

Collagen Biosynthesis in a Case of Epidermolysis bullosa dystrophica recessiva

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Chamson A, Claudy A, Frey J. Collagen biosynthesis in a case of epidermolysis bullosa dystrophica recessiva. Acta Derm Venereol (Stockh) 1987; 67: 16-23.

Collagen metabolism was studied in fibroblast cultures from a patient presenting an epidermolysis bullosa dystrophica recessiva (EBDR) syndrome characterized, in particular, by blistering below the basal lamina observed by electron microscopy. The previously described increase in collagen production was confirmed and several other qualitative modifications of the secreted collagen were observed, including an underhydroxylation of lysine, a decrease in the type III/type I collagen ratio, and an increase in the rapidly degraded collagen. On the other hand, these fibroblasts were able to organize and contract collagen to form a dermal equivalent like normal fibroblasts. Normal keratinocytes can grow and form an epidermal sheet on the surface of these dermal equivalents including normal or pathological fibroblasts. *Key words: Fibroblasts; Collagen lattice.* (Received March 13, 1986.)

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Epidermolysis bullosa (EB) is an inherited disorder of the skin in which blisters form as a result of minor trauma (1). Three categories are defined according to cleavage of the skin: EB simplex in which the split occurs within the epidermis, junctional EB, which was first described by Herlitz (2) and is characterized by the blistering of the skin at the dermo-epidermal junction, and EB dystrophica in which blistering develops below the epidermal basement membrane in the papillary dermis. Both the dominant and recessive forms of epidermolysis bullosa dystrophica are characterized by marked skin fragility, subepidermal blister formation, scarring, milia and nail dystrophy. In addition, in the more severe form of this disease, epidermolysis bullosa dystrophica recessiva (EBDR), there are usually marked growth retardation, deformities of the hands and feet, oropharyngeal and/or esophageal blisters, and an increased risk of developing skin cancer in later life.

The biochemical basis of the recessively inherited form of epidermolysis bullosa dystrophica has been intensively studied. Electron microscopy has demonstrated the degradation of the papillary dermal collagen (3) and the absence of anchoring fibrils (4). Fibroblasts grown from patients with EBDR show increased collagenase synthesis and secretion (5, 6). This collagenase appears to be structurally altered with a lower thermal stability and diminished affinity for calcium, its metal cofactor (7).

In addition, it has also been shown that the total collagen production by EBDR fibroblasts is increased (8, 9).

We prepared fibroblast cultures from a case of EBDR in order to characterize the collagen and to study its biosynthesis. We also studied the ability of these cells to form a collagen lattice and the effect of such a dermal equivalent on the growth of normal keratinocytes.

CASE REPORT

The proband was a full-term infant weighing 2.4 kg at birth. A few minutes after birth, he developed severe bullous eruptions involving the limbs and trunk. Painful blisters continued to occur both

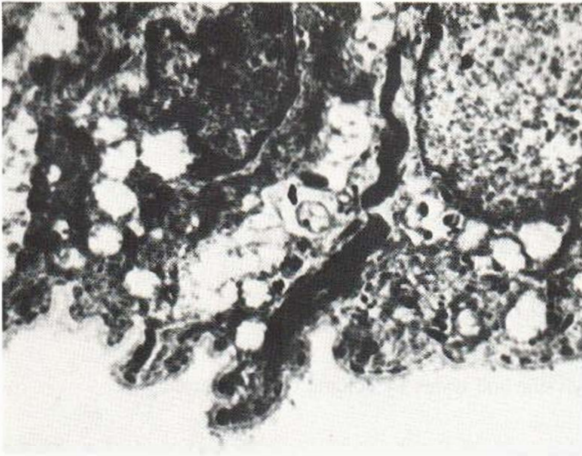


Fig. 1. Electron micrograph: EBDR skin biopsy specimen used for the cell culture. It shows blistering below lamina densa (magnification $\times 60\,000$).

spontaneously and with trauma, and healed with scar formation. The child lost all his fingernails by the age of 18 months. Chronic blistering led to progressive syndactyly, shortening of his fingers a "mitten hand" at 3 years of age. Oral blisters caused difficulty in swallowing and stunted growth.

The parents of this child were unrelated. The first two female children were normal. Our patient was the fourth child. The third child had severe EBDR and died at the age of 17 months. The mother refused prenatal diagnosis at the fourth pregnancy. Histopathological examination showed subepidermal bullae. Bullous pemphigoid antigen, laminin and type IV collagen were expressed in the roof of the blister. Electron microscopic findings showed that blister formation occurred in a plane below the basal lamina (Fig. 1). Anchoring fibrils were scarce and collagenolysis was marked.

Therapy consisted of phenytoin and 13-*cis*-retinoic acid. No clinical improvement was noted.

MATERIALS AND METHODS

Cell culture

Fibroblasts were propagated through serial subcultures from skin explants of the patient. Control cultures were obtained from the skin of healthy subjects after plastic surgery. The fibroblasts were subcultivated by trypsinization (one to two splits). The cultures were plated in T₇₅ plastic Corning tissue culture flasks in Eagle's Minimum Essential Medium (MEM) without glutamine because of its variable effect (10), buffered with bicarbonate (2 g/l) at pH 7.4 and supplemented with 10% calf serum (Flow), penicillin (50 U/ml) and streptomycin (50 µg/ml). The cultures were grown in a 5% CO₂-95% air atmosphere. The culture medium (15 ml/flask) was changed every three days. The cells were studied at the 16th passage. Labelling was carried out on the 9th day after the last trypsinization.

Metabolic studies

Cell labelling. To quantify the collagen and protein production, fibroblasts from the patient and healthy subjects were incubated with L-[U-¹⁴C] proline and [4,5-³H]lysine (Amersham) for 24 hours. The incubation mixture included 5 µCi of ¹⁴C-proline (specific activity: 280 mCi/mmol), 200 µCi of ³H-lysine (specific activity: 85 Ci/mmol), 50 µg/ml of β-amino-propionitrile and 50 µg/ml of ascorbic acid in a final volume of 10 ml culture medium.

After labelling, the medium was removed, the cells were washed with saline and mechanically detached with a rubber policeman. The metabolic studies were performed only on the culture medium mixed with the washing solutions, after dialysis against acetic acid (from 0.1 to 0.5 mol/l), since the cell pellet contained less than 10% of the total collagen produced. DNA was assayed in the cell pellet using Burton's method (11).

Estimation of protein production. The labelled proteins (both collagen and non-collagen proteins) were assayed in the culture medium (500 µl) following precipitation with 95% ethanol (1:4 v/v) for 4 h at +4°C. The precipitate was centrifuged and the pellet was washed three times with 95% ethanol. The pellet was resuspended in water (2 ml) and sonicated (B 12 Sonifer: 40 watts; 4×30 s). 3.5 ml of ready solv EP scintillator (Beckman) were added to 200 µl of protein suspension, and the radioactivity

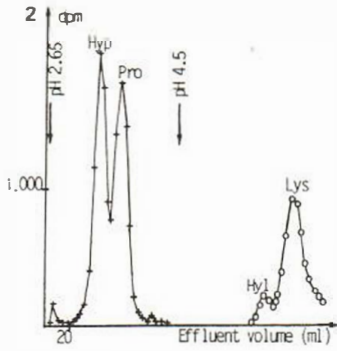


Fig. 2. Separation of hydroxyproline, proline, hydroxylysine and lysine by column chromatography on Dowex WX 8. +, dpm of ^{14}C ; o, dpm of ^3H .

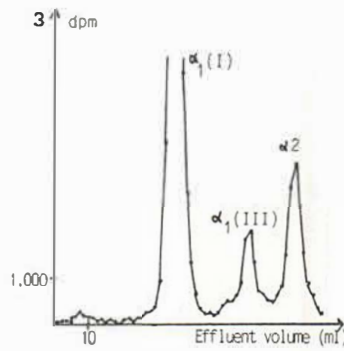


Fig. 3. CM cellulose chromatography of purified labelled collagen.

was counted in a LS 1800 Beckman scintillation counter: a double labelling program was used. The results were expressed in DPM of ^{14}C per μg of DNA.

Estimation of collagen production. The labelled collagen secreted into the culture medium was purified as described by Chandrakajan et al. (12). The non-collagen proteins and incompletely processed collagen were digested with pepsin (Sigma P 7012-1 mg/ml) for 16 h at $+4^\circ\text{C}$. After digestion, the collagen was precipitated with 4.2 mol/l of NaCl (final concentration) for 4 h at $+4^\circ\text{C}$ in the presence of carrier collagen (2.5 mg of calf skin collagen) centrifuged (30 min at 3000 rpm) and resuspended in 0.5 mol/l acetic acid. After 3 h of dialysis against 0.5 mol/l acetic acid, the collagen was precipitated by dialysis against 20 mmol/l of disodium phosphate (six changes over 72 h), centrifuged and redissolved in 0.5 mol/l acetic acid (4 ml). A 200 μl aliquot was used for the determination of the radioactivity in the presence of 3.5 ml of ready solv EP scintillator (Beckman). A double labelling program was used. The results were expressed in DPM ^{14}C per μg of DNA.

Determination of hydroxylation rate of the secreted collagen. A 1 ml aliquot of the purified collagen was hydrolysed with 6 mol/l HCl for 6 h at 100°C (optimal conditions for total amino-acid release). The characteristic collagen amino-acids were separated by ion exchange chromatography. Chromatography was performed on a 18×1.0 column of Dowex WX 8 using two different buffers in turn, at a flow rate of 30 ml/h: first 0.1 mol/l pyridine-acetic acid buffer, pH 2.65, which separates hydroxyproline and proline as previously described (13), and then 0.2 mol/l pyridine-acetic acid buffer, pH 4.5, which separates hydroxylysine and lysine. Fractions (4 ml) were collected and 0.5 ml were counted in 3.5 ml of ready solv EP scintillator (Beckman). A double labelling program was used. The pattern of the separation is shown in Fig. 2. The hydroxylation rate was expressed by the ratios of labelled hydroxyproline/labelled proline for the prolylhydroxylase activity and labelled hydroxylysine/labelled lysine for the lysylhydroxylase activity.

Determination of collagen types. The α chain composition of collagen samples previously purified from culture media was analysed. Collagen chains were separated by carboxymethyl cellulose chromatography (Fig. 3) according to Piez et al. (14). Chromatography was performed on a 10×1.0 column of Whatman CM 32 at 45°C , using 0.06 mol/l sodium acetate buffer pH 4.8, containing 1 mol/l urea with a gradient of 0–0.15 mol/l NaCl at a flow rate of 36 ml/h. Fractions (0.6 ml) were collected and counted in 3.5 ml of ready solv EP scintillator (Beckman).

Rapidly degraded collagen. The amount of rapidly degraded collagen produced during the 24 h incubation was estimated by the difference between the radioactivity of hydroxyproline before and after dialysis (15). The non-dialysable fraction was obtained by exhaustive dialysis of the medium until the dialysate was free of radioactivity. A 1 ml aliquot of both the total medium and the non-dialysable fraction was hydrolysed with 6 mol/l HCl (final concentration) for 6 h at 100°C . Hydroxyproline was then isolated by ion exchange chromatography (13). Hydroxyproline radioactivity was measured by liquid scintillation. The percentage of rapidly degraded collagen was calculated from the ratio of dialysable to total hydroxyproline.

Statistics. All the experiments were carried out several times (n°) in parallel. The results were expressed as means and standard deviation (SD). The differences between the means of the control and the patient were compared using Student's *t*-test.

Table I. Collagen biosynthesis by fibroblasts cultures

	Patient			Control			t-Test
	No.	Mean	SD	No.	Mean	SD	
Collagen DPM ¹⁴ C/ µg DNA	3	53 694	5 296	4	10 229	1 826	p<0.001
Collagen/total proteins, %	3	55.0	8.8	4	19.2	1	p<0.001
Type I collagen, DPM ¹⁴ C/µg DNA	3	46 925	4 591	4	8 602	1 529	p = 0.001
Type III collagen, DPM ¹⁴ C/µg DNA	3	6 769	765	4	1 627	306	p<0.001
Rapidly degraded collagen, %	4	23.5	3.3	5	14.0	4	0.010<p<0.05

Study of the ability of EBDR fibroblasts to form a collagen lattice

We compared the ability of EBDR fibroblasts and control fibroblasts to contract and organize collagen gel using a new cell culture model described by Bell et al. (16) in which acid soluble collagen was contracted by fibroblasts to obtain a collagen lattice including the cells. The collagen lattices were prepared by mixing EBDR fibroblasts (200 000 cells) or control fibroblasts (200 000 cells) with culture medium (1.76 ×, 2.3 ml) calf serum (0.45 ml), calf skin collagen purified without previous enzyme digestion (1.5 ml at 2.5 g/l in 1% acetic acid) and 0.1 mol/l NaOH (0.25 ml) in 60 mm bacteriological Petri dishes.

The kinetics of collagen lattice contraction were estimated by measuring the lattice diameter from the time when the cells were mixed with the collagen solution up to 10 days.

Collagen lattices made with EBDR fibroblasts or with control fibroblasts were used as a dermal equivalent for keratinocyte cultures. Two days after the lattice preparation, epidermis explants obtained after trypsin digestion of normal skin (0.25%, overnight at +4°C) were spread on collagen lattices. The cultures were grown in a 5% CO₂-95% air atmosphere in the culture medium described in Material and Methods (cell culture). After a month, the structure of these dermal equivalents covered with keratinocytes was studied by histological techniques: the lattices were fixed with Bouin's fluid and embedded in paraffin. Sections (4 µm) were stained with hematoxylin safran.

RESULTS

Collagen and total proteins secreted by monolayers of fibroblasts obtained from the patient were compared with those of normal fibroblasts. Table I shows that there was a significant increase in collagen production by the fibroblasts culture derived from the patient. The collagen/total protein ratio showed that the increase in collagen was about 2.8 times that of the total proteins.

The lysine and proline hydroxylation rates of the collagen purified from the culture

Table II. Hydroxylation rate of the purified collagen produced during 24 h by fibroblasts cultures

	Patient			Control			t-Test
	No.	Mean	SD	No.	Mean	SD	
Hyp/Pro	3	1.04	0.06	6	0.94	0.07	NS
Hyl/Lys	3	0.18	0.02	6	0.31	0.007	0.001<p<0.01

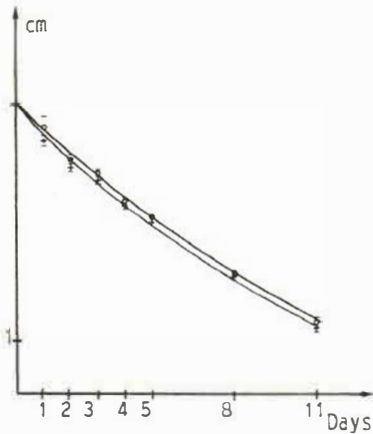


Fig. 4. Kinetics of lattice contraction by (+) 2×10^5 normal fibroblasts, and (o) 2×10^5 EBDR fibroblasts.

medium were then analysed. Table II shows that the proline hydroxylation rate was normal. On the other hand, there was a decrease in the lysine hydroxylation rate which was about 2/3 of that measured in the control fibroblasts.

The α chain composition of the collagens secreted into the culture medium was also analysed by CMC chromatography. Table I shows that there was an increase in both type I and type III collagen in the fibroblast culture derived from the patient, though type III collagen only comprised 12.4% of the total collagen whereas the control values ranged from 15.2 to 16.6%.

We then compared the ability of EBDR fibroblasts and normal fibroblasts to form a collagen lattice. Fig. 4 shows that the EBDR fibroblasts are able to contract and organize collagen gel. The kinetics of collagen lattice contraction by the both types of fibroblasts were not significantly different.

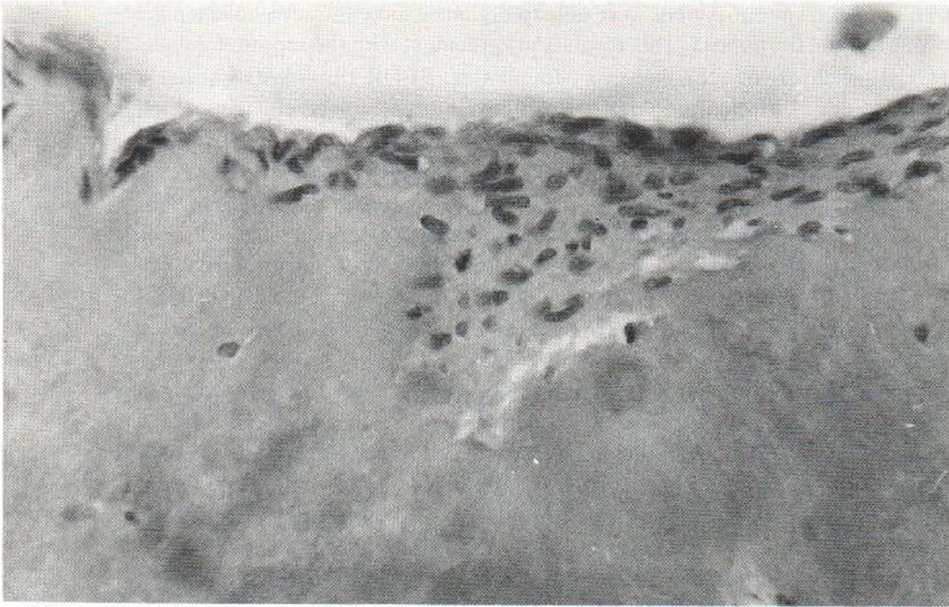
Histological studies showed that when normal epidermis explants were spread onto the collagen lattices, keratinocytes could migrate from the explants and multiply on the lattice surface to form an epidermal sheet with several layers of cells. No difference (Fig. 5) could be seen between the keratinocyte culture on the collagen lattice contracted with normal fibroblasts (Fig. 5 A) and that contracted with EBDR fibroblasts (Fig. 5 B).

DISCUSSION

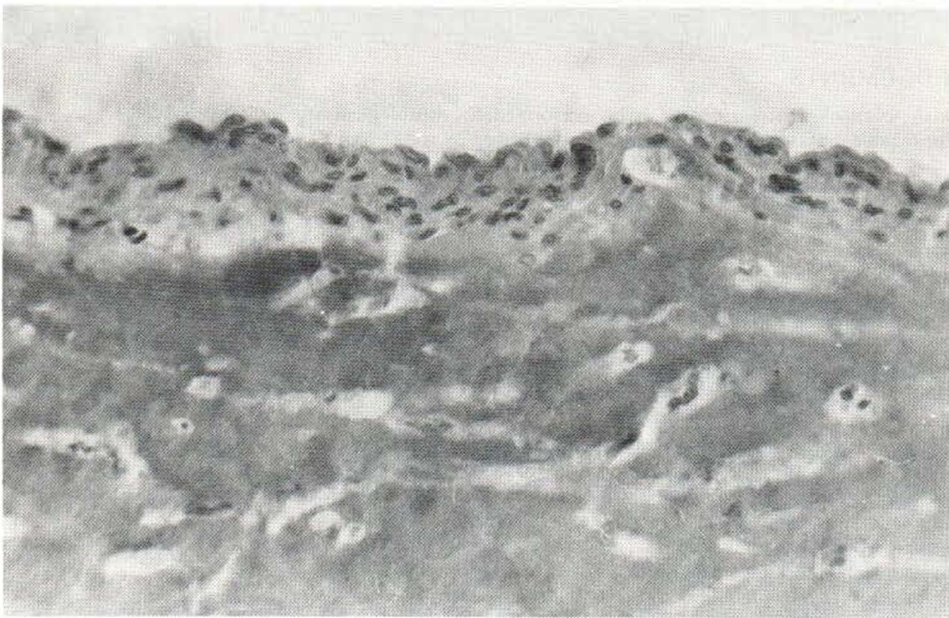
The increase in collagen synthesis demonstrated in these experiments confirms previous reports (8, 9), in which the collagen synthesis by EBDR fibroblasts was found to be several times higher than that of control fibroblasts.

In these previous reports, there was a rise in both collagen and protein secretion but this increase in collagen production was greater than the rise in protein secretion according to our experiment.

Until now, only the collagen synthesis has been studied in EBDR fibroblasts cultures. In our experiment, we reported an increase in the rapidly degraded collagen. This breakdown of the newly synthesized collagen may be a result both of collagenase activity and defective collagen (15). In this paper, we demonstrate several qualitative modifications of the secreted collagen, and in particular, a reduction in the lysyl hydroxylation rate of the secreted collagen although the prolylhydroxylation was normal. This underhydroxylation of the lysine apparently disagrees with the increased level of lysylhydroxylase demonstrated by Kero et al (8) in several lines of EBDR fibroblasts. However, in that report, the



A



B

Fig. 5. Histology of a collagen contracted lattice by normal fibroblasts (A) and by EBDR fibroblasts (B), both were covered by human keratinocytes grown from normal epidermis explants ($\times 200$).

increased level of lysylhydroxylase was variable from one patient to another and was non-existent in one case. On the other hand, the collagen secreted by the fibroblasts cultivated from our patient contained a lower percentage of type III collagen in comparison with the control fibroblasts. This anomaly in the ratio of the different types of collagen may also be responsible for a molecular fragility of the collagen matrix produced by the EBDR fibroblasts.

Fibroblasts cultivated from our patient were able to organize and contract a collagen gel in the same way as normal fibroblasts. This result is in agreement with the report of Adams & Priestley (17); Ehrlich et al. (18), however, found that EBDR fibroblasts caused a delayed lattice contraction. Ehrlich prepared the lattices with pepsinized collagen which can be easily fractionated into its α chain components. These polypeptide chains are sensitive to proteases which are increased in EBDR (19).

Histological studies in which normal epidermis explants were spread on lattices including normal of pathological fibroblasts showed that an epidermis formed on the lattice surface in both cases, with a normal attachment to the "dermis". This similar behavior of keratinocytes cultivated onto dermal equivalent including normal of pathological fibroblasts suggested that the blisters observed in vivo may be caused by another parameter apart from an anomaly in the fibroblasts.

In our experiment, it was not possible to determine the role of defective collagen produced by the EBDR fibroblasts, because in this dermal equivalent the latticed collagen was mainly the added calf skin collagen; the collagen produced by the fibroblasts was only a minor component. But some clear areas around the fibroblasts in the dermal equivalent appeared, when it was populated with EBDR fibroblasts. This phenomenon might be related to a collagenase activity as it was suggested by the metabolic study.

To conclude, this study of fibroblasts isolated from a patient with EBDR demonstrated the importance of connective tissue disturbance in this disease.

ACKNOWLEDGEMENTS

The authors wish to thank Docteur Hervé, who performed the histological studies, and Sylvie Peyroche for her technical assistance. The English was reviewed by the C.N.R.S. translation office.

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