RECESSIVE DYSTROPHIC EPIDERMOLYSIS BULLOSA

Evidence for Increased Collagenase as a Genetic Characteristic in Cell Culture*

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Recessive dystrophic epidermolysis bullosa (RDEB)[1] is a debilitating and often fatal disease characterized by repeated blistering after minor trauma. The morphologic finding of collagen degeneration (1) coupled with increased collagenase activity in explant cultures of skin from RDEB patients (2, 3) suggested that excessive collagenase might be repsonsible for the blistering phenomenon. This postulate was further substantiated by the demonstration of increased tissue levels of immunoreactive collagenase (iHSC) in both the unaffected and blistered skin of RDEB patients (4). Evidence that some abnormality in the regulation of collagenase synthesis or activity was important in the pathogenesis of the disease was obtained when collagenases derived from skin fibroblast cultures of two patients with RDEB were shown to be structurally altered (5). The enzymes, which appeared to be overproduced in cell culture, demonstrated increased thermolability and reduced affinity for Ca^{2+}, a cofactor required both for enzyme activity and thermal stability (6). In addition, there was decreased activity per unit immunoreactive protein, raising the question of the relationship of the altered enzymes to the blistering phenomenon.

If a primary defect in the regulation of synthesis and/or degradation of collagenase were basic to the etiology of RDEB, it might be expected that increased levels of iHSC would be seen in fibroblast cultures. Thus, it seemed essential to determine (*a*) whether a quantitative increase in iHSC and/or collagenase activity could be found in RDEB fibroblast cultures, (*b*) whether such a phenotypic trait persists through many cell passages, (*c*) whether this trait might be utilized to distinguish RDEB from other genetic forms of the disease, and (*d*) the extent to which heterogeneity is manifested within the fibroblast cultures of various RDEB patients.

Materials and Methods

Cell Cultures. RDEB fibroblast cultures were established from a 3-mm skin punch biopsy after obtaining informed consent. Control cultures were initiated from healthy volunteers or were purchased from The American Type Cell Culture Collection, Rockville, Md. As a further

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[1] *Abbreviations used in this paper:* DDEB, dominant dystrophic EB; DEBS, dominant EB simplex; EB, epidermolysis bullosa; HG, high glucose; iHSC, immunoreactive human skin collagenase; RDEB, recessive dystrophic EB; REBL, recessive EB letalis.

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TABLE I

Description of Human Skin Fibroblast Lines

Controls	RDEB	REBL	DDEB	DEBS
WUN 76128 (F 47)*	WUE 7506 (F 12)‡	WUE 7515 (F 30)	WUE 76111 (M 20)	WUE 76103 (F 32)
CRL 1141 (M 3)	WUE 7565 (F 3)§	WUE 7510 (M 15)‖	WUE 7560 (F 12)	WUE 76102 (F 14)
CRL 1187 (M 14)	WUE 76108 (M 4)	WUE 7514 (M 14)‖	WUE 7512 (M 19)	WUE 7567 (M 4)
CRL 1119 (M 15)	WUE 7504 (F 8)		WUE 7513 (M 1)	WUE 7535 (M 10)
CRL 1124 (F 40)	WUE 7564 (F 1)		WUE 7570 (F 25)	WUE 7502 (F 19)
WUN 76130 (F 20)	WUE 77200 (F 4)		WUE 7557 (M 21)	
WUN 76131 (F 27)	WUE 76114 (M 1)		WUE 7503 (F 2)	
CRL 1121 (M 3)	WUE 76124 (F 1 mo)		WUE 7508 (F 10)	
CRL 1106 (fetus)¶	WUE 7505 (F 14)		WUE 7562 (F 64)	
	WUE 7572 (F 34)			

* Donor, sex, and age are noted in parenthesis.

‡ WUE 7506 previously reported as RDEB-FC (5).

§ WUE 7565 previously reported as RDEB-TH (5).

‖ WUE 7510 and WUE 7514 described previously as cases 1 and 2 (7) and as cases 4 and 5 (8), respectively.

¶ Sex unknown (See ATCC Catalog, 1975).

control, fibroblast cultures were established from patients with other genetically distinct forms of epidermolysis bullosa (EB)—recessive EB letalis, dominant dystrophic EB, and dominant EB simplex. All patients were examined by one of us (E.A.B.) and classified based on clinical, genetic, and histologic findings as described previously (4). The cell lines employed in this study are detailed in Table I.

Cells were grown in plastic culture flasks (Corning Glass Works, Science Products Div., Corning, N.Y.) in Dulbecco's modified Eagle's medium-high glucose + glutamine (Microbiological Associates, Walkersville, Md.) with 0.03 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (pH 7.6), 20% fetal calf serum, and 200 U of penicillin and 200 μg of streptomycin per ml at 37°C. In most of the experiments to determine the concentration of iHSC in the culture medium, fibroblasts were grown to early confluence, a cell density at which collagenase accumulation is greatest (9). Serum-containing medium was then removed, the cells were washed four times with Hanks' balanced salt solution, and the cultures were maintained in serum-free Dulbecco's modified Eagle's medium-HG + glutamine for 24 h. This serum-free medium was then dialyzed against 4,000 vol of distilled water, lyophilized, and stored at −80°C until assayed.

In studies to assess the specific activity of collagenase in RDEB fibroblast cultures, cells were grown in plastic roller bottles (490 cm^2, Corning Glass Works) or in 75 cm^2 plastic culture flasks (BioQuest, BBL & Falcon Products, Becton, Dickinson, & Co., Cockeysville, Md.). At confluence, the cells were maintained for 24–48 h in serum-free medium, after which the medium was concentrated and assayed for collagenase activity and immunoreactive enzyme protein.

Enzyme Assays. Human skin procollagenase was activated proteolytically with trypsin as described previously (10). For each enzyme preparation, a range of trypsin concentrations (0.1–2.0 μg trypsin per 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 approximately 3,000 cpm per substrate gel (11).

The levels of collagenase in certain RDEB fibroblast cultures were compared with those of β-glucuronidase, a lysosomal enzyme, and lactate dehydrogenase, a cytoplasmic enzyme. In these studies, after harvesting the serum-free culture medium, the cells were washed with Hanks' balanced salt solution, lysed with a Sonic Dismembrator for 30 s at an intensity of 60 dB and assayed for protein, β-glucuronidase (12), and lactate dehydrogenase (13) using kits obtained commercially (Sigma Chemical Co., St. Louis, Mo.).

Collagenase Radioimmunoassay. iHSC was measured by a slight modification of the double antibody radioimmunoassay previously reported (14). The procollagenase used as the unlabeled standard and for iodination in the radioimmunoassay was purified to homogeneity from cell culture medium as described by Stricklin et al. (15). This same enzyme preparation was used

to produce monospecific antiserum to the enzyme (16). Standard radioimmunoassay curves were performed in duplcate with disposable plastic microfuge tubes (Beckman Instruments, Inc., Fullerton, Calif.) that contained a 1:2,500 dilution of the gamma globulin fraction of antiserum to human skin collagenase, ^{125}I-labeled human skin collagenase (approximately 20,000 cpm/tube) and 0–100 ng of electrophoretically homogeneous human skin collagenase in a total vol of 250 μl. After incubation for 24 h at 4°C, goat anti-rabbit IgG was added in excess. The resulting precipitates were isolated, washed, and counted in a single channel gamma scintillation spectrometer. Unknowns, consisting of the various collagenase preparations, were reconstituted in a small vol of 0.05 M Tris-0.15 M NaCl buffer and assayed for immunoreactive enzyme protein in an identical fashion using 100-μl portions of serial doubling dilutions of the enzymes. Controls in which nonimmune rabbit gamma globulin was substituted for specific antiserum were routinely included.

Protein Synthesis Studies. General protein synthesis was measured in control and representative RDEB fibroblast cultures. In these studies, the cells were grown to confluence, washed, and placed in serum-free medium containing 10 μCi/ml of a [^{14}C]labeled amino acid mixture. After 24 h at 37°C, the cultures were harvested and total protein synthesis was determined as the amount of [^{14}C]labeled amino acids incorporated into 10% trichloroacetic acid-insoluble material in the medium and cells.

Other Assays. Mean population doubling times of each cell line were determined by seeding cultures from a single large pool at low density. Replicate cultures were fed serum-containing medium every 2 days for the length of the experiment. Duplicate cultures were harvested daily for cell counts. Protein (17) and DNA (18) were determined using established methods.

Results

In initial studies iHSC levels were determined in the fibroblast culture media of 10 patients with RDEB, 3 with recessive EB letalis, 9 with dominant dystrophic EB, and 5 with dominant EB simplex. As shown in Fig. 1, the mean concentration of iHSC in the cultures of the 10 RDEB patients was 328% of the control cultures ($P < 0.001$). The range of values for iHSC content of the RDEB culture medium was 104–645% of control, suggesting considerable heterogeneity among the RDEB patients. In contrast, iHSC was found to be 95% of control in recessive EB letalis (range, 70–115%), 91% of control in dominant dystrophic EB (range, 39–138%), and 96% of control in dominant EB simplex (range, 79–130%). In no case were the levels of enzyme protein significantly different from those of control cultures (Fig. 1).

Although the RDEB cultures as a group had significantly increased iHSC concentrations, the wide range of values found for the iHSC levels suggested that heterogeneity in the quantity of iHSC being produced was present. This possibility was explored by comparing the iHSC production by each RDEB fibroblast line with that of control lines within a series of experiments rigidly controlled for growth conditions. In these experiments the cells were placed in serum-free medium only at 80–90% confluence. Furthermore, to minimize any variability in collagenase expression which might be caused by pH differences,[2] a single preparation of culture medium was utilized for all 7 experiments. As a group the RDEB cell lines displayed an approximate threefold increase in iHSC/mg protein ($P < 0.01$) (Table II). Individually, however, there was significant variability in iHSC production with 8 of the 10 fibroblast lines having significant increases in iHSC (Table II). Several lines had iHSC levels which were up to fivefold increased, but two lines, WUE 7504 and WUE 76124, failed to demonstrate any increase in iHSC concentration.

[2] D. B. Busiek and E. A. Bauer. 1978. Environmental pH modulation of collagenase in normal human fibroblast cultures. Manuscript submitted for publication.

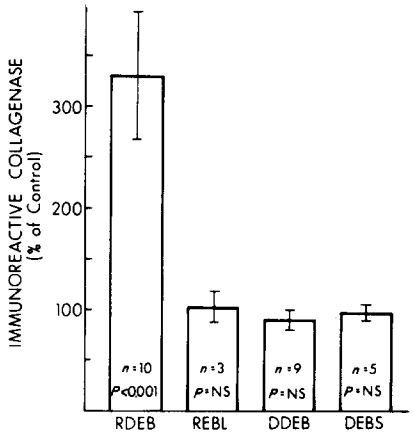

FIG. 1. Immunoreactive collagenase concentration of EB fibroblast cultures. Collagenase was quantitated as ng iHSC/mg protein in the culture medium of each EB group in a total of 21 different experiments. RDEB represents 10 different cell lines; recessive EB letalis (REBL), 3 different cell lines; dominant dystrophic EB (DDEB), 9 different cell lines; dominant EB simplex (DEBS), 5 different cell lines. The values are presented as percent of control, so that comparisons can be made between experiments. Statistical analysis was made using an analysis of variance after it was determined that variances within groups were homogeneous by Bartlett's test.

TABLE II

Production of Immunoreactive Collagenase by Normal and RDEB Fibroblast Cultures

Culture	Number*	Immunoreactive collagenase	*P*
		ng/mg protein‡	
Controls	22	1,274.0 ± 199.2	—
RDEB			
WUE 7506	5	2,376.9 ± 263.6	<0.01
WUE 7565	2	2,645.1 ± 100.8	<0.025
WUE 76108	12	1,978.5 ± 305.5	<0.05
WUE 7504	4	1,380.6 ± 210.6	NS§
WUE 7564	4	5,768.5 ± 1,414.1	<0.0125
WUE 77200	4	2,831.0 ± 799.1	<0.005
WUE 76114	5	6,439.9 ± 1,615.5	<0.001
WUE 76124	3	1,230.1 ± 327.7	NS§
WUE 7505	4	2,293.5 ± 778.0	<0.05
WUE 7572	2	6,268.2 ± 468.0	<0.001

* Number of cultures examined in each cell line. Controls represent 22 cultures examined in seven different experiments.

‡ Expressed as mean ± SE; the statistical significance of each value is expressed relative to the control cultures using Student's *t* test.

§ NS, not significant.

Previous studies of the collagenases from two RDEB cell lines showed decreased activity per unit immunoreactive protein (5). Thus, this parameter was also investigated in eight of the RDEB cell lines (Table III). Collagenase activity displayed significant variation between fibroblast lines, perhaps due to variable loss of enzyme activity in structurally comprised enzymes during the 24-h serum-free incubation, as suggested previously (5). It is noteworthy, however, that in five of the eight cell lines

TABLE III

Specific Activity of Collagenase from RDEB Fibroblast Cultures

Culture	Collagenase activity*	Immunoreactive collagenase‡	A/I§
	%C	*%C*	*%C*
WUE 7506	138.5 ± 46.4	234.0 ± 42.9	59.2
WUE 7565	20.6 ± 11.7	188.3 ± 17.4	10.9
WUE 76108	112.8 ± 56.6	340.3 ± 92.1	33.1
WUE 7504	66.4 ± 5.9	127.5 ± 41.3	52.1
WUE 7564	102.4 ± 35.6	644.8 ± 121.8	15.9
WUE 76114	138.6 ± 6.8	424.1 ± 75.8	32.7
WUE 7505	20.0	242.9 ± 37.1	8.2
WUE 7572	146.4 ± 29.7	668.6 ± 280.9	21.9

* Collagenase activity determined as cpm, [^{14}C]collagen solubilized per mg protein in 5 h at 37°C and expressed as mean ± SE % of control for all experiments performed with each line.

‡ Immunoreactive collagenase was quantitated as ng per mg protein as detailed in Fig. 1 and expressed as mean ± SE % of control.

§ Ratio of activity per immunoreactive protein.

TABLE IV

Comparison of Collagenase Activity with Lysosomal and Cytoplasmic Enzymes in Normal and RDEB Fibroblast Cultures

Culture	Collagenase		β-Glucuronidase	Lactate dehydrogenase
	cpm/μg DNA	*cpm/μg protein*	*U/μg DNA*	*U/μg DNA*
Exp. 1				
Controls*	768.3 ± 393.3	119.2 ± 68.9	2.12	406.7
WUE 7572	4,047.6	580.4	2.36	508.2
	(526.8%C)	(487.1%C)	(111.3%C)	(125.0%C)
WUE 7506	2,217.5	360.0	3.75	334.1
Exp. 2	(288.6%C)	(302.1%C)	(176.9%C)	(82.1%C)
Controls*	704.1 ± 134.9	157.5 ± 72.3	1.06	244.5
WUE 7572	2,947.0	593.3	1.00	269.9
	(418.6%C)	(376.7%C)	(94.3%C)	(110.4%C)
WUE 7506	1,370.7	252.0	1.34	222.8
	(194.7%C)	(160.0%C)	(126.4%C)	(91.1%C)

* Controls represent mean ± SE values from two different cell lines.

examined mean collagenase activity, determined as activity per mg protein, was normal or increased. Thus, in most instances it appeared that the disproportionate increase in immunoreactive enzyme protein was largely responsible for the decrease in collagenase activity per unit immunoreactive protein. Normal human skin fibroblast lines displayed little variability in either collagenase activity or immunoreactive protein. The mean activity was 75.6 ± 20.5 cpm/μg protein (mean ± SE) while immunoreactive enzyme was 1,003.7 ± 309.6 ng/mg protein. The mean variability for control cultures between experiments was 27.4%.

In two representative RDEB lines a comparison was made between the enzymatic activity of collagenase and that of β-glucuronidase, a lysosomal enzyme, and lactate dehydrogenase, a cytoplasmic enzyme. As depicted in Table IV, one RDEB line, WUE 7572, showed a four- to fivefold increase in collagenase activity in these

TABLE V

Effect of Cell Passage on the Expression of Immunoreactive Collagenase

Culture	Passage	Immunoreactive collagenase*
		%C
WUE 7506	8	376.4
	11	198.6
	12	416.1
	13	322.1
WUE 7565	5	213.5
	9	228.6
WUE 76108	4	250.2
	6	949.6
	7	220.4
	10	975.5

* iHSC content of the cultures was determined as ng/mg protein and expressed as the percent of control.

experiments, while β-glucuronidase and lactate dehydrogenase activities were essentially the same as those of control cultures. A second RDEB line, WUE 7506, displayed up to a threefold increase in collagenase activity in these particular experiments while having at greatest a 1.8-fold increase in β-glucuronidase. Lactate dehydrogenase was equal to that of the control cultures. These findings illustrate that in any given experiment marked increases in collagenase activity can be observed, again suggesting that the differences in enzyme activity between experiments are related to variable loss of activity in structurally altered enzymes (5).

As a further measure of the relative capacity of these RDEB fibroblast cultures to produce collagenase when compared to normal control lines, total protein synthesis was quantitated. Although there was a 2.9-fold increase in collagenase in line WUE 7506, total protein synthesis was equal to that found in control cultures (202,041 cpm/mg cell protein; 91.5% of control). The other RDEB line, WUE 7572, had approximately two times greater total protein synthesis, (506,361 cpm/mg cell protein; 229.3% of control), but this increase did not account for the greater than fivefold increase in collagenase seen in the experiment. In addition, mean population doubling times were determined in control and RDEB fibroblast cultures and found to be 2.20 ± 0.57 days (mean ± SE) for the control cultures and 1.82 ± 0.16 and 2.63 ± 0.51 days for WUE 7572 and WUE 7506, respectively (P = NS). Thus, the increased concentration of iHSC in the RDEB fibroblast cultures was not due to differences in the kinetics of growth of the mutant cells.

If increased production of iHSC were a genetic characteristic of RDEB cells, it might be expected that this trait would be expressed throughout several cell passages. Three lines were examined in both low and high serial passages (Table V). Although each line reflected the intrinsic variability seen between experiments, there was no apparent loss of the ability to synthesize increased amounts of iHSC.

Discussion

The present study indicates that cell cultures of RDEB patients express an increase in immunoreactive collagenase protein analogous to that seen in vivo. It appears that this parameter may serve as a genetic marker for at least some of the RDEB patients, since 8 out of 10 cell lines studied had significant elevations in iHSC (Table II). In

TABLE VI

Comparison of in Vivo and in Vitro Immunoreactive Collagenase in EB Patients

Patient	In vivo iHSC*	In vitro iHSC‡
	%C	%C
RDEB		
WUE 76114	383.9	424.1
WUE 7572	300.4	668.6
REBL		
WUE 7510	169.2	100.0
WUE 7514	292.8	70.1

* Determined as ng/mg tissue dry weight (4).

‡ Determined as ng/mg protein and expressed as the mean percent of control for all experiments performed with each cell line.

addition, in five of the eight cell lines examined enzyme activity as well as iHSC was increased.

It is important to emphasize that increased collagenase was not seen uniformly in fibroblast culture, a finding that suggests that all patients who phenotypically appear to have RDEB may not have the same biochemical defect. Since all of the patients in this study classified as RDEB were sporadic cases within a kindred, the two patients with normal in vitro production of immunoreactive collagenase might represent new mutants for dominant dystrophic EB, although this possibility may prove unlikely on clinical grounds (4, 19, 20).

It is of interest that 4 of the 27 patients whose cells were examined in this study also had previous in vivo determinations of iHSC (4). The in vivo and in vitro iHSC levels of these patients have been summarized in Table VI, showing that the elevated tissue levels of collagenase in the RDEB patients are reflected in vitro. Despite the increased in vivo iHSC in certain patients with recessive EB letalis, a genetically different form of EB, in vitro collagenase was not increased. The reason for the increased tissue levels of collagenase protein is not known, but it is possible the in vivo elevation is reflective of a chronic wound healing process in these severely affected recessive EB letalis patients which is not expressed in fibroblast cultures.

The underlying mechanism for the increased in vitro concentrations of collagenase in RDEB fibroblasts is at present unknown. The highest concentrations of iHSC were seen most consistently in late log phase growth, a time during which the concentrations of many enzymes are most reflective of synthesis and protein degradation is, in general, lowest (21). Until detailed studies of the biosynthesis and degradation of collagenase in cell cultures are available, however, both overproduction and/or decreased degradation of enzyme protein represent possible mechanisms for iHSC accumulation.

The increased concentration of collagenase appears to be a specific manifestation of the mutant RDEB cells. When a comparison was made between the ability of these cells to express collagenase, measured as enzyme activity or immunoreactive material, and their ability to express prototypic lysosomal (β-glucuronidase) and cytoplasmic (lactate dehydrogenase) enzymes, collagenase was significantly greater (Table IV). Furthermore, neither a change in mean population doubling times nor in general protein synthesis appeared to be of sufficient magnitude to account for the greater enzyme concentrations.

Our previous studies utilizing cells from two RDEB patients (WU 7506, WUE 7565) indicated the collagenases were structurally altered as manifested by decreased thermal stability, diminished affinity for Ca^{2+} and decreased activity per immunoreactive protein (5). Despite the increased in vivo and in vitro concentrations of enzyme protein, the finding of functionally altered enzymes raises the question of the relationship of the enzyme abnormalities to the disease. As shown in Table III, six more patients, in addition to those previously reported (WUE 7506 and WUE 7565), have been identified in whose cells collagenase activity per immunoreactive material is decreased. The collagenase from one of the additional patients (WUE 76108) also manifests diminished thermal stability and altered cofactor and substrate kinetics (unpublished observations), establishing that this enzyme is structurally altered like those of the two previous patients (5). Although the collagenases from the remaining patients have not yet been purified and examined for thermal stability and cofactor requirements, if the apparent lower catalytic efficiency (activity/immunoreactive protein) is the result of a structurally altered enzyme, this parameter in conjunction with increased accumulation of immunoreactive material would represent an important genetic marker for RDEB. Indeed, the data indicate that in at least three patients with RDEB there is overproduction of a structurally defective collagenase. In a similar fashion, overproduction of altered enzymes has been observed in other genetic disorders (22–24).

Although structurally aberrant collagenases occur in vitro, it is clear that if some collagenases are functionally near normal in vivo, as suggested by the enzyme activities (Table III), overproduction may well have important pathogenetic links to connective tissue destruction and blistering. In addition, it is possible that such an increase in collagenase may in part explain the decreased or absent anchoring fibrils observed in vivo (25, 26).

Kanan et al. (27) using electron microscopy have recently reported that cultured fibroblasts from RDEB patients contain an increased number of lysosomal bodies akin to those seen in mucopolysaccharide storage diseases. They postulated that alterations in lysosomal function might explain the abnormal collagen metaoblism seen in RDEB. Except for the collagenase from polymorphonuclear leukocytes (28, 29), there is no evidence to suggest that human collagenases are lysosomal enzymes. Indeed, a nonparallel relationship was seen between collagenase and β-glucuronidase in the RDEB lines examined. Thus, the relationship between increased iHSC and the increased lysosomal bodies seen on electron microscopy remains to be determined.

Summary

Fibroblast cultures from patients with recessive dystrophic epidermolysis bullosa (RDEB) demonstrated an increased capacity to synthesize and secrete collagenase. This phenotypic trait appeared to distinguish RDEB from other genetically distinct forms of epidermolysis bullosa. The finding of increased collagenase may be a specific manifestation of these cells in that prototypic lysosomal and cytoplasmic enzymes were present in approximately normal concentrations. In addition, this trait persisted through many cell passages, suggesting that the property was genetically determined. The elevated concentrations of immunoreactive collagenase in fibroblast cultures of patients with RDEB reflected those previously observed in vivo (4) and support the concept of a pathogenetic role for the enzyme in the blistering phenomenon. In three of the cell lines, the increase in enzyme protein occurred in association with a

structurally defective enzyme. The data suggest that this may be a characteristic of all RDEB cells.

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