

Collagen Synthesis and Degradation by Epidermolysis bullosa Fibroblasts

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Collagen synthesis was measured in fibroblasts cultured from the skin of six patients with epidermolysis bullosa simplex, from six patients with dystrophic epidermolysis bullosa and six age-matched controls without skin disease. Both groups of patients' fibroblasts synthesized approximately twice as much collagen (dpm/cell) as the controls. Synthesis of other proteins showed a smaller increase. Collagenase activity in culture media from four fibroblast lines per group was measured, using a ^3H -collagen substrate, both before and after trypsin treatment to activate procollagenase. As expected, the dystrophic group had the highest activity (30% more than controls) and the result was little affected by trypsin: the enzyme appeared to be in active form. Enzyme activity in the simplex group was increased from 67% to 114% of control values by trypsin treatment. The excessive collagen synthesis in both dystrophic and simplex fibroblasts may be a consequence of their greater collagenase activity and suggests an unsuspected dermal involvement in epidermolysis bullosa simplex. Our data confirm an excessive secretion of collagenase by dystrophic fibroblasts but suggest that the enzyme's state of activation may be the important aetiological feature of the dystrophic disease. *Key words: Collagen; Simplex; Dystrophic.* (Received December 14, 1984.)

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Epidermolysis bullosa (EB) is the collective name for a group of hereditary blistering diseases. The three broad categories within it are defined by the level at which separation occurs: in EB simplex (EBS), the split is within the epidermis; in junctional EB, separation is between the epidermis and underlying dermis; in dystrophic EB, which may be inherited through either dominant or recessive autosomal genes, blisters form within the dermis and scarring follows.

Collagenase abnormalities have been implicated in the aetiology of dystrophic recessive EB (EBDr) since Pearson's suggestion (1) that collagenolysis might be occurring, and since the initial collagenase investigations of Eisen (2). In 1977 Bauer, Gedde-Dahl and Eisen (3) demonstrated that the enzyme was produced in excess (some four-fold compared with the amounts of collagenase protein present in normal-appearing skin), and also in an aberrant form with reduced specific activity (4). The abnormality in collagenase production arises at the level of transcription (5).

Until recently there has been less interest in the target of the collagenase enzyme in any of the EB types, due to the apparent absence of any collagen abnormality. This impression of normality may be misleading, however, and here we substantiate our preliminary report (6) of abnormal collagen metabolism in EBDr and EBS skin fibroblasts.

MATERIALS AND METHODS

Cell lines

Skin fibroblast cultures were established, as previously described, (7) from the forearm skin of six healthy volunteers (mean age 21 ± 2 [S.E.] years), of six patients with EBS (aged 19 ± 6 years) and of six with EBDr (aged 20 ± 7 years) after informed consent; details of the patients are given in Table I.

Table I. Details of the patients donating skin fibroblasts

S (W-C) = Simplex Weber-Cockayne type, Dr (H-S) = Dystrophic Hallopeau-Siemens type, p = punch (either 4 or 5 mm) biopsy, i = incisional biopsy, f = forearm, th = thigh

No.	Age	Sex	Type	Biopsy	Site	Blistering
EB9	42	M	S (W-C)	p	f	Feet: mild
EB10	31	F	S (W-C)	p	f	Hands+feet: mild
EB11	12	F	S (W-C)	p	f	Hands+feet: mild
EB12	8	M	S (W-C)	p	f	Hands+feet: moderate
EB23	3	F	S (W-C)	p	f	Hands+feet: mild
EB30	19	F	S (W-C)	p	f	Hands, feet+friction sites: mild
EB1	15	F	Dr (H-S)	p	th	Generalised: severe
EB2	13	F	Dr (H-S)	i	th	Generalised: severe
EB5	47	F	Dr (H-S)	p	f	Generalised: severe
EB14	2	F	Dr (H-S)	i	f	Generalised: Died aged 3 years
EB18	36	F	Dr (H-S)	i	f	Generalised: moderate
EB31	4	M	Dr (H-S)	i	th	Extremities, localised type

Fibroblasts were cultured in Nunc plastic flasks or dishes in Dulbecco's medium plus 10% foetal calf serum, 4 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Gibco Ltd, Paisley, Scotland). Newly seeded cultures were flushed with 5% CO₂/95% air before incubation at 37°C and medium was changed three times weekly.

Collagen synthesis

We measured the incorporation of ³H-proline into collagenase-sensitive protein, as previously described (8), except that ³H-proline was added in culture medium containing 2% foetal calf serum. Cell lines were used between passages 5-10. Each of the six collagen assays involved the comparison, in each of five replicate dishes, of a control with an age-matched EBDr and EBS cell line, permitting statistical analysis by Student's paired *t*-tests.

Collagenase activity

Four age-matched control lines were compared with four EBS and four EBDr cell lines; the cell lines were between passages 4 and 9.

Calf skin collagen (Sigma, Poole, U.K.) was labelled with ³H-acetic anhydride (Amersham, U.K.; 500 mCi/mmol), as described by Lefevre et al. (9), to produce the radioactive substrate for the assay. The specific activity was 2.5 × 10⁷ dpm/mg and its relative susceptibility to bacterial collagenase and trypsin was 9:1. Medium from three confluent culture flasks (175 cm² growth area) of a fibroblast cell line was removed and the cells washed five times with phosphate buffered saline. Serum-free medium (20 ml) was added to each flask before incubation at 37°C for 72 hours. The media were then pooled

Table II. Collagen and non-collagenous protein synthesis in control and EB fibroblasts

Mean values (± SE) are shown from 6 cell lines

	Control	EBS	EBDr
dpm collagen/10 ⁵ cells	4 314 ± 664	8 672 ± 1 845	6 778 ± 1 030
dpm other protein/10 ⁵ cells	29 189 ± 842	33 648 ± 8 871	38 077 ± 9 240
R.C.S. ^a	3.2 ± 0.5	5.1 ± 0.9	4.1 ± 0.8

^a R.C.S., relative collagen synthesis: the percentage of total protein synthesis which is collagen, as calculated by Diegelmann & Peterkofsky (16).

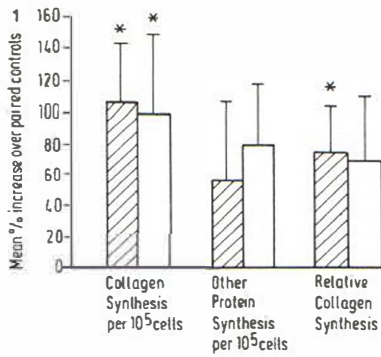


Fig. 1. Protein synthesis in EBS ■ and EBDr fibroblast lines as mean percentage increases (\pm SE) over paired controls. Six lines were used per group. Statistical comparisons with controls were by Student's paired *t*-test ($*p < 0.05$).

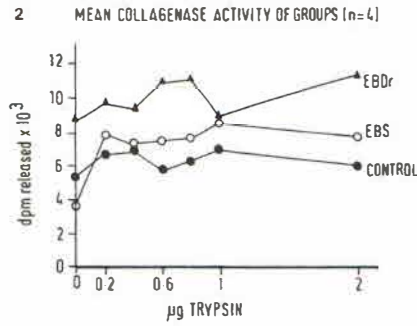


Fig. 2. Collagenase activity before and after activation with trypsin in EBDr (\blacktriangle), EBS (\circ) and control (\bullet) groups (4 lines per group).

and the total cell number estimated by counting aliquots of resuspended cells in a Coulter Counter. Pooled medium was concentrated 10-fold by vacuum dialysis at 4°C through Millipore immersible-CX ultrafilters (nominal pore size 10 000 daltons). The concentrate was dialysed against 50 mM Tris-HCl 5 mM CaCl₂ buffer (pH 7.5) for 24 hours at 4°C. Collagenase activity was then measured following trypsin activation (10). 100 µl aliquots of the final supernatant, containing the radioactivity released from the substrate, were diluted in 1 ml of water and added to 10 ml of scintillant composed of Triton X-100 and NE233 (Nuclear Enterprises, Edinburgh, U.K.) in a 2:1 ratio for scintillation counting. Collagenase activity was expressed as the ³H-collagen radioactivity released, per 10⁶ cells in the original culture, in a 100 µl aliquot of the reaction mixture (total volume 975 µl).

RESULTS

Table II shows collagen and non-collagenous protein synthesis in the EB and control groups. These overall mean values for each group do not, however, accurately reflect the differences between control and EBDr or EBS cell lines *within* each of the six experiments, which were higher than those given in Table II. In order to do this the difference between the EB line and its control in each experiment was expressed as a percentage. The Student's paired *t*-test was then used to test the significance of the mean percentage differences between control and EB cell lines (Fig. 1).

Expressed in this way, collagen synthesis per 10⁵ cells is seen to have been elevated by approximately the same amount in both the EBS (108%) and EBDr groups (100%) over controls ($p < 0.05$). That is to say, collagen synthesis per cell was approximately double that of controls. Synthesis of other proteins per 10⁵ cells varied between experiments and the differences were not statistically significant. The increase in relative collagen synthesis was only significant for the EBS group ($p < 0.05$), though it approached significance in the EBDr group ($p < 0.1$).

Fig. 2 illustrates the mean collagenase activities of the four lines in each group following activation of the pro-collagenase by various concentrations of trypsin. Although there was considerable variability between cell lines, the EBDr group had the highest collagenase activity before and after activation by trypsin compared to the control group, but activation made little difference to the enzyme activity. In contrast, the EBS enzyme was considerably affected by activation, the change being about 2.5 times that observed in the control cell lines and 6 to 7 times that seen in EBDr (Fig. 3). The level of the activated EBS

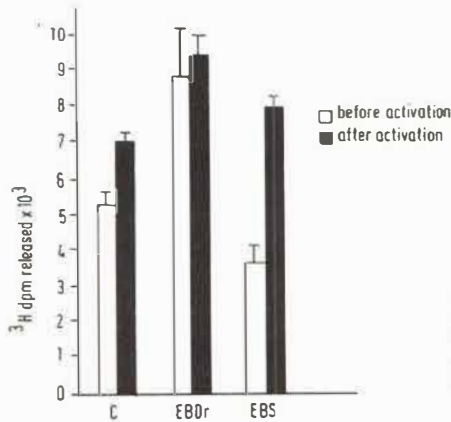


Fig. 3. Effect of trypsin activation on collagenase activity. Values for activated enzyme (solid columns) are averaged from all the trypsin data in Fig. 2 and are shown with standard errors ($n=4$).

collagenase was slightly higher than control levels but lower than that observed in the EBDr group. Stated in another way, Figure 3 shows that over 50% of the EBS enzyme, and 26% of the control enzyme, but only 7% of the EBDr enzyme, was in the inactive form requiring release by trypsin.

DISCUSSION

These experiments demonstrate an abnormality of collagen synthesis in both EBDr and EBS fibroblasts. This was particularly unexpected in EBS where no disturbance of the dermis is apparent. However, our data indicate that collagen synthesis in both EBS and EBDr fibroblasts was approximately double that of matched control fibroblast lines. Kero et al. have recently reported several-fold increases in collagen synthesis in comparisons of fibroblasts from two patients with EBDr and six control lines (11).

The 30% higher total collagenase activity in our EBDr fibroblasts compared to controls (Fig. 3) agrees well with the 28% reported by Bauer and Eisen (12). They also found substantial variation between their EBDr lines, but the same amount of collagenase protein, irrespective of activity, in controls and EBS fibroblasts. We suggest that the crucial feature of the EBS collagenase is the high proportion present as inactive procollagenase: trypsin treatment increased activity by 124±26% in EBS fibroblast medium whilst the control group showed only a 30±18% increase ($p<0.05$; paired *t*-test). The smaller change in the EBDr group on activation (9±2%), means that virtually all the enzyme was already in the active form. This could represent autoactivation (10), either before or during preparation of the samples, or a deficiency in an inhibitor of the pro-enzyme, in each case restricted to the EBDr group. The concept that it is the state of activation of the collagenase which is important, rather than the total amount of enzyme protein, is supported by the findings of Kero et al. (11): fibroblasts from their two EBDr patients had less enzyme activity after activation than controls, but higher proportions of free enzyme (39 and 81% against only 6% in controls).

Manabe et al. (13) demonstrated that blister fluid from patients with EBS and EBDr can produce the characteristic intraepidermal or intradermal separations in normal skin in vitro. Their findings may help to resolve the principal controversy concerning EB aetiology: is the primary abnormality excessive protease activity or a deficiency in protein structure(s) (14, 15) which make the skin susceptible to protease attack? There is evidence favouring both possibilities. In our data we regard the large amount of free enzyme activity

in EBDr fibroblasts as the primary factor, with increased collagen synthesis as a compensatory response by the fibroblast. The slight increase in collagenase in EBS fibroblasts also seems to provoke greater collagen synthesis and a favourable balance which ensures protection of the dermis *in vivo*.

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