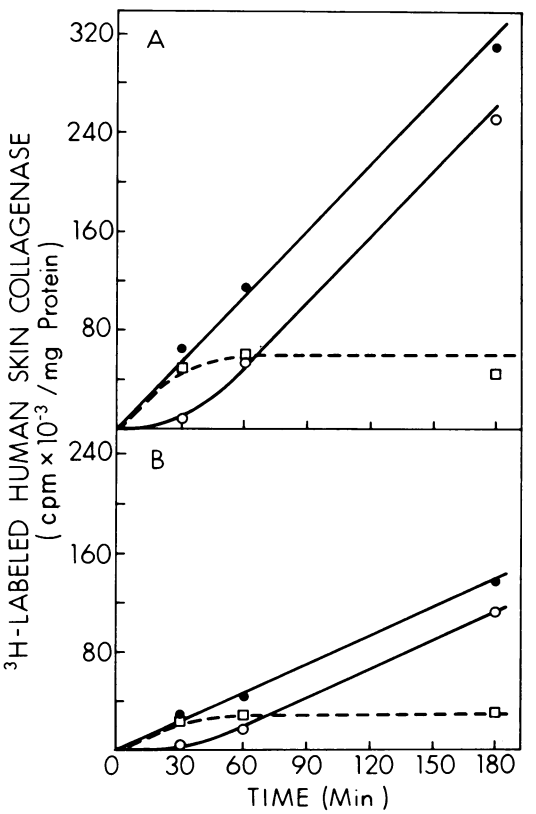

FIGURE 5 Short term synthesis and secretion of ^{3}H-labeled collagenase. Replicate culture flasks were labeled and processed as noted in Fig. 3. A. WUE 7572; B. control. ●, total ^{3}H-labeled collagenase; ○, extracellular ^{3}H-labeled collagenase; □, intracellular ^{3}H-labeled collagenase. The cell protein content was 0.32–0.39 mg/flask for WUE 7572 and 0.29–0.39 mg/flask for the control.

TABLE II
Fate of Intracellular ^{3}H-Labeled Human Skin Collagenase

Culture	Intracellular ^{3}H-labeled collagenase at zero time	Secreted ^{3}H-labeled collagenase after 2-h chase	Calculated loss due to degradation	
	cpm	*cpm*	*cpm*	*%*
Experiment*				
Control	10,625	9,990	635	6.0
WUE 76114	43,125	36,384	6,741	15.6
WUE 7572	70,506	43,898	26,608	37.7
Experiment 2‡				
Control	6,660	5,408	1,252	18.8
WUE 76114	8,700	6,120	2,580	29.7
WUE 7572	20,000	15,520	4,480	22.4

* Replicate 150-cm^2 culture flasks were labeled with 50 μCi/ml of [^{3}H]leucine for 6 h before processing. Intracellular ^{3}H-labeled collagenase was determined after partial purification of the immunoreactive enzyme protein in the cell lysates. For determination of secreted enzyme protein, a 1,000-fold excess of cold leucine was added and the medium was harvested after a 2-h chase period. The cell protein was 2.2–3.0 mg/flask for the control, 2.3–2.9 mg/flask for WUE 76114, and 2.3–2.9 mg/flask for WUE 7572.
‡ Replicate 75-cm^2 culture flasks were labeled with 50 μCi/ml of [^{3}H]leucine for 6 h and were processed as noted in experiment 1. The cell protein was 1.5–1.7 mg/flask for the control, 1.6–1.8 mg/flask for WUE 76114, and 1.8–1.9 mg/flask for WUE 7572.

at the beginning of the chase period in one set of cultures with that recovered 2 h later after secretion into the medium in a replicate set of cultures (Table II). In the control cultures, a mean value of 88% of the labeled intracellular collagenase protein was secreted into the medium as macromolecular material, whereas ~12% was apparently lost through intracellular degradation or exists in a form not readily recoverable with this method. Similarly, in the RDEB cell lines, ~74% of the ^{3}H-labeled collagenase was secreted as macromolecular material, whereas ~26% existed as putatively degraded compounds. These findings suggest that in the RDEB cultures, the intracellular ^{3}H-labeled collagenase has been lost, presumably through degradation, to an extent equal to or greater than that of the control fibroblast cultures, and that the differences in accumulation of immunoreactive material cannot be attributed to diminished intracellular degradation.

Because increased accumulation of collagenase in the cell culture medium might also reflect decreased extracellular degradation, we investigated the extracellular stability of both newly secreted ^{3}H-labeled proteins and ^{3}H-labeled collagenase. For these studies, the [^{3}H]leucine was chased with an excess of cold leucine (1 mM final concentration) and both trichloroacetic acid-precipitable and antibody-precipitable protein were measured at varying time points (Table III). Once secreted, both the total ^{3}H-proteins and the ^{3}H-labeled collagenase were stable in the medium in both control and RDEB fibroblast cultures for periods of up to 20 h. Thus, altered extracellular stability of the collagenase, at least as determined immunochemically, in RDEB cultures apparently did not account for the increased accumulation.

Because the rate of incorporation of radioactive leucine into protein is affected by several factors, other control experiments were performed to exclude possible sources of misinterpretation. In particular, the specific radioactivity of the intracellular leucine pool in the RDEB fibroblast cultures was found to be 10,507±1,379 cpm/nmol of leucine (mean±SE, n = 4) compared with 10,764±2,442 cpm/nmol (n = 4) in the control cells (P = NS). In addition, the growth kinetics of control and RDEB fibroblasts were measured. No significant differences in growth were observed with the control lines (n = 3), showing a mean population doubling of 2.20±0.57 d (mean±SE), whereas WUE 76114 and WUE 7572 had values of 2.18±0.24 and 1.82±0.16 d, respectively (P = NS).

To exclude the possibility that [^{3}H]leucine was being preferentially incorporated into collagenase in RDEB, we measured collagenase in the culture medium of a series of replicate cultures by three different methods—antibody precipitation, radioimmunoassay, and enzyme activity. As shown in Table IV, the ratios of ^{3}H-labeled collagenase to radioimmunoassayable collagenase and to collagenase activity in RDEB were essentially identical to those seen in

TABLE III
*Stability of Extracellular ^{3}H-Labeled Proteins**

Culture	Time‡	^{3}H-labeled proteins§	^{3}H-labeled collagenase‖
	h	*cpm* × 10^{-6}	*cpm* × 10^{-3}
Experiment 1			
Control	0	8.9	997.2
	3	10.1	1,001.7
	7	9.8	944.2
	20	9.0	ND¶
WUE 7572	0	7.3	1,001.6
	3	9.2	1,082.1
	7	9.7	946.0
	20	9.0	ND
Experiment 2			
Control	0	1.8	69.3
	4	2.6	83.7
	13	2.8	60.9
	20	2.7	88.2
WUE 76114	0	2.7	260.1
	4	3.5	320.3
	13	3.7	326.2
	20	3.5	305.7

* Confluent 75-cm^2 culture flasks were labeled either for 25 h (experiment 1) or 6 h (experiment 2) with 50 μCi/ml [^{3}H]leucine after which an excess of unlabeled leucine (1 mM) was added and portions of the culture medium were harvested at various time points from the same culture flask.

‡ Time elapsed after an excess of nonradioactive leucine was added.

§ 10% trichloroacetic acid-precipitable proteins in the culture medium.

‖ ^{3}H-labeled collagenase was quantitated as net counts per minute precipitated above an ovalbumin-antiovalbumin control.

¶ ND, assay not done.

the control preparations. Thus, the increase in ^{3}H-labeled collagenase in RDEB was not the result of preferential [^{3}H]leucine incorporation into the enzyme.

Because our studies indicated that there was no further accumulation of intracellular enzyme protein after the first 60–90 min and that the rate of secretion was largely representative of *de novo* synthesis (Fig. 3), we used the extracellular labeled enzyme to approximate the relative synthesis of collagenase (Table V). For these experiments, the amount of ^{3}H-labeled collagenase in the medium was measured in relation to the total newly synthesized trichloroacetic acid-precipitable protein in the medium and cells. In each case, despite an overall 1.5- to 2.7-fold increase in ^{3}H-labeled collagenase, the RDEB cells displayed no increase in general protein synthesis. Thus, the increase in synthesis of the enzyme cannot be attributed only to enhanced protein synthesis.

DISCUSSION

In the present study, we have used a sensitive, highly specific immunoprecipitation method (8) to explore the mechanisms involved in increased accumulation of collagenase in skin fibroblast cultures derived from patients with RDEB. These biosynthetic techniques have provided evidence indicating that the excessive accumulation of collagenase is the result of enhanced synthesis. First, the initial rate of accumulation of intracellular labeled material was greater in RDEB cells than in control fibroblast cultures (Figs. 3–5). Second, secretion of ^{3}H-labeled collagenase into the medium occurred at significantly greater rates than in the control lines (Figs. 3–5). Third, the turnover of labeled enzyme protein in the RDEB cultures was equal to or greater than that seen with control cells, indicating that diminished enzyme degradation could not account for the difference between control and RDEB cultures. Fourth, no preferential incorporation of [^{3}H]-leucine into RDEB collagenase was observed as evidenced by the close correlation in quantitation of collagenase whether measured by enzyme activity, radioimmunoassay, or direct immunoprecipitation of ^{3}H-labeled enzyme (Table IV).

The increased synthesis of collagenase appeared to be a specific manifestation of the RDEB cells. Although the rate of synthesis of collagenase was consistently found to be 1.5–3 times greater in RDEB cells than in the control lines (range, up to nine times), total protein synthesis was not enhanced (Table IV and V). Furthermore, other potential sources for misinterpretation, such as enhanced growth kinetics or altered specific radioactivity of the intracellular leucine pool, were equal to those of control cultures.

Our previous studies indicated the existence of an altered form of collagenase in the fibroblast cultures of at least three patients with RDEB, as manifested by altered thermal stability in the partially purified enzyme. This finding was correlated with an increased apprent K_m for Ca^{2+}, a thermostabilizing cofactor (7). In addition, the RDEB collagenase displayed an apparent diminished catalytic efficiency (7), a finding that our recent observations (unpublished) suggest can also be ascribed to marked lability of the enzyme. Despite these differences, the RDEB collagenases were quite similar to control preparations in their cation exchange behavior and immunologic properties (7). In the present study we further explored the degree of structural alteration in the RDEB collagenases by assessing the electrophoretic behavior of the antibody-precipitated enzyme protein. The finding of electro-

TABLE IV

*Ratio of ^{3}H-Labeled Collagenase to Collagenase Quantitated by Radioimmunoassay and Enzyme Activity**

Culture	A	B		C	
	^{3}H-labeled collagenase‡	Radioimmunoassayable collagenase§		Collagenase activity‖	
	cpm/mg	*ng/mg*	*ratio (A/B)*	*cpm/mg*	*ratio (A/C)*
Experiment 1					
Control	140,816	ND¶	ND	85,700	1.6
WUE 7572	730,679	ND	ND	536,200	1.4
Experiment 2					
Control	29,621	1,045	28.4	7,607	3.9
WUE 7572	191,264	6,713	28.5	47,632	4.0

* Replicate 75-cm^2 culture flasks (experiment 1) or 10-cm^2 culture plates (experiment 2) were incubated for 12 h before harvesting the medium for determination of collagenase by direct precipitation, radioimmunoassay, or enzyme activity.

‡ ^{3}H-labeled collagenase is expressed as total counts per minute per milligram of cell protein above an ovalbumin-antiovalbumin control.

§ Radioimmunoassayable collagenase protein is expressed as total nanograms per milligram cell protein and as the ratio of ^{3}H-labeled collagenase to radioimmunoassayable collagenase (A/B).

‖ Collagenase activity is expressed as the total counts per minute, ^{14}C-collagen solubilized at 37° per milligram cell protein and as the ratio of ^{3}H-labeled collagenase to collagenase activity (A/C).

¶ ND, assay not done.

phoretic mobilities identical to those seen in the control enzyme preparations (Fig. 1) further supports our earlier postulate of the existence of only a small, but functionally significant, difference in structure (7). In addition, it is noteworthy that the intracellular species of RDEB procollagenase were also electrophoretically similar, if not identical, to the control preparations (Fig. 2). It remains possible, however, that by using a system of cell-free synthesis with mRNA harvested from RDEB fibroblasts, we may be able to detect minor variations in either the primary or posttranslationally modified gene products.

Taken together, the data indicate that enhanced synthesis of collagenase is an important pathophysiologic aspect of RDEB. This characteristic is expressed both in vitro and in vivo (5–7). Indeed, the rates of collagenase biosynthesis observed in these two particular RDEB patients' cells in vitro (1.4- to 9-fold increased) correlate well with the amount of immunoreactive collagenase found previously in vivo (5, 6) in their normal-appearing skin (three to fourfold increased). Although the precise mutation leading to overproduction of collagenase is at present unknown, the data suggest a defect in a regulatory gene or in a gene coding for another cellular function that modulates collagenase expression. For at least some RDEB patients, however, this explanation is inadequate because there is evidence for the existence of a structural gene mutation associated with oveproduction (7). Indeed, preliminary studies (unpublished) using culture medium from WUE 76114 strongly suggest the existence of a structurally altered form of collagenase in this patient as well.

Although increased enzyme activity has been observed in a variety of genetic circumstances, both primary and secondary mechanisms have been cited as the basis for such increases (20–26). In a situation perhaps more directly analogous to RDEB, examples of genetically increased synthesis of abnormal gene products —both intracellular and extracellular—have also been described (27–30). The most detailed study to date has been in the Hektoen variant of glucose-6-phosphate dehydrogenase in which a single amino acid substitution in the enzyme was found to be associated with a fourfold increase in synthesis of the gene product (31), representing a so-called quantitative mutant (32). As discussed by Yoshida (31) and in a later review by Goldberger (33), the data suggest the possibility of defective autogenous regulation of synthesis, a mechanism known to exist in bacteria and fungi and recently described in the biosynthesis of mouse myeloma protein (34, 35).

Irrespective of whether such a mechanism exists in RDEB, it should be noted that increased collagenase

TABLE V

Accumulation of ^{3}H-Labeled Human Skin Collagenase in the Fibroblast Culture Medium

Culture	Time*	Cell protein	^{3}H-labeled proteins‡	^{3}H-labeled human skin collagenase§	
	h	*mg*	*cpm × 10^{-6}/mg*	*cpm × 10^{-3}/mg*	*% of total*
Experiment 1‖					
Control 1	1.5	0.29	30.5	44.4	0.15
	4.0	0.29	58.9	229.9	0.39
	7.5	0.31	75.2	338.7	0.45
Control 2	1.5	0.27	27.2	37.3	0.14
	4.0	0.25	46.7	180.1	0.39
	7.5	0.27	74.6	366.5	0.49
WUE 7572	1.5	0.36	28.1	79.4	0.28
	4.0	0.33	53.6	313.9	0.59
	7.5	0.35	69.6	620.0	0.89
Experiment 2¶					
Control	3.0	0.33	33.2	61.5	0.19
	10.0	0.29	90.6	168.6	0.19
WUE 7572	3.0	0.25	29.7	95.6	0.32
	10.0	0.26	89.1	333.1	0.37
Experiment 3**					
Control 1	6.0	1.64	ND	46.2	ND
	12.0	1.58	37.0	68.5	0.19
Control 2	6.0	2.80	ND	23.1	ND
	12.0	2.74	22.3	32.8	0.15
WUE 76114	6.0	1.67	ND	51.5	ND
	12.0	1.64	26.4	97.1	0.37
Experiment 4‡‡					
Control 1	5.0	1.73	12.1	35.3	0.29
	10.0	1.59	24.0	85.0	0.35
Control 2	5.0	1.26	10.5	101.4	0.97
	10.0	1.06	16.6	327.3	1.97
WUE 76114	5.0	1.08	7.4	132.2	1.79
	10.0	1.15	12.6	561.2	4.45

* Time elapsed after [^{3}H]leucine was added to the cultures.

‡ 10% trichloroacetic acid precipitable ^{3}H-labeled proteins in the medium plus cells.

§ Total net radioactivity (specific minus nonspecific) precipitated from the culture medium with antiserum to human skin collagenase per milligram cell protein. The data are also presented as a percent of the total (medium plus cells) trichloroacetic acid-precipitable radioactivity in the culture.

‖ Replicate 10-cm^2 culture plates were labeled with 70 μCi/ml of [^{3}H]leucine. Control 1, WUC 1124; control 2, WUN 77248.

¶ Replicate 10-cm^2 culture plates were labeled with 50 μCi/ml of [^{3}H]leucine. Control, WUN 76130.

** Replicate 75-cm^2 culture flasks were labeled with 11 μCi/ml of [^{3}H]leucine. Control 1, WUG 3348; control 2, WUE 7567. (ND, assay not done).

‡‡ Replicate 75-cm^2 culture flasks were labeled with 20 μCi/ml of [^{3}H]leucine. Control 1, WUN 76130; control 2, WUN 77248.

synthesis is genetically unique for RDEB (6), and that other genetic types of EB are not characterized by alterations in collagenase expression (6). This fact was emphasized in the present study by the observation of normal rates of collagenase biosynthesis by cells derived from a patient with dominant EB simplex (Fig. 4). Furthermore, our recent observations in cell cultures derived from patients with the albopapuloid form

of dominant dystrophic EB (36) indicate that this variety of EB is genetically characterized by a disorder in glycosaminoglycan synthesis. The use of these in vitro biochemical markers should now permit us rationally to address the problems of genetic counseling and therapeutic intervention in this debilitating and often fatal group of diseases.

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