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Analysis of Matrix Protein Components of the Dermis-Like Structure Formed in a Long-Term Culture of Human Fibroblasts: Type VI Collagen Is a Major Component[1]

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Formation of a dermis-like structure by a long-term culture of fibroblasts in the presence of ascorbic acid is a potential model for tissue organization or wound healing, and has its practical use as a skin graft. In the present study, solubilization of the dermis-like structure without pepsin treatment was attempted for analysis of pepsin-labile matrix components that might be involved in the formation of the dermis-like structure, as well as quantification of mutated type I collagen that could be susceptible to pepsin. The whole dermis-like structure was dissolved in a Tris buffer containing SDS and urea at 80°C. Analysis of the extract by SDS-PAGE revealed several protein bands that were not found in the pepsin-treated extract. Among them, the polypeptide band migrating at 140k under reducing condition showed a similar intensity of protein staining to the α2(I) chain band. The N-terminal amino acid sequences of cyanogen bromide peptides derived from the 140k polypeptide band as well as the amino acid composition of the band suggested that the band essentially consisted of α1(VI) and α2(VI) chains. The results demonstrated that the type VI collagen was a major component, being a comparable in amount to type I collagen, in the dermis-like structure.

Key words: β-aminopropionitrile, L-ascorbic acid 2-phosphate, fibroblast, type I collagen, type VI collagen.

Grinnell *et al.* reported that fibroblasts produced an organized matrix (dermis-like structure), composed of type I collagen as a major constituent, in a long-term culture supplemented with ascorbic acid, due to the stimulation of synthesis, procollagen processing, and fibril formation of type I collagen (*1*). Other related studies also reported that the dermis-like structure was formed by a long-term culture with L-ascorbic acid 2-phosphate (asc 2-p), a phosphate derivative that is biochemically very stable, retaining ascorbic acid activity for a long time. A long-term culture of fibroblasts with asc 2-p can form the dermis-like structure by accumulation of extracellular matrix components without frequent replacement of the culture medium (*2-4*). Formation of the dermis-like structure in a long-term culture of fibroblasts with ascorbic acid or asc 2-p is regarded as a potential model for tissue organization or wound healing, as well as having practical use as a skin graft, particularly for diabetic ulcer (*5*). Furthermore, production and deposition of a mutated type I collagen were also examined in a long-term culture of fibroblasts derived from osteogenesis imperfecta (OI) patients (*6, 7*).

For quantification of the collagenous proteins of the dermis-like structure, pepsin treatment has been used, since the collagenous triple-helical structures generally show resistance to pepsin treatment and become soluble in acidic solution after the treatment. Since the mutation of the type I collagen might cause increased susceptibility to pepsin treatment due to concomitant destabilization of the protein conformation, as suggested in previous studies (*8, 9*), quantification of the protein recovered after pepsin treatment may not reflect the amount of the type I collagen deposited by the fibroblasts derived from OI patients (*7*). The dermis-like structure appeared to have a distinct effect in the treatment of skin ulcer, particularly of the patients suffering from diabetes mellitus, from other dermis equivalent such as type I collagen gel containing fibroblasts, although the analysis of the proteins solubilized by pepsin treatment indicated that a major component is type I

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Abbreviations: asc 2-p, L-ascorbic acid 2-phosphate; BAPN, β-aminopropionitrile; BPB, bromophenol blue; CBB, Coomassie Brilliant Blue R-250; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FN, fibronectin; OI, osteogenesis imperfecta; PBS, Dulbecco's phosphate-buffered saline; PVDF, polyvinylidene difluoride; TSU, 50 mM Tris-HCl, pH 7.5, containing 0.5% SDS and 8 M urea.

collagen in either dermal replacement (*5*). In general, analysis of the extracts by means of pepsin treatment can not provide information on pepsin-labile or insoluble components, such as type VI collagen, type IV collagen, fibronectin, fibrillin, and decorin.

Compositional analysis of the extracellular matrix in tissues to date has encountered apparently contradictory problems: proteolytic or any other degrading treatments may lose information about the intact proteins which are susceptible to the treatment, while the complete solubilization of the materials, particularly type I collagen fibrils, appears to be hardly possible without degrading treatment due to the covalently bonded crosslinks between the molecules. In the present study, we attempted to analyze protein components including pepsin-labile ones of the dermis-like structure deposited during long-term culture of fibroblasts. For the biochemical analysis, the dermis-like structure was formed by minimizing intermolecular crosslinks so that the components could be solubilized more readily. That is, in the cell culture, the activity of lysyl oxidase, which catalyzes the oxidative deamination of lysine or hydroxylysine ε-amino groups to yield aldehyde, which eventually reacts with other amino or aldehyde groups to form intermolecular covalent crosslinks, was inhibited by addition of β-aminopropionitrile (BAPN) to the culture medium without altering the synthesis and deposition of the type I collagen (*1, 10*). In fact, about 95% of the type I collagen in the dermis-like structure was extractable with dilute acetic acid (*1*). Nevertheless, a substantial amount of insoluble residue was left after the acetic acid extraction. Hence, we attempted to analyze the whole components, including pepsin-labile components, by total solubilization.

MATERIALS AND METHODS

Materials—The following materials were purchased. Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) from Nissui. Trizma base, β-aminopropionitrile (BAPN) fumarate, and phenylmethylsulfonyl fluoride from Sigma Chemical. Cyanogen bromide, magnesium salt of L-ascorbic acid 2-phosphate (asc 2-p), and sodium azide from Wako Pure Chemical Industries. Urea (specially prepared reagent) and *N*-ethylmaleimide from Nacalai Tesque. Streptomycin and penicillin from GIBCO. Fetal bovine serum (FBS) from Bio Cell. Pepsin from Worthington Biochemical. Monoclonal antibody (FN12-8) against human plasma fibronectin from Takara. Horseradish peroxidase-conjugated antibody to mouse IgG and SDS-PAGE Standards, High Range, from Bio-Rad. PVDF membrane from Millipore. Human plasma fibronectin was a generous gift from Dr. Fumio Fukai (Science University of Tokyo, Tokyo). Type I collagen was extracted from rat tail tendon with 0.5 M acetic acid. Human type I and III collagens were prepared by salt fractionation of the extracts from pepsin-treated human placenta following the procedure of Epstein Jr. (*11*).

Cells and Cell Culture—Human dermal fibroblasts were obtained from a normal donor at age 18 as described previously (*12*). Human dermal fibroblasts (OIK-130) were established from a baby patient who had died at birth. From X-ray film, the patient was diagnosed as a type II osteogenesis imperfecta (lethal perinatal) with crumbled long bones, beaded ribs, and chest deformity. Cells were seeded onto a 35 mm dish at a density of 5×10^4/dish and grown in DMEM supplemented with 50 μg/ml streptomycin, 50 U/ml penicillin, 10% FBS, 0.2 mM asc 2-p, and 250 μM BAPN. In some cultures, where indicated, BAPN was omitted from the medium. The culture medium was replaced with fresh medium twice a week. The cells were cultured for 3-5 weeks.

Preparation of the Dermis-Like Structure as Starting Material for Biochemical Analysis—The dermis-like structure was washed twice with PBS, following the removal of the medium of the fibroblast culture. The dermis-like structure collected by scraping with a rubber policeman was suspended in 0.02 M Na_2HPO_4 containing a protease inhibitor cocktail composed of 5 mM EDTA, 1 mM *N*-ethylmaleimide, and 100 μM phenylmethylsulfonyl fluoride, with 0.02% (w/v) sodium azide, overnight at 4°C. The suspension was then centrifuged at $16,000\times g$ for 10 min. The pellet was resuspended in distilled water containing the protease inhibitor cocktail overnight at 4°C, then centrifuged at $16,000\times g$ for 10 min. The pellet was lyophilized.

Dissolution of the Dermis-Like Structure with Pepsin Treatment—The lyophilized materials (0.75 mg in dry weight) were treated with pepsin (0.1 mg/ml) in 300 μl of 0.5 M acetic acid for 24 h at 4°C, followed by centrifugation at $16,000\times g$ for 30 min. The supernatant was neutralized with Tris to inactivate the pepsin, and then an aliquot was subjected to SDS-PAGE analysis. The precipitate was dissolved in 300 μl of 50 mM Tris-HCl, pH 7.5, containing 0.5% SDS and 8 M urea (TSU) at 80°C for 5 min; afterwards, no precipitate was visible on centrifugation at $16,000\times g$ for 10 min. An aliquot was subjected to SDS-PAGE.

Dissolution of the Dermis-Like Structure in a Tris Buffer Containing SDS and Urea with Heating—The lyophilized materials (0.75 mg in dry weight) were suspended in 300 μl of TSU and heated at 80°C for 5 min. The treatment appeared to cause the suspension to form a clear solution without visible sediment after centrifugation at $16,000\times g$ for 10 min. In order to check whether any components adhered to the tube or whether any transparent sediment remained at the bottom, 20 μl of TSU containing β-mercaptoethanol (final conc.; 5% v/v) was added to the centrifuge tube (washing fraction). The extract with TSU or the washing fraction thus recovered was subjected to SDS-PAGE analysis.

SDS-PAGE—In all the experiments, aliquots of the sample solutions with or without β-mercaptoethanol (final conc.; 5% v/v) were subjected to SDS-PAGE after they had been heated at 80°C for 5 min, mixed with glycerol and BPB, and centrifuged at $7,000\times g$ for 30 s. SDS-PAGE was performed on a 5% separating gel with a 3% stacking gel on top in a discontinuous buffer system (*13*). In some experiments, proteins were separated on 5% polyacrylamide gel containing 3.6 M urea. Separated polypeptides on the gel were stained with Coomassie Brilliant Blue R-250 (CBB) in acetic acid-methanol and destained with acetic acid-methanol. Most collagenous polypeptides were stained red or red-purple in this staining procedure, while noncollagenous proteins were stained blue or blue-purple. Protein standards used for relative molecular mass estimation in poly-

acrylamide gels were as follows: phosphorylase *b*, 97,400; β-galactosidase, 116,250; myosin, 200,000; human plasma fibronectin (FN) monomer, 230,000; human plasma FN dimer, 460,000. In some experiments, interrupted gel electrophoresis was carried out according to the method of Sykes *et al.* (*14*). That is, after BPB had penetrated into the separating gel (1 cm below the interface between the stacking gel and the separating gel), electric power was switched off, and the sample wells were filled with 50% (v/v) glycerol containing 0.5 M DTT. The power was switched on to reinitiate electrophoresis after 30 min of incubation.

Immunoblotting—Proteins resolved on a polyacrylamide gel were electrophoretically transferred to a nitrocellulose sheet (2 mA/cm² for 2 h) (*15*). The nitrocellulose sheet was cut in halves, and one half was treated with amido black to stain proteins. The other half was incubated for 3 h at 30°C with a monoclonal antibody against human plasma fibronectin (FN12-8) diluted 1:5,000 in PBS containing 0.1% (v/v) Tween 20 after the sheet had been treated with PBS containing 3% (w/v) BSA at 4°C overnight, and then the sheet was washed with PBS containing 0.1% Tween 20 at room temperature. Then it was incubated with a horseradish peroxidase-conjugated antibody to mouse IgG diluted 1:1,000 in PBS containing 0.1% Tween 20 for 2 h at 30°C. After having been washed with PBS containing 0.1% Tween 20, then with PBS, the sheet was incubated with PBS containing diaminobenzidine and H_2O_2 for color development at room temperature. The anti-FN monoclonal antibody showed a positive reaction with human plasma FN but negative reactions with human type I or III collagen-derived polypeptides.

Cyanogen Bromide Peptide Mapping and N-Terminal Amino Acid Sequence Analysis—The protein band with $M_r = 140$k separated on 5% polyacrylamide gel was subjected to CNBr treatment according to the method described by Barsh *et al.* (*16*). Briefly, the protein band on the gel stained with CBB for a short time was cut out. The gel slice corresponding to the 140k polypeptide band was treated with CNBr at 32°C for 2 h. Then, the strip was placed on top of the second slab gel, composed of 12.5% polyacrylamide, subjected to SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was stained with CBB, and the CNBr peptide bands were cut out into strips. These strips were directly subjected to protein sequencing (Applied Biosystems model 473A or Beckman LF3000) (*17*).

The Amino Acid Analysis—The amino acid analysis was performed according to the method described by Takio *et al.* (*18*). Briefly, protein bands on the gel were electrophoretically transferred onto PVDF membrane, and the membrane was washed twice with distilled water, then stained with CBB. The stained sheet was washed with distilled water and dried. Each stained band was cut out and treated with 6 M HCl for 24 h at 110°C for hydrolysis to amino acids. The PVDF strip was then incubated in 0.1 M HCl/30% (v/v) methanol at room temperature for extraction of the resulting amino acids. The extraction was repeated three times. The extracts were combined, dried *in vacuo* at room temperature, and then subjected to amino acid analysis. The amino acid analysis was carried out by HPLC on a reverse-phase column CAPCELL PAK C_{18} SG 120Å (Shiseido) using pre-column derivatization with phenyl isothiocyanate.

RESULTS

Formation of Dermis-Like Structure in Long-Term Cultures of Fibroblasts with Asc 2-p—A long-term culture (up to one month) with asc 2-p of human skin fibroblasts, including the fibroblasts derived from a patient with osteogenesis imperfecta (OIK-130), formed a thick sheet-like layer, reminiscent of dermis, as reported by Grinnell *et al.* (*1*) regardless of the presence of BAPN. A portion of the periphery of the layer was often detached from the culture dish. In the absence of asc 2-p, the whole cell layer was not recovered as a sheet from the dish, since it was very thin and physically fragile. Asc 2-p was effective in allowing the development of macroscopically dermis-like structures during long-term cultures of all the fibroblasts examined.

Analysis of Protein Components by Pepsin Treatment of the Dermis-Like Structure—Since asc 2-p is a potent stimulator of type I collagen production by fibroblasts, the dermis-like structure formed by a long-term culture with asc 2-p may well be composed of physically strong type I collagen fibrils, as has been reported (*1-4*). Treatment with pepsin in acetic acid gave rise to a slightly opaque suspension in 0.5 M acetic acid. The suspension was clarified by centrifugation. The sediment, pepsin-insoluble fraction, dissolved to afford a clear solution upon heating in TSU. The whole dermis-like structure was thus recovered as solubilized components. The pepsin-insoluble fraction varied in amount from one sample to another for unknown reasons, but they all dissolved in heated TSU.

SDS-PAGE analysis under nonreducing conditions of the pepsin-treated dermis-like structure formed in the presence of asc 2-p and BAPN indicated that type I collagen α1(I), α2(I), and β(I) chains, type III collagen γ(III) chain, and type V collagen α1(V) and α2(V) chains were recovered as major components (Fig. 1, lane 1). This result was consistent with the previous reports (*1, 2, 6*), in that the pepsin-resistant proteins of the dermis-like structure were composed of collagenous proteins which contain large triple-helical domains in the molecules (type I collagen, type III collagen, and type V collagen). Strongly stained protein bands with higher molecular masses than collagen γ chain (the trimer of collagen α chains) were detected under nonreducing condition in the solution containing pepsin-insoluble fraction (Fig. 1, lane 2).

Under reducing conditions, the band above the γ chain disappeared in the pepsin-soluble or pepsin-insoluble fraction (Fig. 1, lanes 4 and 5). Instead, red-purple stained bands were detected with heavy staining above and at the gel front, extending even further below in both pepsin-soluble and insoluble fractions (in contrast to no staining below the gel front in the extract solubilized by nonproteolytic treatment: lane 6). Previous reports did not describe such an observation of a large amount of small polypeptides migrating at the gel front and further below in the pepsin-treated extract. The front bands might be derived from fragments of the fibrillar collagens, shorter collagenous domains of other types of collagen or other pepsin-resistant domains of proteins in the dermis-like structure. Red-purple staining color at the gel front implies the presence of collagenous polypeptides (*19-21*).

Analysis of Protein Components Solubilized by Nonproteolytic Treatment of the Dermis-Like Structure—The

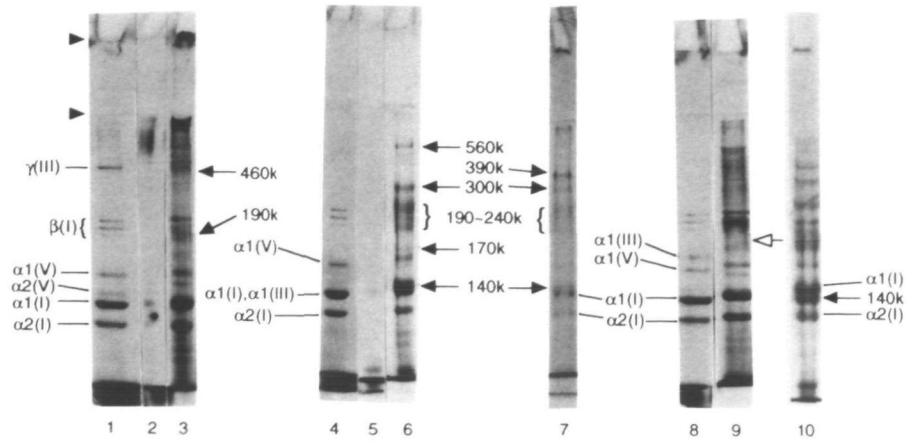

Fig. 1. **SDS-PAGE analyses of the protein components of the dermis-like structure deposited by long-term cultures of human skin fibroblasts with asc 2-p and BAPN.** The preparation of the sample solutions is described in "MATERIALS AND METHODS." Lanes 1, 4, and 8: the supernatant after centrifugation of the pepsin-treated dermis-like structure; lanes 2 and 5: the SDS/urea extract at 80°C from the sediment of the pepsin-treated dermis-like structure; lanes 3, 6, and 9: the supernatant after centrifugation of the SDS/urea extract with heat at 80°C for 5 min; lane 7: the washing fraction obtained by addition of heated TSU containing β-mercaptoethanol with a 1/15 volume of the supernatant; lane 10: SDS/urea extract without centrifugation. Sample solutions (lanes 1-9) of the same volume, 20 μl each, except for lane 10 (15 μl), were applied. Protein bands were stained with CBB. Lanes 1-3: under nonreducing conditions; lanes 4-7: under reducing conditions; lanes 8 and 9: Sykes's interrupted gel electrophoresis (*14*) with the addition of DTT after the BPB band had penetrated into the separating gel; lane 10: SDS-PAGE on a polyacrylamide gel containing 3.6 M urea under reducing conditions. Arrowheads point to the top of the stacking gel and the interface between stacking gel and separating gel. Arrows indicate the blue-stained bands not found in the pepsin-treated extract. The molecular masses of the bands (140k, 170k, 190-240k, 300k, 390k, 460k, and 560k) were denoted on the basis of globular protein standards (lanes 3, 6, and 7). In lane 10, the migration of the 140k band was not affected by the presence of urea on a polyacrylamide gel, while the migration of α1(I) band was retarded. An open arrow indicates the lower limit of broadened staining (blue color).

lyophilized materials of the dermis-like structure formed in the presence of BAPN were suspended in TSU. Upon heat treatment at 80°C for 5 min, the materials were apparently dissolved in the solution (hereafter called SDS/urea extract). In order to see whether any materials adhered to the centrifuge tube, a small amount of the heated TSU (1/15 volume of the supernatant) was added, together with β-mercaptoethanol, to the centrifuge tube after removal of the supernatant (washing fraction). The SDS-PAGE analysis of the washing fraction showed essentially the same bands as the supernatant of the SDS/urea extract, except for the band with $M_r = 390$k, plus faint protein bands with $M_r = 140$k, 190-240k, and 300k as well as α1(I) and α2(I) (Fig.1, lane 7). Protein content in the washing fraction (Fig. 1, lane 7) was less than 1% of that in the supernatant extracted from the dermis-like structure (Fig. 1, lane 6), based on the staining intensities of the protein bands. The solubilization with heated TSU made it possible to analyze major protein components of the dermis-like structure on SDS-PAGE with 5% gel, since essentially all of the polypeptides penetrated into the polyacrylamide gel under reducing conditions (Fig. 1, lanes 6 and 10).

Under nonreducing conditions, the SDS/urea extract (Fig. 1, lane 3) contained several polypeptide bands which were not found in the pepsin-treated extracts (Fig. 1, lanes 1 and 2), particularly the blue-stained bands remaining at the tops of 3% stacking gel and 5% separating gel and those corresponding to 190k and 460k. Upon reduction with β-mercaptoethanol, the bands on the gel tops and the 460k band disappeared, and blue-stained bands corresponding to $M_r = 140$k (140k polypeptide), 170k, 190-240k, 300k, and 560k appeared (Fig. 1, lane 6). The blue staining of the bands implied that they did not contain the proteins that constituted the large collagenous domains, since collagenous polypeptides are stained red-purple with CBB followed by destaining in acetic acid-methanol solvent. The blue-stained broad band migrated behind the pepsin-treated α1(III) chain on interrupted gel electrophoresis, where the reduction was performed after BPB penetrated into the separating gel (Fig. 1, lanes 8 and 9). The results suggested that the 140k polypeptide band may have come from disulfide-bonded molecules or aggregates of varying sizes, larger than pepsin-treated γ chain of type III collagen.

Relative Content of the 140k Polypeptide and Type I Collagen—The 140k polypeptide band in the SDS/urea extracts from the dermis-like structure formed by the culture of normal fibroblasts comigrated with α1(I) chain band, and thus the relative amount of the 140k to type I collagen could not be determined. However, in the gel containing 3.6 M urea, these bands migrated separately (Fig. 1, lane 10). Similar intensities of protein staining of the α2(I) chain band and the 140k polypeptide band implied comparable contents of the type I collagen and the 140k polypeptide in the SDS/urea extract of the dermis-like structure.

Characterization of the 140k Polypeptide Band—The 140k polypeptide band with the strongest staining was further characterized. Incidentally, we observed that the SDS/urea extract from the dermis-like structure formed by the culture of the fibroblasts derived from a patient with osteogenesis imperfecta (OIK-130) with asc 2-p in the absence of BAPN showed essentially only the blue-stained bands under reducing conditions, and neither α1(I) nor α2(I) chain band (Fig. 2) was present.

The CNBr peptide mapping of the 140k polypeptide band

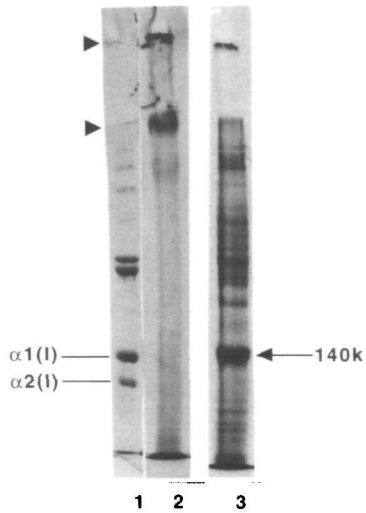

Fig. 2. **SDS-PAGE of the SDS/urea extract from the dermis-like structure produced by human skin fibroblasts derived from an osteogenesis imperfecta patient.** A dermis-like structure formed by a long-term culture of human skin fibroblasts, OIK-130, derived from a patient with osteogenesis imperfecta in the absence of BAPN was treated with heated TSU to extract the components. Protein bands were stained with CBB. Lane 1: type I collagen from rat tail tendon as a reference; lanes 2 and 3: the supernatant after centrifugation of the suspension at 7,000×*g* for 30 s. Lanes 1 and 2: under nonreducing conditions; lane 3: under reducing conditions. Arrowheads point to the top of the stacking gel and the interface between the stacking gel and the separating gel.

from the dermis-like structure deposited by the culture of OIK-130 fibroblasts indicated that the 140k polypeptide band was composed of the same polypeptides as those constituting the 140k polypeptide band from dermis-like structure obtained from the culture of normal dermal fibroblasts (data not shown).

The 140k polypeptide band electrophoretically separated from the extracts of the dermis-like structure of OIK-130 fibroblasts was transferred to PVDF membrane and subjected to amino acid analysis. The band contained small amounts of hydroxyproline and hydroxylysine, and relatively large amounts of glycine (Table I), suggesting that the 140k polypeptide band might well contain collagenous peptides. Since the 140k polypeptide band appeared only on SDS-PAGE under reducing conditions, the constituent polypeptides were derived from disulfide-bonded aggregates. From the previous studies, it was anticipated that the polypeptides migrating at 140k upon reduction on SDS-PAGE might contain α1(III), α1(VI), and α2(VI) chains among collagenous proteins.

Hence, the N-terminal sequences of seven CNBr peptides obtained from the 140k polypeptide band were determined (Fig. 3). All the peptides showed sequences found in either α1(VI) or α2(VI). In particular, the 13 and 15 amino acid sequences of bands 4 and 5 matched the sequence of the α1(VI) chain, and the 13 amino acid sequence of band 7 matched the sequence of the α2(VI) chain. Large peptides with strong staining, bands 1 and 2, showed low recoveries in the determination of amino acid sequences, implying that the band may contain mixed peptides. Both the sequences start with G, followed by E, but further residues showed low recoveries of various

TABLE I. **The amino acid composition of the 140k polypeptide band.** (residues/1,000)

	140k	Human α1(VI)[a]	Human α2(VI)[b]	α1(VI) and α2(VI)[c]	Human α1(III)[d]
Asp	93	70	70	70	42
Asn	N.D.	28	33	31	N.D.
Glu	109	68	66	67	71
Gln	N.D.	40	42	41	N.D.
Ser	63	50	53	52	39
Gly	137	154	152	153	350
His	17	14	21	18	6
Ala	70	71	58	65	96
Tyr	19	28	18	23	3
Val	60	64	59	62	14
Ile	41	42	40	41	13
Leu	76	65	61	63	22
Thr	48	39	44	41	13
Arg	54	58	67	62	46
Met	2	10	12	11	8
Cys	12	19	20	19	2
Phe	22	33	39	36	8
Lys	39	53	51	52	30
Hyl	39	—	—	—	5
Pro	79	91	90	90	107
Hyp	20	—	—	—	125
Trp	N.D.	3	4	3	N.D.

The SDS-PAGE of SDS/urea extract was performed as in lane 3 of Fig. 2. The protein band was electrophoretically transferred to PVDF membrane. The 140k band strip cut out was treated with 6 M HCl at 110°C for 24 h for hydrolysis of the polypeptides. The hydrolysate was extracted from PVDF membrane with 0.1 M HCl/30% methanol and then subjected to amino acid analysis. [a]From PIR database; pir: CGHU1A, accession number: S05377. [b]From PIR database; pir: CGHU2A, accession number: S05378. [a,b]The data are based on the amino acid sequences deduced from the nucleotide sequences. [c]The compositions for the combined polypeptides are based on a 1:1 ratio of the α1(VI) to α2(VI) chain. [d]From Ref. *24*. N.D., not determined.

amino acid residues including G and E. Peptides beginning with MGE can be assigned to many protein primary structures including α1(VI) or/and α2(VI) chains. On the other hand, no specific sequences corresponding to the α1(III) chain were found. The amino acid composition of the 140k polypeptide band was consistent with a combined amino acid composition calculated for the α1(VI) and α2(VI) chain sequences, as deduced from nucleotide sequences (Table I). These results suggested that the 140k polypeptide band mainly consisted of α1(VI) and α2(VI) chains.

Characterization of Other Pepsin-Labile Proteins in the Dermis-Like Structure—Other polypeptide bands that were detected with significant intensity of protein staining only in the SDS/urea extract, but not in the pepsin-treated extract, were bands corresponding to 170k, 190-240k, 300k, and 560k in addition to the peptide 190k, which was found under nonreducing conditions as well. Since polypeptides of fibronectin (FN), which is known to be present in the cell culture deposit, have a molecular weight of ~230k after reduction (*25*), the polypeptides resolved on the gel were subjected to immunoblotting with the anti-FN antibody. The band corresponding to the 220-240k bands under reducing conditions was specifically stained (Fig. 4, lane 3). The immuno-staining intensities with the monoclonal antibody of the 220-240k bands of the SDS/urea extract (Fig. 4, lane 3) were about the same as that of the 230k band of plasma FN which was loaded with an amount of 0.2

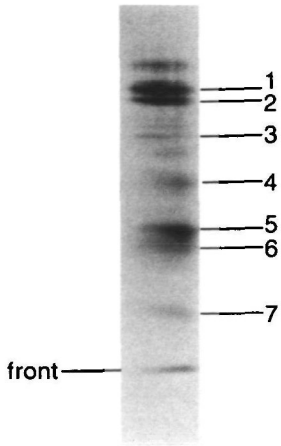

Fig. 3. **Amino-terminal sequences of the CNBr peptides from the 140k polypeptide band.** Top: The 140k polypeptide band was separated by SDS-PAGE of the SDS/urea extract as in lane 3 of Fig. 2. The band was treated with CNBr. Then the reaction products were separated on 12.5% polyacrylamide gel and transferred to PVDF membrane as described under "MATERIALS AND METHODS." The CNBr peptides as numbered in the figure were subjected to amino acid sequence analyses from the amino-terminals. Bottom: Amino acids are represented by the one letter code. When more than two amino acids were detected in the sequencing cycle, they are represented by characters in parentheses. Capital letters in parentheses mean that the signals were the strongest among the amino acids detected in that cycle. After major amino acids residues were aligned, remaining amino acids were aligned so as to match the amino acid sequences deduced from cDNA. The sequences start from the methionine residues with the assumption that CNBr should have cleaved specifically the peptide bonds containing the methionine residue at the C-terminus. The amino acid sequences deduced from DNA base sequences that matched those of CNBr peptides are underlined. Bands 4 and 5 were mixtures of at least 3 peptides. All the peptides showed sequences found in either α1(VI) or α2(VI) chain. Z, hydroxyproline; X, unidentified residue. a: From Refs. *22* and *23*.

Bands	Amino Acid Sequences of CNBr peptides	Corresponding Amino Acid sequences deduced from cDNA[a]
1	GE	
2	GE(PZ)GEZ	α2(VI):GN^{272}MGEPGEPGQ
3	GEXGEZ	
4	(Aip)(Gk)(GN)(Tn)(FVd)(ATE)GEALQY	
	AGGTFTGEALQY	α1(VI):QW^{697}MAGGTFTGEALQYTR
	ikNnVE	α1(VI):VD^{238}MIKNNVEQVCCS
	p dA	α1(VI):TR^{106}MPGGRDALKSSV
5	(Aep)(Gd)(Gvl)(Trl)(Fd)(Atp)(Gdl)(Ekp)(Asq)(Lsi)(QV)(Yd)(TAp)(RV)	
	AGGTFtGEALQYTR	α1(VI):QW^{697}MAGGTFTGEALQYTRDQ
	p rdAlkssVdAV	α1(VI):TR^{106}MPGGRDALKSSVDAVKY
	edvl pdpqi p	α2(VI):DD^{800}MEDVLCPDPQIVCPDPQ
	l	
6	E(DN)VLEP	α2(VI):DD^{800}MEDVLCPDPQIVCPDPQ
7	(Kp)QFVPQF(Ik)SQ(LV)XN	α2(VI):FH^{70}MKQFVPQFISQLQNEF

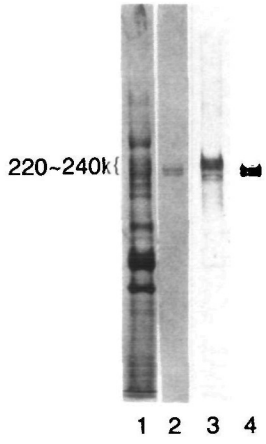

Fig. 4. **Immunoblotting of the SDS/urea extract from the dermis-like structure with a monoclonal antibody against fibronectin.** Protein bands were transferred to a nitrocellulose membrane after electrophoresis under reducing conditions. The strips cut out were stained with amido black (lanes 1 and 2) or immunostained with a monoclonal antibody against human plasma FN (lanes 3 and 4). Lanes 1 and 3: the SDS/urea extract from the dermis-like structure, 50 μg each of the lyophilized extract; lanes 2 and 4: human plasma FN, 0.6 μg and 0.2 μg, respectively.

μg (Fig. 4, lane 4). Assuming that the affinities of plasma FN and cellular FN with the antibodies are the same, the FN content in the 220-240k bands of the SDS/urea extract (Fig. 4, lane 1) corresponded to about 0.2 μg of the plasma FN, an amount which is scarcely stained with amido black. The FN content in the SDS/urea extract was about a thirtieth to fiftieth of that of type VI collagen, as judged from the staining intensity. A positive staining with anti-fibrillin antibody was obtained for the band with $M_r = 460$k after reduction and alkylation (data not shown).

The 300k band showed the strongest protein intensity among the bands found only in the SDS/urea extract, except for the 140k polypeptide band (Fig. 1, lane 6). Hence, the 300k band could be derived from α3(VI), if the type VI collagen molecules were to consist of the trimer with a chain composition of α1(VI), α2(VI), and α3(VI). If α3(VI) has alternative sizes distributed in the range from $M_r = 180$k to $M_r = 260$k as reported in previous studies (*26, 27*), and if the amount of α3(VI) chain is equivalent to that of α1(VI) or α2(VI) in molar ratio, the protein bands corresponding to 190k-240k could be α3(VI) or might contain a polypeptide(s) derived from α3(VI). The characteristics of the pepsin-labile polypeptides in the dermis-like structure are summarized in Table II.

TABLE II. **The characterization of pepsin-labile polypeptides in the tissue-like structure.**

Size of polypeptides after reduction	Staining intensity with CBB	Protein identified or proposed
140k	+++	α1(VI) and α2(VI)
300k	++	α3(VI)
190-240k	++	Alternative spliced or processed form of α3(VI) and others
170k	+	—
390k[a]	+	—
460k[b]	+	—
560k	+	—
220-240k	N.D.	Fibronectin
460k[c]	N.D.	Fibrillin

[a]The 390k polypeptide was only found in the washing fraction. [b]The 460k polypeptide was only found under nonreducing conditions. [c]The 460k polypeptide was found under reducing condition and alkylation. N.D.: Not detected with protein staining, but detected with immunostaining. —: Unidentified.

DISCUSSION

The matrix components, including pepsin-labile ones, were semiquantitatively analyzed in the dermis-like structure formed by a long-term culture of fibroblasts in the presence of BAPN. The analysis was made possible by the total solubilization of the materials without degradative treatment. The SDS-PAGE patterns of the solubilized components revealed several protein bands that were not found in the extract obtained by pepsin treatment. One of the polypeptide bands with a strong staining was a 140k polypeptide band that migrated at the same position as α1(I) chain under reducing conditions. Three lines of evidence indicated that the 140k polypeptide band consists of α1(VI) and α2(VI). (i) The type VI collagen α chains showed the same mobilities as α1(I) or α1(III) on SDS-PAGE under reducing conditions (*26*). The 140k polypeptide band showed a faster mobility than α1(I) or α1(III) in urea-containing SDS-PAGE, as would be expected for the α1(VI) and α2(VI) chains, which have large globular domains (*22, 23, 28*). The α1(I) and α1(III) chains are known to be retarded in comparison with globular proteins with similar molecular masses in urea-containing SDS-PAGE (*28*). The 140k polypeptide band behaved like polypeptides containing globular domains. (ii) The amino acid composition of the 140k polypeptide band matched the combined composition of the α1(VI) and α2(VI) chains. (iii) The CNBr peptide mapping of the 140k polypeptide band and that of GP140, which was identified as α1(VI) and α2(VI) by Crawford *et al.* (*29*), were similar to each other with a correlation coefficient of 0.98. All seven CNBr peptides from the 140k polypeptide band contained amino acid sequences shared with α1(VI) and α2(VI) chains. The results led us to conclude that the type VI collagen-derived polypeptides, α1(VI) and α2(VI), are the major components of the dermis-like structure, as well as type I collagen, since the protein staining intensity of the 140k polypeptide band was as strong as that of the α2(I) chain.

Removal of most type I collagen from the dermis-like structure by solubilization with 50 mM Tris-HCl, pH 8.5, containing 2 M urea and 0.15 M NaCl or 0.5 M acetic acid at 4°C still left apparent residues, suggesting that components other than the type I collagen, including type VI collagen, are major constituents of the dermis-like structure. Furthermore, the dermis-like structure formed by the fibroblasts derived from an osteogenesis imperfecta (OIK-130) patient showed no macroscopic difference, though it contained a smaller amount of the type I collagen than the type VI collagen (unpublished data). Hence, we speculate that type VI collagen, representing a major protein component of the dermis-like structure, might be directly involved in organizing the framework of the dermis-like structure.

We propose that asc 2-p highly stimulated the overall production and deposition of type VI collagen, as well as type I collagen, in the fibroblast culture, resulting in the dermis-like structure that contained comparable amounts of type VI collagen and type I collagen. The reports that the synthesis and secretion of type VI collagen were greatly enhanced by ascorbic acid are consistent with this proposal (*30-32*). On the other hand, it was reported that the deposition reaction of type VI collagen was not affected by the presence of ascorbic acid (*33*), although the deposition of type I collagen, in addition to its production, was promoted by asc 2-p (*1, 2, 6*). Thus, it may be that the increase in the deposited amount of the type VI collagen with asc 2-p could be ascribed to increased production. Consequently, the relative amount of type VI collagen to type I collagen in the deposit would be much higher in the absence of asc 2-p or at a certain timing of culture in the presence of asc 2-p, since deposition of type I collagen may require ascorbic acid. Our preliminary studies, in fact, indicated that the 140k polypeptide band or type VI collagen appeared to be the only major protein in the SDS/urea extract from the cell layer of the fibroblast culture without asc 2-p and also that the relative content of type VI collagen to type I collagen was higher at an early stage of culture (6 days).

The previous reports (*1, 2, 6*) on the analysis of the dermis-like structure after pepsin treatment did not refer to type VI collagen as a major component or to the possible involvement of type VI collagen in the formation of the dermis-like structure. Type VI collagen α chains contain short collagenous domains with three interruptions which might cause susceptibility to pepsin (*22, 23, 34, 35*). Thus, the pepsin-resistant polypeptide fragments derived from the type VI collagen may be low in molecular weight, migrating at the 5% gel front, particularly after extensive pepsin treatment. Strong staining with red-purple color at the gel front in the SDS-PAGE analysis of a pepsin-treated extract from the dermis-like structure may imply that the front bands contained such type VI collagen-derived collagenous peptides (Fig. 1, lanes 1, 2, 4, and 5). The analyses of collagenous components in the dermis-like structure by the use of pepsin treatment may tend to cause underestimation of the total amount of type VI collagen.

The immuno-staining of the cultured fibroblasts reveals fibrillar FN around the cells (*25*). The deposited amount of FN protein with 230k monomer polypeptide was estimated from the immunoblotting analysis. It was less than a thirtieth of the amount of type VI collagen, suggesting that asc 2-p may not stimulate production or accumulation of fibrillar FN as much as type I collagen and type VI collagen.

A large amount of microfibrils that were reactive with anti-fibrillin monoclonal antibody was found in the deposit formed on a nylon filtration mesh by a fibroblast culture in the presence of ascorbic acid (*36*). Previous reports suggested that fibrillin proteins were present as disulfide-bonded aggregates, which may not be dissociated into monomers even under reducing conditions, in the matrix deposited by fibroblast culture (*37*). The immunoblotting analysis of the SDS/urea extract after reduction and alkylation showed that a polypeptide band migrating close to the dimeric fibronectin band (460k) and faster than the 560k band, reacted with anti-fibrillin monoclonal antibody (a generous gift from Dr. M. A. Gibson, University of Adelaide, South Australia, Australia) (data not shown), suggesting that fibrillin is one of the pepsin-labile protein components in the dermis-like structure.

Some of the protein bands migrating between α1(I) and β(I), regardless of reduction, may be due to type V collagen-derived polypeptides, since the bands with slightly higher migration were also found in the pepsin extract. On the other hand, the band which may correspond to α1(III) chain was not detected in the SDS/urea extract on interrupted gel electrophoresis for as yet unclarified reasons. It might be that the type III collagen polypeptide without proteolytic treatment does not have a discrete size.

In contrast to the previous reports suggesting that asc 2-p-promoted formation of the dermis-like structure was due to the enhanced synthesis and accumulation of type I collagen, the present findings demonstrate that a number of other pepsin-labile proteins, type VI collagen in particular, may also contribute to the formation of the dermis-like structure. Our studies also suggested that the pepsin-labile proteins might account for the clinical effectiveness in the treatment of diabetic ulcers. Type VI collagen, in particular, may be one of the effective factors in the treatment, since a previous report showed that type VI collagen α chains are abundantly expressed in the early phase of wound healing (*38*).

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