Characterization of a type VI collagen-related M_r -140000 protein from cutis-laxa fibroblasts in culture

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The precise biochemical defects in connective-tissue metabolism that are responsible for the laxity of skin seen in the syndrome of cutis laxa are largely unknown. We have studied fibroblasts cultured from skin explants of a 2-year-old male with the syndrome. Electron-microscopic examination of this skin revealed decreased amounts of amorphous elastin and an increase in elastin-associated microfibrils. Although the cultured fibroblasts were similar to control skin fibroblasts in morphology, growth rate and total protein synthesis, there was a 4–6-fold increase in accumulation of a collagenous protein of M_r 140000 in both the culture medium and in the cell layer. This protein was structurally distinct from collagen types I, III, IV, V and VIII. It was found to be related to a cell-surface-associated glycoprotein, GP140, by both antigenic cross-reactivity and peptide mapping. Our data support observations that GP140 is a precursor of at least one form of pepsin-extracted type VI collagen.

Cutis laxa is a rare syndrome of abnormal connective tissues marked by a loss of elasticity of the skin. It exists in both acquired and inherited forms. Although microscopically the skin shows no diagnostic features, some cases of inherited cutis laxa do display deficient elastin bundles and excessive accumulations of elastin-associated microfibrils (Holbrook & Byers, 1982). The biochemical defects responsible for this syndrome are likely to be heterogeneous and are so far unknown.

We have studied the ultrastructure of the dermis and the proteins synthesized by skin fibroblasts cultured from a 2-year-old male with cutis laxa. Ultrastructurally, the patient's skin contained increased amounts of microfibrillar structures as compared with age- and sex-matched controls. The 10–12 nm-diameter microfibrils were typical of those studied in bovine tissues and in human aorta and placenta. The cultured skin fibroblasts from this patient were analysed as a possible source of secreted microfibrillar protein. Electron-microscopic examination of the cultured cells revealed a relative decrease in extracellular matrix compared

with control skin fibroblasts. No microfibrillar structures were observed. However, metabolic labelling and fractionation of cellular culture medium proteins showed a selective increase in secretion of a collagenous protein, of M_r approx. 140000 on the basis of globular-protein standards. This protein was also extracted from the radio-labelled cell layers in greater amounts, compared with control fibroblasts.

The collagen was shown to be related to the cell-surface-associated glycoprotein GP140, described by Carter (1982*a,b*) and Carter & Hakomori (1981) by both immunological reactivity and CNBr-cleavage peptide mapping. We believe this protein to be similar to other 140000- M_r glycoproteins isolated from elastin-rich bovine aortic and ligamentum-nuchae tissues (Cleary & Gibson, 1983; Gibson & Cleary, 1982) and from cultured bovine ligamentum-nuchae fibroblasts (Sear *et al.*, 1981). A 140000- M_r glycoprotein from these tissues has been shown to generate peptides similar to type VI collagen after digestion with pepsin (Jander *et al.*, 1984).

Our work also supports the relationship between GP140 and type VI collagen suggested by Heller-Harrison & Carter (1984*a,b*) and Hesse & Engvall

Abbreviations used: CLF, cutis-laxa fibroblasts; SDS, sodium dodecyl sulphate.

(1984). The cultured cutis-laxa fibroblasts (CLF) examined here are the first human cells to reveal an abnormality in production of this collagenous protein.

Experimental

The subject was a 2-year-old Caucasian male with cutis laxa noted since birth. Clinically there was severe skin involvement with loss of normal elasticity; subglottic stenosis, mild laryngomalacia and a functional cardiac murmur were also noted. Two years later the patient was noted to have easy bruisability and a dilated pulmonary artery and aortic arch. The parents and an older sibling were clinically normal. The mode of inheritance in this case was not clearly defined.

Cell culture and metabolic labelling

Fibroblasts were grown from tissue explants of skin biopsies from the extensor surface of the upper arm of the subject and of disease-free patients of similar age, as well as from the foreskin of clinically normal infants. Cells were plated at approx. 10^6 cells/100 mm plastic tissue-culture dish (Falcon) and passaged at confluent densities by EDTA/trypsin treatment. Cultures were grown in Dulbecco's minimal essential medium (Grand Island Biological Co.) containing penicillin G (100 units/ml), streptomycin sulphate (100 µg/ml), 3-hydroxybutyrate (0.4 µg/ml), glutamine (584 µg/ml), sodium ascorbate (50 µg/ml) (added fresh at each passage) and 10% (v/v) foetal bovine serum (Hyclone) at 37°C in a mixture of CO₂/air (1:19).

Cultures between the fifth and the tenth passage were metabolically labelled as previously described (Sage & Bornstein, 1982). Cells that had just reached confluence were preincubated in serum-free medium containing sodium ascorbate and β-aminopropionitrile fumarate (64 µg/ml) at 37°C for 30 min. The medium was removed and replaced with fresh preincubation medium containing either 10 or 25 µCi of [2,3-³H]proline (35 Ci/mmol; New England Nuclear)/ml. After 24 h at 37°C, the culture medium was removed and transferred to plastic centrifuge tubes containing freshly prepared mixtures of proteinase inhibitors (Sage & Bornstein, 1982). Cell layers were rinsed with 0.2 mM-phenylmethanesulphonyl fluoride in phosphate-buffered saline (0.15 M-NaCl/0.008 M-sodium phosphate buffer, pH 7.4), and this rinse was added to the labelled medium. This medium was then clarified in a clinical centrifuge at 400 g and subsequently kept at 4°C. For some experiments, cells were exposed to radioisotope in the presence of 0.5% foetal bovine serum to facilitate the identi-

fication and fractionation of culture-medium proteins.

Isolation of culture-medium proteins

Culture-medium proteins were precipitated differentially with (NH₄)₂SO₄ at 4°C in silicone-treated glassware. Solid (NH₄)₂SO₄ (Ultrapure; Bethesda Research Laboratories) was added over a 2 h period to a final concentration of 20% (w/v), and the solution was gently stirred overnight. The resulting precipitate was isolated by centrifugation at 48 000 g for 20 min. This procedure was repeated by adding (NH₄)₂SO₄ to the supernatant to a final concentration of 50% (w/v) and isolating the additional precipitated proteins by centrifugation.

The precipitated proteins were fractionated by ion-exchange chromatography on DEAE-cellulose as described previously (Sage & Bornstein, 1982). Pooled fractions were dialysed at 4°C against 0.1 M-acetic acid in the presence of 10 µg of pepstatin A (Protein Research Foundation; 100 mg/ml in dimethyl sulphoxide), freeze-dried, and stored at 4°C.

For some procedures, proteins were further fractionated on CM-cellulose as previously described (Sage & Bornstein, 1979).

Isolation of cell-layer proteins

Sequential extraction of fibroblast proteins was performed by modification of the procedure described by Carter (1982a). After removal of the culture medium, the cells were scraped with a 'rubber policeman' into a non-ionic detergent solution containing 0.2% (v/v) Triton X-100, Tris/HCl, pH 7.4 (50 mM), EDTA (20 mM), *N*-ethylmaleimide (100 mM) and phenylmethanesulphonyl fluoride (2 mM) at 4°C, stirred for 15 min, and centrifuged at 20 000 g. The insoluble material was then extracted in similar fashion with 50 mM-NaHCO₃ in 8 M-urea, followed by centrifugation. This pellet was analysed by SDS/polyacrylamide-gel electrophoresis in the presence of dithiothreitol (Laemmli, 1970). This procedure resulted in the appearance of two major bands of apparent *M*_r 220 000 and 140 000, corresponding to fibronectin and GP140 respectively (Carter, 1982a).

Immunocytochemistry

Antigenic reactivity of DEAE-cellulose-fractionated culture-medium proteins was assessed autoradiographically by electrophoretic transfer, after SDS/polyacrylamide-gel electrophoresis, to nitrocellulose filters (Schleicher and Schuell, 0.45 µm pore size), and incubation with specific antisera, followed with ¹²⁵I-labelled staphylococcal protein A (New England Nuclear; 10⁷ c.p.m./20 ml) (Towbin *et al.*, 1979). Antiserum to GP140 was prepared and provided by Dr.

William Carter (Carter, 1982b) (Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.) and type VI collagen by Dr. Beat Trüb (University of Washington, Seattle, WA, U.S.A.).

Electron microscopy

For electron-microscopic studies, biopsy specimens were fixed in Karnovsky's fixative (Karnovsky, 1965) followed by 1% OsO₄ in distilled water. Portions were dehydrated in ethanol, embedded in Epon (Luft, 1961), and thin sections stained with 1% phosphotungstic acid, uranyl acetate and lead citrate. Cultured cells were similarly fixed as described previously (Henning & Holbrook, 1983).

Other procedures

Bacterial-collagenase and pepsin digests were performed as described previously (Sage *et al.*, 1980). For determination of the extent of synthesis of collagenous protein, bacterial-collagenase digestion assays were performed on soluble fractions of culture media and cell layers (Sage *et al.*, 1979). Total protein synthesis was assessed by incorporation of [2,3-³H]proline and specific radioactivity determined as described by Peterkofsky *et al.* (1982). Hydroxyproline/proline ratios were determined for radiolabelled proteins resolved on SDS/polyacrylamide-gel electrophoresis and excised from the gel as described previously (Sage *et al.*, 1980).

Peptide mapping of samples fractionated by CM-cellulose was performed by CNBr digestion according to Crouch & Bornstein (1978). The digestion products were resolved by SDS/polyacrylamide-gel electrophoresis and the peptide patterns compared with those of types I, III, IV, V and VIII collagens (Sage *et al.*, 1980). CNBr digestion was performed on samples excised from gels after SDS/polyacrylamide-gel electrophoresis of proteins from both cell layers and culture media by a modification of a procedure previously described (Barsh *et al.*, 1981). Digestion was limited to 2 h and the digestion products were analysed subsequently by SDS/polyacrylamide-gel electrophoresis with 0.5M-urea.

Results

Skin morphology

Electron-microscopic examination of the skin from the subject with cutis laxa revealed collagen fibrils that appeared normal in size and configuration. There was, however, a marked decrease in the amorphous elastin component of elastic fibres compared with age-matched controls. The elastin present was surrounded by large amounts of 10–12 nm diameter microfibrillar structures that pro-

duced an overall speckled appearance (Fig. 1). These findings were most pronounced near fibroblasts. Elastin adjacent to smooth-muscle cells appeared more typical with substantial amorphous component surrounded by a rim of microfibrils.

Cell cultures

The morphology of cultured fibroblasts from the patient appeared similar to fibroblasts from clinically normal age-matched controls and to human foreskin fibroblasts by phase-contrast microscopy. In addition, the cells from the patient and those from control had identical growth curves when cultured at similar densities (results not shown).

In 7-day-old cultures that had been plated at similar densities and maintained at confluence for 3 days there appeared to be relatively less extracellular matrix deposited by the CLF than by normal cells (Fig. 2). Neither microfibrillar structures nor elastin bundles were evident by transmission-electron-microscopic study of any of the cultured fibroblasts from normal or cutis-laxa skin.

Culture-medium proteins

Metabolically radiolabelled culture-medium proteins were fractionated initially at concentrations of 20% and 20–50% (NH₄)₂SO₄ in the presence of proteinase inhibitors. The 20%-(NH₄)₂SO₄ precipitates appeared to contain primarily type I procollagens and their processed derivative molecules, as well as fibronectin, after analysis by SDS/polyacrylamide-gel electrophoresis. There were no differences in the relative proportions of these products among the cells examined.

The culture-medium proteins that were precipitated at a 20–50% (NH₄)₂SO₄ concentration were fractionated by DEAE-cellulose chromatography (Fig. 3). A significant proportion of the [³H]-proline-labelled proteins did not bind to the DEAE-cellulose, and most of the bound proteins were eluted as a broad peak early in the gradient. Although the elution profile of radiolabelled proteins was similar, SDS/polyacrylamide-gel electrophoresis of pooled fractions revealed several differences (Fig. 3 insets). One of the major radiolabelled proteins from the CLF culture medium migrated on SDS/polyacrylamide-gel electrophoresis, both in the presence and in the absence of dithiothreitol, with an apparent *M_r* of 140 000 (Fig. 3a inset, lane IV) on the basis of globular-protein standards. Similar fractions from control fibroblasts contained several different proteins, in addition to a minor species of *M_r* 140 000 (Fig. 3b inset, lane IV).

*Characterization of 140 000-*M_r* protein*

The 140 000-*M_r* protein, isolated from CLF culture medium by DEAE-cellulose chromato-

Fig. 1. Transmission electron micrograph of an elastic fibre in the reticular dermis of the affected individual (a) and a normal age-matched control (b)

Note the abundance of the microfibrils and the paucity of elastin in the fibres from the patient compared with the control. Magnification $\times 11350$.

Fig. 2. Cultured fibroblasts from the patient (a) and a normal control (b) showing the comparative density of cells plated at equal densities and grown for 7 days

Note the apparent absence of fibrous matrix in the culture from the affected individual. Magnification $\times 410$.

Fig. 3. DEAE-cellulose chromatography of [^3H]proline-labelled fibroblast culture-medium proteins

Labelled protein was precipitated from culture medium with 20–50% $(\text{NH}_4)_2\text{SO}_4$ and chromatographed on DEAE-cellulose in 6M-urea and 50mM-Tris/HCl, pH 8.0. Gradient elution was performed with 0–200mM-NaCl. Conductivities were read at 4°C. Pooled fractions (indicated by Roman numerals) were dialysed against 0.1 M-acetic acid in the presence of 10 μg of pepstatin, freeze-dried and analysed by SDS/polyacrylamide-gel electrophoresis (3/6/10% composite gels) under reducing conditions, followed by autoradiography (insets). Samples containing protein from approximately equal numbers of cells were loaded on the gels. Migration of appropriate standards is indicated (FN, fibronectin). (a), CLF culture-medium proteins; (b), control skin-fibroblast culture-medium proteins from age- and sex-matched subject. Cells were of similar passage number.

graphy, was digested specifically by highly purified bacterial collagenase (Fig. 4). The protein was also degraded extensively by pepsin at 4°C within 2 min (Fig. 5, lanes 3–5) under conditions that produced collagen from type I procollagen (Fig. 5, lanes 1–2). Hydroxyproline/proline ratios for the 140000- M_r protein averaged 0.45–0.50, compared with the value of 0.80–0.85 obtained for type I pro-

collagen that was also secreted into the culture medium by these cells.

This collagenase-sensitive 140000- M_r protein was compared with collagen types I, III, IV, V and VIII after cleavage with CNBr. For this procedure, additional purification of the 140000- M_r protein was achieved by CM-cellulose chromatography. As shown in Fig. 6, the CNBr-cleavage

Fig. 4. SDS/polyacrylamide-gel electrophoresis of 140000- M_r protein after incubation with highly purified bacterial collagenase

CLF culture-medium proteins containing 140000- M_r protein were chromatographed on DEAE-cellulose (peak IV, Fig. 3a) and digested as described in the Experimental section at 37°C for 2 h. The digest was analysed on a composite (3/6/10%) gel under reducing conditions, and the bands were detected by autoradiography.

peptide pattern obtained from the 140000- M_r protein was clearly different from those corresponding to type I procollagen, type III procollagen, type IV procollagen, type V collagen and type VIII collagen. Attempts to digest type VI collagen, obtained by pepsin extraction of bovine aorta, with CNBr failed to yield peptide fragments, probably because of oxidation of methionine residues.

Although the 140000- M_r protein was distinct from the collagen types commonly secreted by fibroblastic cells in culture, it appeared to be similar to a fibroblast cell-surface-associated glycoprotein (GP140) (Carter, 1982a). High- M_r precursor(s) to type VI collagen, some of which may relate to GP140, have been described in fibroblast culture medium (Heller-Harrison & Carter, 1984a,b; Hesse & Engvall, 1984; Trüeb & Bornstein, 1984). We therefore investigated the relationship of the CLF culture-medium protein to GP140 and to type VI collagen. Immunoblotting with antibody against cell-surface-associated GP140 revealed antigenic cross-reactivity with

Fig. 5. SDS/polyacrylamide-gel electrophoresis of 140000- M_r protein after incubation with pepsin

Samples were dissolved in 0.1 M-acetic acid at 4°C and incubated with pepsin for designated time periods as described in the Experimental section. Subsequently, the samples were immediately frozen on solid CO₂, freeze-dried and analysed on composite (3/6/10%) gels. Detection of bands was by autoradiography. Lanes 1 and 2, control skin-fibroblast culture-medium proteins, precipitated with 20% (NH₄)₂SO₄, and incubated with pepsin for 0 (lane 1) and 60 min (lane 2). Lanes 3–5, CLF culture-medium proteins, containing 140000- M_r protein, were obtained by chromatography on DEAE-cellulose (peak IV, Fig. 3a) and incubated with pepsin for the indicated times. Digestion for up to 60 min failed to yield further changes. Type I procollagen and collagen chains are identified.

several preparations of the CLF 140000- M_r protein (Fig. 7, lanes 2–4) and with type VI collagen from bovine aorta (Fig. 7, lane 1). The sample of type VI collagen contained several cross-reacting species. Two bands that corresponded to α1(VI) and α2(VI) were apparent on SDS/polyacrylamide-gel electrophoresis, as well as a faint band that comigrated with the lower border of the GP140 extracted from cell layers (lane 1, arrow). The cross-reacting material in lane 2 (Fig. 7) was prepared from CLF by extraction of the cell layer with Triton X-100 followed by 50 mM-NaHCO₃ in 8 M-urea (Carter, 1982a). Incubation of this preparation with anti-GP140 sera revealed a broad band, possibly a doublet, as well as a faint doublet that just entered the 6%-polyacrylamide gel (lane 2, GP140 and double arrows). CLF culture-medium proteins, obtained by precipitation at 20–50%

Fig. 6. *Comparative CNBr digests of collagen types*

Collagen chains, purified by CM-cellulose chromatography, were incubated with CNBr as described in the Experimental section, and the cleavage products were chromatographed over a Sephadex PD-10 column before SDS/polyacrylamide-gel electrophoresis on composite (5/12.5%) gels under reducing conditions. Peptides were detected by fluorescence autoradiography or Coomassie Blue staining. (a) Lane 1, CLF 140000- M_r protein, starting material; lane 2, CLF 140000- M_r protein cleaved with CNBr; lane 3, human skin $\alpha 1(I)$. (b) Lane 1, human $\alpha 1(I)$; lane 2, bovine aortic endothelial-cell pro $\alpha 1(III)$; lane 3, HT-1080 (human fibrosarcoma) type IV procollagen; lane 4, A-204 (human rhabdomyosarcoma) type V collagen; lane 5, bovine aortic endothelial-cell type VIII. CNBr-cleaved peptides from $\alpha 1(I)$ are identified.

$(NH_4)_2SO_4$ and purified by CM-cellulose chromatography, contained a single antigenically cross-reacting component that co-migrated with the radiolabelled 140000- M_r collagenous protein and migrated somewhat more slowly than GP140 on SDS/polyacrylamide-gel electrophoresis (Fig. 7, lane 3). The CLF culture-medium proteins that were precipitated with 20% $(NH_4)_2SO_4$ contained a single cross-reactive species that co-migrated with GP140 (Fig. 7, lane 4). This protein displayed a slightly higher mobility on SDS-polyacrylamide-gel electrophoresis than did the protein isolated from the 20–50% $(NH_4)_2SO_4$ precipitate. The relation between the two forms of the CLF 140000- M_r protein is unclear. Since both culture-medium 140000- M_r species were unaffected by reducing

agents (results not shown), it is unlikely that disulphide-bonded aggregates of GP140 could account for differences in precipitability.

Similar immunoblotting experiments with antibodies to pepsin-treated bovine type VI collagen failed to reveal antigenic cross-reactivity with either GP140 from the cell layer or the culture-medium 140000- M_r protein purified by CM-cellulose chromatography (results not shown). These antibodies are known to react with the $\alpha 1(VI)$ and $\alpha 3(VI)$ chains of pepsin-extracted bovine type VI collagen but not with the $\alpha 2(VI)$ chain (Trüb & Bornstein, 1984).

The relationship between GP140 and the culture-medium 140000- M_r protein was examined by SDS/polyacrylamide-gel electrophoresis of pep-

Fig. 7. Immunoblot of proteins that had been detected with antibodies directed against GP140

Proteins were separated by SDS/polyacrylamide-gel electrophoresis under reducing conditions, transferred to nitrocellulose paper, and allowed to react with anti-GP140 (1:200 dilution) followed by ^{125}I -labelled staphylococcal protein A as described in the Experimental section. Lane 1, bovine aortic type VI collagen (pepsin-extracted); lane 2, CLF cell-layer GP140; lane 3, CLF 140000- M_r protein (CM-cellulose chromatography); lane 4, CLF culture-medium proteins precipitated with 20% $(\text{NH}_4)_2\text{SO}_4$. GP140 and the type VI chains are indicated. Additional cross-reacting species are indicated by single and double arrows.

tides generated by CNBr cleavage. As shown in Fig. 8, the pattern obtained from the culture-medium 140000- M_r protein secreted by CLF fibroblasts (Fig. 8*b*, lane 3) was very similar to that of cell-layer GP140 (Fig. 8*b*, lane 2). The GP140 protein obtained from the CLF (lane 2) also appeared similar to that from the control fibroblasts (compare Fig. 8*b*, lanes 1 and 2). The differences in

peptides in the high- M_r range in Fig. 8(*b*) (lane 3) as compared with those in Fig. 6(*a*) (lane 2) could be explained by the incomplete CNBr cleavage of protein in the polyacrylamide gel.

Protein synthesis

Estimates of the relative amounts of the 140000- M_r protein that was secreted were obtained by incubating cells, plated at equal densities, with [^3H]proline (10 $\mu\text{Ci}/\text{ml}$) for 18 h. Proteins, precipitated successively with 20% and 20–50% $(\text{NH}_4)_2\text{SO}_4$ concentrations, were resolved by SDS/polyacrylamide-gel electrophoresis and subsequently quantified by scanning densitometry. More than 90% of the radiolabelled proteins from the control skin-fibroblast culture medium was found in the 20%- $(\text{NH}_4)_2\text{SO}_4$ precipitate, and 0.60% of all precipitated radioactivity was present in the 140000- M_r protein of the 20–50%- $(\text{NH}_4)_2\text{SO}_4$ precipitate. In contrast, only 75% of the CLF culture-medium proteins were recovered in the 20%- $(\text{NH}_4)_2\text{SO}_4$ -precipitated fraction. Moreover, the 140000- M_r protein accounted for 3.67% of the total protein, a 6-fold increase over control (Table 1). In the 20%- $(\text{NH}_4)_2\text{SO}_4$ precipitate, a discrete band corresponding to the 140000- M_r protein could not be resolved by SDS/polyacrylamide-gel electrophoresis. The protein also could not be detected after fractionation on DEAE-cellulose. We therefore consider that it is unlikely that the 20%- $(\text{NH}_4)_2\text{SO}_4$ precipitate contained significant amounts of the 140000- M_r protein.

Extracts of the radiolabelled cell layers containing GP140 and fibronectin were also resolved by SDS/polyacrylamide-gel electrophoresis and scanning densitometry. The extracts from CLF cell layers represented a larger percentage of the total cellular protein than did those from controls (4.10 versus 1.20%). The radiolabelled band at M_r 140000 represented a 3.7-fold higher proportion of total cellular proteins in the CLF cells (0.75 versus 0.20%) (Table 1).

When incorporation of [^3H]proline into cellular and culture-medium proteins was expressed on a per-cell basis, it was found that the CLF protein contained one-half the total radioactivity of the controls. However, the specific radioactivity of proline (c.p.m./ μmol of proline) in the CLF cells was also determined to be one-half that of the control cells, when cellular and culture-medium proteins were analysed for content of total proline by amino acid analysis. This was a consistent observation whether or not serum was present in the medium at the time of labelling. When corrected for the difference in specific radioactivity of proline, the total protein synthesis was similar. The distribution of newly synthesized protein between

Fig. 8. Comparative CNBr digests of GP140 and the 140000-*M_r* protein from CLF

Radiolabelled proteins were separated by SDS/polyacrylamide-gel electrophoresis (composite 3/6% gels) under reducing conditions, and the bands of interests were cut from the gel. Proteins in the gel were incubated with CNBr (50mg/ml in 70% formic acid), re-equilibrated to pH6.8, and the digestion products analysed by subsequent SDS/polyacrylamide-gel electrophoresis (composite 5/12.5% gel), followed by autoradiography. (a) First-dimension separation: lane 1, human foreskin-fibroblast cell layer after extraction with Triton X-100 followed by 50mM-NaHCO₃ in 8M-urea buffer [fibronectin (FN) and GP140 are identified]; lane 2, CLF cell layer after extraction with Triton X-100 and 50mM-NaHCO₃ in 8M-urea buffer; lane 3, CLF 140000-*M_r* protein present in culture-medium protein. (b) CNBr-digestion products of 140000-*M_r* proteins represented in (a): lane 1, human foreskin-fibroblast cell-layer GP140; lane 2, CLF cell-layer GP140; lane 3, CLF culture-medium 140000-*M_r* protein. The positions of CNBr-digest products co-migrating with $\alpha 1(I)$ -CB7 and $\alpha 1(I)$ -CB8 are indicated. These peptides can be identified as migrating separately from those of GP140, and probably represent p_N $\alpha 1(I)$ that was co-isolated from the cell layer. All radiolabelled proteins in the region indicated as fibronectin (FN) have similar CNBr-digest products (results not shown) and were confirmed as derivatives of fibronectin.

Table 1. Distribution of fractionated [³H]proline-labelled medium and cellular protein

Values are expressed as percentage of radioactivity of (NH₄)₂SO₄- (medium) or trichloroacetic acid- (cellular) precipitable [³H]proline-labelled proteins, and represent the means for two experiments. Control cells were human foreskin fibroblasts of identical passage number as CLF.

	Medium		Cell layer		
	20%- (NH ₄) ₂ SO ₄ ppt.	20-50%- (NH ₄) ₂ SO ₄ ppt.	140000- <i>M_r</i> protein*	Triton/urea extracted†	GP140*
Control	92.2	7.8	0.60 (0.41-0.79)	1.20	0.20 (0.19-0.21)
CLF	73.7	26.3	3.67 (2.64-4.70)	4.16	0.75 (0.41-1.09)

* On the basis of gel-scanning densitometry; range is indicated in parentheses. The data are expressed as percentages of total proline-labelled protein.

† Insoluble cell layer after extraction with Triton X-100 followed by 50mM-NaHCO₃ in 8M-urea as described in the Experimental section.

cell layer and culture medium in all cells examined was also similar (67 and 33% respectively). Finally, the percentage of collagenous proteins synthesized by the cells, as measured by collagenase-sensitive radioactivity in the medium and cell layers, was similar. The data suggest that the CLF in culture exhibit an increase in the production of GP140-like proteins relative to other synthesized proteins, both in the cell layer and in the culture medium, in comparison with control skin fibroblasts.

Discussion

The precise biochemical defects in connective-tissue metabolism responsible for the syndrome of cutis laxa are not known. Although Byers *et al.* (1980) demonstrated a deficiency in lysyl oxidase activity in cultured fibroblasts from two cases classified as X-linked recessive cutis laxa, it now appears more appropriate to classify these cases as subtype IX of the Ehlers-Danlos syndrome with abnormal copper metabolism (Kuivaniemi *et al.*, 1982; Peltonen *et al.*, 1983). It is likely that a heterogeneous group of metabolic derangements is involved in producing the laxity of skin seen in cutis laxa.

The skin of our subject with cutis laxa was of interest because of the predominance of microfibrillar structures associated with the elastin bundles. Microfibrils have been studied in various tissues and have been postulated to serve a variety of functions (Cleary & Gibson, 1983). Ross & Bornstein (1969), in a combined ultrastructural and biochemical study, found that microfibrils were relatively less abundant with increasing age of bovine ligamentum nuchae and proposed that microfibrils may direct subsequent elastin fibre deposition in developing tissues. Interesting clues to the potential biochemical functions of the microfibrils have subsequently emerged. Serafini-Fracassini *et al.* (1981) suggested that amine oxidase activity was associated with a guanidinium chloride extract of bovine ligamentum nuchae, and extracts of bovine aortic tissue containing 'microfibril-related glycoproteins' induced von Willebrand-factor-dependent platelet aggregation (Fauvel *et al.*, 1983). Of major concern in the interpretation of studies of 'microfibril-related' proteins, however, has been the lack of substantiation that these preparations were homogeneous.

In the present study, metabolic labelling of cultured skin fibroblasts revealed a collagenous protein of apparent M_r 140 000 in the culture medium. This protein precipitated at high $(\text{NH}_4)_2\text{SO}_4$ concentrations, had no intermolecular disulphide bonds, and was structurally distinct from collagen types I, III, IV, V and VIII by analysis of CNBr-

digestion products. The culture-medium 140 000- M_r protein was closely related to GP140, the fibroblast cell-surface-associated glycoprotein described by Carter (1982a). In accord with our findings, Carter has described a 'released' form of GP140 in fibroblast culture medium (Carter, 1984). Unlike matrix GP140, this 'released' form is non-disulphide-bonded (Carter, 1984). Pepsin digestion of GP140 was reported to result in peptides similar to type VI collagen (Heller-Harrison & Carter, 1984a,b). A relationship between collagen type VI and GP140 was supported by Hessle & Engvall (1984), who isolated proteins of M_r 240 000 and 140 000 from fibroblast cell layers and culture medium, with monoclonal antibodies to extracellular-matrix components. Since these antibodies reacted with type VI collagen, those authors proposed that these immuno-isolated proteins were precursors of this collagen type. Since antibodies to pepsin-extracted $\alpha 1(\text{VI})$ and $\alpha 3(\text{VI})$ (Trüeb & Bornstein, 1984) do not react with GP140 on immunoblotting, it is possible that GP140 is related to the $\alpha 2(\text{VI})$ chain.

Sear *et al.* (1981) immunoprecipitated a 300 000- M_r non-collagenous protein (MFP-II) and a 150 000- M_r collagenous protein (MFP-I) from bovine ligamentum-nuchae-fibroblast culture medium with the use of an 'anti-microfibrillar protein' serum. This antiserum was raised against guanidinium chloride/collagenase extracts of ligamentum nuchae. MFP-I appears similar to the CLF 140 000- M_r protein with respect to $(\text{NH}_4)_2\text{SO}_4$ -solubility and sensitivity to bacterial collagenase. More recently, this MFP-I glycoprotein has been shown to yield peptides similar to type VI collagen upon pepsin digestion (Knight *et al.*, 1984).

Gibson & Cleary (1982) also isolated a 140 000- M_r collagenous protein from elastin-rich tissues; however, on the basis of tissue localization studies with monospecific antibodies, this component appeared to be unrelated to microfibrils. On the other hand, Jander *et al.* (1984) demonstrated that this 140 000- M_r collagenase-sensitive glycoprotein is related to type VI collagen. Pepsin-generated peptides were similar to those of type VI collagen by the criteria of electrophoretic mobility, antigenic cross-reactivity and electron-microscopic appearance. The relationship of this 140 000- M_r protein and GP140 to other fibroblast-derived proteins of similar size described by Sear *et al.* (1981) and by Hessle & Engvall (1984) appears likely, but this apparent identity will require further study.

Collagenase digestion of the CLF 140 000- M_r protein would have been expected to result in non-collagenous peptide fragments, although none were detected in our gels. Such peptides would probably contain relatively little proline and would

consequently not appear on an autoradiograph. It may be noted that Sear *et al.* (1981) found collagenase-resistant peptides of MFP-I when fucose rather than proline was used in their radiolabelling studies. We also did not detect any pepsin-resistant peptides from the CLF 140000- M_r protein. These peptides were likely to be obscured by other low- M_r radiolabelled proteins in the 10%-polyacrylamide portion of the gel.

Although the cultured fibroblasts from our subject with cutis laxa were shown to have identical growth rates and similar protein-synthetic rates, the deposition of extracellular matrix was decreased and the protein-synthetic patterns were altered from those of normal skin fibroblasts. The concentration of the 140000- M_r protein was increased 6-fold in the culture medium from the CLF as compared with that from control cells. Likewise, a higher percentage of extractable GP140 was present in the CLF cell layer. More precise quantification of cell-layer GP140 and culture-medium 140000- M_r proteins was not possible by immunochemical analysis because radioimmune precipitation with this antibody was incomplete; moreover, the poor solubility of isolated GP140 was a limiting factor in enzyme-linked immunosorbent assays. Although the differences in the relative quantities of CLF GP140 and culture-medium 140000- M_r protein that were isolated might be due to differences in solubility, our data do not support structural differences between these proteins since the CNBr-cleavage products of GP140 from CLF and normal cells were essentially the same.

The relative decrease in extracellular matrix in cell culture may reflect the relative decrease in interstitial collagen production by the CLF, although abnormalities in deposition of specific matrix components cannot be excluded. Fibroblasts in culture have been reported to produce microfibrils under appropriate conditions (Lamburg *et al.*, 1980; Sear *et al.*, 1978). The lack of microfibril production in our cultures may be a function of time in culture, cell density and the presence of ascorbate, which has been shown to suppress elastin and microfibril formation (Jones *et al.*, 1980). Therefore we cannot exclude the skin fibroblasts as a source of the increased microfibrils seen in the subject's skin. A relationship between the relative increase in accumulation of the 140000- M_r proteins and the increase in dermal microfibrils must, however, await procedures such as immunoidentification with specific antibodies to these components.

Since current evidence supports structural homology between collagen type VI and GP140, this case of cutis laxa may be the first human disease associated with abnormalities in deposition of type VI collagen.

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