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Molecular composition of type VI collagen

Evidence for chain heterogeneity in mammalian tissues and cultured cells

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The chain composition and relative abundance of type VI collagen synthesized by cells cultured from foetal bovine nuchal ligament and skin were compared with those of the type VI collagen present in these foetal tissues. Immunoprecipitation of intact collagen VI from medium and cell layers of nuchal ligament fibroblasts and skin fibroblasts at confluence revealed collagen type VI molecules with a chain composition consistent with an [α1(VI)α2(VI)α3(VI)] monomeric assembly. Maintenance of cells in a post-confluent quiescent state promoted a marked phenotypic change in these ratios, with increased concentrations of assemblies composed of equimolar ratios of α1(VI) and α2(VI) chains detected in the medium of these cultures. Analysis of steady-state concentrations of mRNA for α1(VI) and α2(VI) chains revealed these species to be present in increased abundance at post-confluence in all the cultures, but no corresponding increase was observed in the α3(VI) mRNA. In order to assess the physiological significance of these observations, the chain composition of the collagen VI content of the corresponding foetal tissues was assessed by Western blotting after extraction in guanidinium isothiocyanate under reducing conditions. Extracts of nuchal ligament revealed a collagen VI chain composition consistent with a heterotrimeric chain assembly. In contrast, the skin extracts revealed an abundance of α1(VI) and α2(VI) chains with only traces of the α3(VI) chain detected. Increased equimolar concentrations of the α1(VI)-chain and α2(VI)-chain mRNAs in skin again reflected the increased concentrations of these polypeptide chains. Type VI collagen was present in greater abundance both in the nuchal ligament and in the corresponding nuchal-ligament fibroblast cultures. The results indicate that the chain composition of type VI collagen is subject to modulation at the level of transcription as a result of variations in the proliferative state of the cells, and demonstrate that different isoforms of collagen VI occur in foetal development.

INTRODUCTION

Type VI collagen represents a significant component of soft connective tissues and is believed to play a central role in cell–matrix communications (Bonaldo & Colombatti, 1989). Such a role is one for which this collagen is uniquely suited, with its unusual structure and supramolecular organization. The type VI collagen monomer contains a particularly short triple-helical region (105 nm) and large globular *N*-terminal and *C*-terminal domains, and the constituent chains have significantly different M_r values. Whereas the α1(VI) and α2(VI) chains have an M_r of 140000, the α3(VI) component is much larger, with an M_r of 260000–280000 (Engvall *et al.*, 1986; Trueb & Winterhalter, 1986; Colombatti *et al.*, 1989). A number of tissue extraction and biosynthetic studies have demonstrated that these chains generally occur in stoichiometric proportions (Jander *et al.*, 1983; Chu *et al.*, 1987). Intracellular assembly of the monomers into dimers and subsequently tetramers precedes secretion (Engvall *et al.*, 1986), and once in the extracellular space these structures interact to form an extensive microfibrillar network that is predominantly stabilized by disulphide bonding. These structures have been seen at the ultrastructural level to be associated with basement membranes as well as with the major fibrillar collagens (Bruns *et al.*, 1986; Sakai *et al.*, 1986; Keene *et al.*, 1988). These associations have given rise to speculation that the primary role of type VI collagen is one of anchorage of cells to the skeletal framework of the extracellular matrix. Broad-banded fibrils composed of type VI collagen have also been identified in a number of normal tissues, including intervertebral disc (Cornah *et al.*, 1970; Wu *et al.*, 1987), synovium (Levick & McDonald, 1990) and iris and ciliary body (Rittig *et al.*, 1990), and in fibroblast cultures (Bruns, 1984; Bruns *et al.*, 1986), but are more frequently observed in pathological tissues such as tumours (Timpl & Engel, 1987).

The precise chain composition of the monomers incorporated into the assembled microfibrils remains a controversial aspect of type VI collagen structure and function. Structural considerations have resulted in the prediction that the [α1(VI)α2(VI)α3(VI)] heterotrimer is the composite with maximum disulphide-bonding and highest stability, and that aggregates comprising monomers of different chain compositions would be less stable entities with enhanced susceptibility to proteinase attack. However, a significant body of data has now accumulated, principally at the level of gene expression, to suggest that alternative type VI collagen chain composites might be assembled and secreted under particular biologically or pharmacologically induced experimental conditions, and possibly in certain heritable diseases (Crawford *et al.*, 1985). For example, non-co-ordinated regulation of gene expression either of α3(VI) chain or of α1(VI) and α2(VI) chains has been observed in human skin fibroblasts grown in collagen gels (Hatamochi *et al.*, 1989) or treated with γ-interferon (Heckmann *et al.*, 1989), and for corneal fibroblasts and some tumour cells (Chu *et al.*, 1987). It has been suggested that this pattern of regulation may reflect the chromosomal localization of the human collagen VI genes, with the structural genes encoding the α1(VI) and α2(VI) chains separated by no more than 200 kb on chromosome 21 whereas the α3(VI) gene is on chromosome 2 (Cutting *et al.*, 1988; Weil *et al.*, 1988). Regulation of transcription of the type VI collagen structural genes can also be exerted by a co-ordinate mechanism, as demonstrated in

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experiments on transformed fibroblasts showing simultaneous depletion of all type-VI-collagen-specific mRNAs (Chu *et al.*, 1987). Less is known about whether the chain variations at the message level are transmitted to the protein level. Recently, down-regulation of the mRNA encoding a single chain of type VI collagen by γ-interferon was shown to lead to an overall reduction in biosynthesis and matrix deposition of collagen VI (Heckmann *et al.*, 1989). In the present paper we describe for the first time variations in the chain composition of intact collagen VI molecules present in foetal bovine cells and tissues. These observations lead us to propose that type VI collagen exists as a heterogeneous family of isoforms with potential functional variations, the expression of which is apparently controlled primarily at the level of transcription.

EXPERIMENTAL

Materials

Foetal calves were obtained from the local abattoir within 1 h of maternal death. Culture medium, foetal-calf serum and antibiotics were obtained from Gibco BRL Ltd., Paisley, Scotland, U.K. Pepsin (EC 3.4.23.1) from pig stomach mucosa, phenylmethanesulphonyl fluoride, *N*-ethylmaleimide, L-ascorbate, dithiothreitol, Nonidet P-40, high-M_r non-collagenous standards and agarose (type 1) were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Tween 20 was obtained from BDH Chemicals, Poole, Dorset, U.K. CsCl was supplied by Boehringer Mannheim, Lewes, East Sussex, U.K. Guanidinium isothiocyanate was supplied by Fluka Chemicals, Glossop, Derbyshire, U.K.

L-[^{35}S]Methionine (1443 Ci/mmol) and [^{32}P]dCTP were purchased from Amersham International, Amersham, Bucks., U.K. Protein A–Sepharose CL-4B was obtained from Pharmacia Fine Chemicals, Milton Keynes, Bucks., U.K. Polyclonal and monoclonal antisera to human fibronectin were obtained from Calbiochem, San Diego, CA, U.S.A., and Sigma Chemical Co. respectively.

The following type VI collagen cDNA probes were utilized in this investigation: a 1.9 kb insert of pUC19 p18 coding for collagen α1(VI), a 2.3 kb insert of pUC19 p1 coding for collagen α2(VI), and a 1.2 kb insert of pUC19 p24 coding for collagen α3(VI) (Chu *et al.*, 1988). These clones were obtained from Dr. M.-L. Chu, Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA, U.S.A. The cDNA clone Fn421 encoding a fragment of human fibronectin was obtained from Dr. M. J. Humphries of this Department.

Preparation and maintenance of cultures

All cell lines were propagated by explant culture from freshly excised tissues obtained from foetal calves of 140 days gestation. Stock cultures were routinely maintained in Dulbecco's modified Eagle's medium supplemented with L-glutamine at 2 mM, penicillin (100 units/ml), streptomycin (50 μg/ml) and 10% (v/v) foetal-calf serum. Cells were subcultured by trypsinization with a split ratio of 1:3. Cultures that were newly confluent or 8–10 days post-confluent to be labelled were pre-incubated for 24 h either in Dulbecco's modified Eagle's medium or in Eagle's minimum essential medium without methionine, depleted of foetal-calf serum but containing ascorbate (50 μg/ml). The culture media were obtained from Gibco BRL Ltd. Labelling was carried out in fresh medium containing [^{35}S]methionine (10 μCi/ml) for 24 h.

Isolation of newly synthesized proteins

Medium containing the newly synthesized proteins was briefly centrifuged to remove cell debris before the addition of proteinase inhibitors (2 mM-phenylmethanesulphonyl fluoride, 10 mM-*N*-ethylmaleimide, 25 mM-6-aminohexanoic acid and 25 mM-EDTA). The medium was either analysed immediately or stored at −20 °C. Labelled medium proteins were concentrated by precipitation with $(NH_4)_2SO_4$ at 30% saturation at 4 °C. Precipitates were redissolved in 50 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-EDTA, 40 mM-NaCl and 1% Nonidet P-40 (NET buffer).

Cell layers were extracted in the presence of proteinase inhibitors for 24 h at 4 °C in 50 mM-Tris/HCl buffer, pH 8.0, containing 8 M-urea. The extracts were dialysed extensively against 50 mM-Tris/HCl buffer, pH 8.0, containing 5 mM-EDTA and 40 mM-NaCl, and Nonidet P-40 to 1% was added before immunoprecipitation. The insoluble residues were also retained for analysis.

Immunoprecipitation of intact native type VI collagen

Two initial sequential immunoprecipitation steps were carried out on all fractions with commercially available polyclonal and monoclonal anti-(human fibronectin) sera in a stepwise manner. Type VI collagen was then immunoprecipitated with two well-characterized polyclonal antisera to bovine and pig type VI collagen respectively (Ayad *et al.*, 1989; King *et al.*, 1989). Immunoprecipitations were carried out by following the method described by Cooper *et al.* (1981). Normal rabbit serum was used as a control. Briefly, to 500 μl of sample was added 5 μl of antiserum or normal rabbit serum. After incubation at room temperature for 1 h, 30 μl of a 50% (w/v) suspension of Protein A–Sepharose in NET buffer was added and the incubation was continued at 4 °C overnight with end-over-end mixing. The complex of antigen, antibody and Protein A–Sepharose was collected by centrifugation, and was washed twice with the above buffer and twice with 10 mM-Tris/HCl buffer, pH 6.8. Bound proteins were released for electrophoretic analysis by heating at 100 °C for 3 min in Laemmli sample buffer containing 50 mM-dithiothreitol (Laemmli, 1970). Iodoacetamide to 10 mM was subsequently added to the samples before electrophoresis. For pepsin digestion experiments, proteins were initially released into 50 mM-Tris/HCl buffer, pH 7.5, containing 6 M-urea, before extensive dialysis against 0.5 M-acetic acid and overnight digestion with pepsin (100 μg/ml) at 4 °C. Type VI collagen assemblies remain stable in these conditions as a result of their highly disulphide-bonded nature (Engvall *et al.*, 1986).

Tissue extraction of intact type VI collagen

Equal wet weights of 140-day foetal bovine nuchal ligament and skin were initially extracted into Laemmli sample buffer containing 2 M-urea and 50 mM-dithiothreitol. Extracts were centrifuged for 5 min at 14000 *g*, and the supernatants and insoluble residues were retained for electrophoretic analysis. Tissues were also homogenized into 4 M-guanidinium isothiocyanate containing 0.1 M-2-mercaptoethanol, and the homogenates were subjected to centrifugation through CsCl at a density of 1.0 g/ml. The solubilized protein fractions recovered from the top of the gradient were dialysed extensively against distilled water before freeze-drying and subsequent analyses.

Electrophoretic analysis

Labelled immunoprecipitated polypeptides from culture medium and cell layers, and tissue-extracted proteins, were analysed by discontinuous SDS/6.5%-PAGE and fluorography (Bonner & Laskey, 1974; Laskey & Mills, 1975), staining with Coomassie Brilliant Blue or the periodic acid/Schiff reaction, or Western blotting (Towbin & Martin, 1982), as appropriate. M_r values were determined by reference to both collagenous and non-

collagenous standards, and these included types I, II and V collagens, myosin (M_r 200000), phosphorylase *b* (M_r 95000), BSA (M_r 69000), ovalbumin (M_r 46000) and carbonic anhydrase (M_r 30000). Fluorograms were scanned by a laser densitometer (LKB 2202 Ultroscan).

Isolation of RNA and Northern-blot and slot-blot hybridization

Total RNA was prepared from cultured cells and tissues by extraction in 4 M-guanidinium isothiocyanate followed by centrifugation over a cushion of 5.7 M-CsCl for 20 h (Chirgwin *et al.*, 1983). The resulting pellets were dissolved in 10 mM-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA, extracted in phenol/chloroform (1:1, w/v) and after precipitation with ethanol dissolved in water. The amount of total RNA in each sample was determined spectrophotometrically at 260 nm. Polyadenylated RNA was isolated by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972). For Northern-blot analysis, RNA was electrophoresed in 1% agarose gels containing 2.2 M-formaldehyde, transferred to nitrocellulose filters and hybridized with cDNAs oligolabelled with [α-^{32}P]dCTP. For slot-blot hybridizations, total RNA was loaded on to nitrocellulose filters in various concentrations and the filters were hybridized with radioactive cDNAs utilizing the same conditions employed for the Northern-blot analysis, followed by fluorography at −80 °C in the presence of intensifying screens. The amounts of mRNA were then quantified by scanning densitometry.

RESULTS

Isolation of intact native type VI collagen from foetal bovine fibroblast cultures

Primary cultures of nuchal-ligament fibroblasts and skin fibroblasts were established from 140-day bovine foetuses, and their morphological and growth characteristics indicated that they were expressing their normal behavioural patterns in culture. The cells were labelled with [^{35}S]methionine in the presence of ascorbate upon reaching confluence, and the proteins synthesized and secreted by the two cell types under these conditions were analysed by SDS/PAGE and fluorography. The newly synthesized proteins secreted into the medium were initially fractionated with $(NH_4)_2SO_4$ at 30% saturation, a procedure that specifically precipitates native collagens, their precursors and other high-M_r proteins (Fig. 1*a*). The cell layers were extracted with 8 M-urea at neutral pH. Major species present were fibronectin and types I and III collagens, as previously described by Ayad *et al.* (1986). The presence of type VI collagen was not, however, readily detected, and further specific fractionation by immunoprecipitation was necessary in order to characterize the type VI collagen both secreted into the medium and laid down in the cell layer.

The glycoprotein fibronectin not only represents a major biosynthetic product of these cultures, but is of similar M_r and electrophoretic mobility to the α3(VI) component. Consequently, to simplify the characterization of the type VI collagen syn-

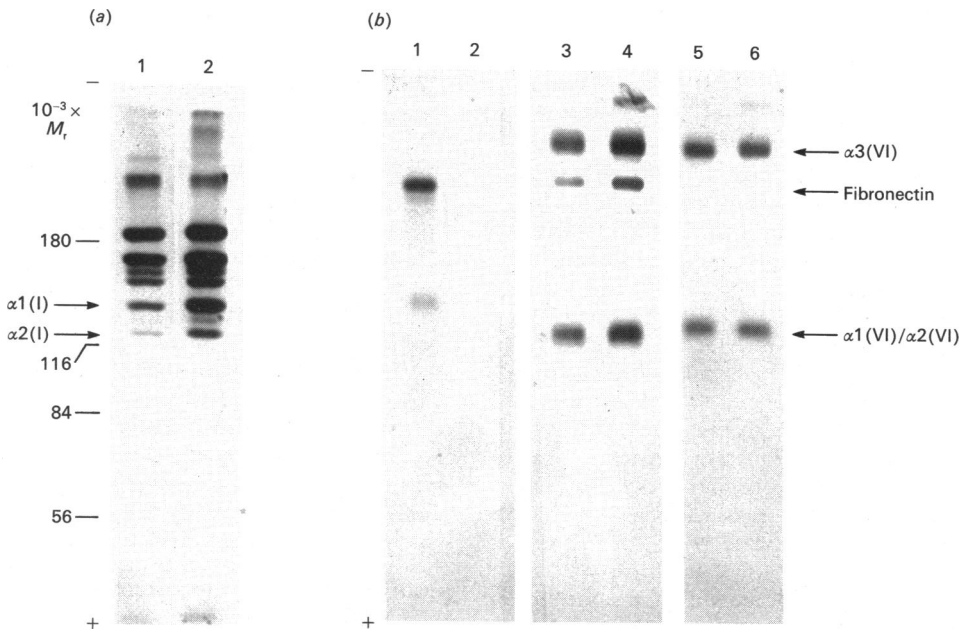

Fig. 1. Fluorograms of newly synthesized [^{35}S]methionine-labelled proteins secreted into the medium by confluent cultures of nuchal-ligament fibroblasts and skin fibroblasts

(*a*) Cells at confluence were labelled for 24 h with [^{35}S]methionine, and the labelled medium proteins were concentrated by precipitation with $(NH_4)_2SO_4$ at 30% saturation. Precipitates were separated by SDS/PAGE on 6.5% (w/v) gels under reducing conditions, and the bands were detected by fluorography. Track 1, nuchal-ligament fibroblasts; track 2, skin fibroblasts. (*b*) The 0–30%-saturation-$(NH_4)_2SO_4$ medium fractions were sequentially immunoprecipitated with polyclonal and monoclonal antisera to fibronectin followed by either of two anti-(type VI collagen) sera as described in the Experimental section. Tracks 1–4 represent immunoprecipitates from nuchal-ligament fibroblast culture medium, and tracks 5 and 6 represent immunoprecipitates from skin fibroblast culture medium. Tracks 1 and 2, first and second fibronectin immunoprecipitates; tracks 3 and 5, type VI collagen immunoprecipitated with antiserum to bovine type VI collagen; tracks 4 and 6, type VI collagen immunoprecipitated with antiserum to pig type VI collagen serum. The migration positions of the α-chains of collagens I and VI and of the non-collagenous proteins of known M_r are indicated.

thesized in culture, attempts were made to remove this species from the various fractions by two sequential immunoprecipitation steps utilizing a polyclonal antiserum and a monoclonal antiserum. All of these immunoprecipitates contained a major fibronectin component of M_r 250000, and a lower-M_r species (170000) was also occasionally observed (Fig. 1*b*, tracks 1 and 2). In most cases, the second precipitation brought down only traces of fibronectin. Intact type VI collagen was then immunoprecipitated by either of two well-characterized polyclonal antisera to type VI collagen. The collagen VI immunoprecipitates, when analysed under reducing conditions, were shown to comprise a band of 140000 [the α1(VI) and α2(VI) chains, which comigrate] and several closely spaced bands with an M_r range of 260000–280000 [corresponding to the α3(VI) variants]. These results demonstrate that newly synthesized type VI collagen molecules are present in the culture medium at confluence, and that these species can be isolated in the intact form by immunoprecipitation (Fig. 1*b*). Traces of fibronectin were also detected in some of these immunoprecipitates (particularly in those of nuchal ligament) despite rigorous preceding steps for its removal, which is suggestive of a pool of fibronectin in direct association with collagen VI. Collagen VI molecules can also be demonstrated in the cell-layer extracts by using similar methodology (results not shown). Densitometric scanning of gels loaded with total immunoprecipitable radioactive material showed 50–60% of the newly synthesized type VI collagen to be present in the cell layer, and most of this could only be extracted with urea. There was no evidence of processing of the type VI collagen α-chains, which were of identical mobility in medium and cell-layer fractions. Analysis of the proportions of radioactivity recovered in the immunoprecipitates indicated that in the nuchal-ligament fibroblast cultures 4–5% of the newly synthesized protein occurred as type VI collagen, whereas in the skin fibroblast cultures the value was approx. 2%.

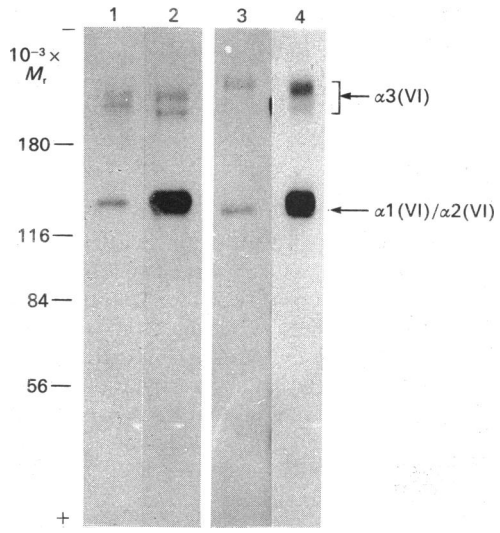

Fig. 2. Fluorograms comparing the [^{35}S]methionine-labelled type VI collagen immunoprecipitated from the culture medium of nuchal ligament fibroblasts and skin fibroblasts at confluence and at 10 days post-confluence

Cells were labelled for 24 h with [^{35}S]methionine, and the 0–30%-saturation-$(NH_4)_2SO_4$ fractions were immunoprecipitated with anti-(pig type VI collagen) serum before analysis by SDS/PAGE on 6.5% (w/v) gels under reducing conditions. Tracks 1 and 3 contain immunoprecipitates from the medium of confluent cultures, and tracks 2 and 4 contain immunoprecipitates from the medium of 10-day post-confluent cultures. Tracks 1 and 2, nuchal-ligament fibroblasts; tracks 3 and 4, skin fibroblasts. The migration positions of the α-chains of type VI collagen and of the non-collagenous proteins of known M_r are indicated.

Comparison of the chain composition of the type VI collagen immunoprecipitated from confluent and post-confluent cultures

In preliminary experiments where type VI collagen synthesis was studied in cells at confluence and at 8–10 days post-confluence, there was not only a marked increase in the levels of newly synthesized collagen VI in the post-confluent cultures (up to 20% as judged by immunoprecipitable radioactive material), but also pronounced variations in the collagen VI chain composition in these cultures. A further detailed study was then undertaken. Scanning of fluorograms revealed that the fractions obtained from both nuchal-ligament and skin fibroblast cultures at confluency contained type VI collagen assemblies that exhibited an $(\alpha1+\alpha2)/\alpha3$ chain ratio approaching 1:1 (Fig. 2), which on the basis of the known methionine contents of collagen VI chains is consistent with chain proportions 1:1:1. However, at 10 days post-confluence, significant deviations from these chain proportions could be demonstrated (Fig. 2). The post-confluent cultures showed evidence of a sharp increase in the concentration of the $(\alpha1+\alpha2)$ band present in the medium, where ratios of between 3:1 and 4:1 were recorded. In some experiments the skin fibroblast culture medium exhibited even higher chain ratios of up to 7:1. These increases were not apparent in the urea extracts of the cell layers (results not shown), where chain ratios similar to those obtained in the confluent cultures were recorded. Thus the type VI collagen secreted into the medium of the post-confluent cultures is not representative of the total type VI collagen synthesized by these cells.

The chain composition of the radiolabelled native triple-helical type VI collagen molecules secreted into the medium of confluent and post-confluent cultures was also assessed by pepsin digestion of immunoprecipitates and subsequent analysis of the resistant domains by SDS/PAGE and fluorography. Since these domains of the individual chains of collagen VI are electrophoretically separable, this approach provided an opportunity to confirm the elevation in the concentration of α1(VI) and α2(VI) chains in the post-confluent cultures, as well as to obtain information on their relative abundance. Fig. 3 demonstrates that in the medium of the post-confluent cultures there was a marked increase in the intensity of the α1(VI) and α2(VI) chains concomitant with an apparent decrease in concentration of the α3(VI) chain. The relative abundance of these polypeptide chains synthesized by the fibroblasts was assessed by densitometric analysis of fluorograms (Table 1 part *a*). The $\alpha1/\alpha2$ ratio remained constant at confluence and post-confluence in all cases. Both of the cell types responded to conditions of diminished proliferation by expressing $(\alpha1+\alpha2)/\alpha3$ ratios elevated above those observed upon reaching confluency.

Abundance and chain composition of intact type VI collagen extracted from foetal bovine tissues

Bovine nuchal-ligament and skin samples from 140-day foetuses were extracted by using procedures capable of effecting the virtually complete solubilization of the extracellular matrix. This methodology was adopted because it facilitates an evaluation both of the chain composition and of the relative abundance of the type VI collagen present in these tissues. In some experiments tissues were homogenized directly into Laemmli sample buffer containing 2 M-urea under reducing conditions and solubilized proteins were analysed by SDS/PAGE in combination with staining or Western blotting. In most experiments, however, we utilized a procedure originally designed for the

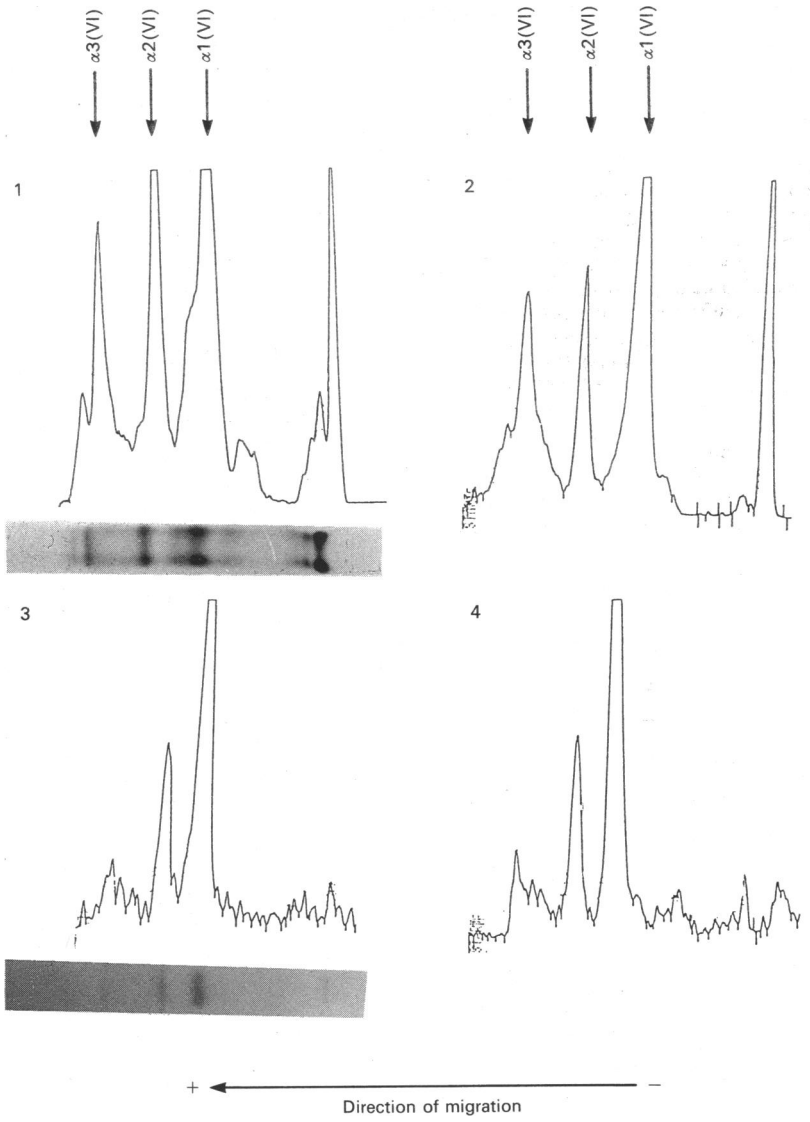

Fig. 3. Electrophoresis of pepsin-digested [^{35}S]methionine-labelled type VI collagen immunoprecipitated from the culture medium of confluent and post-confluent nuchal-ligament fibroblasts and skin fibroblasts

Type VI collagen immunoprecipitated from the medium of confluent and 10-day post-confluent cultures of nuchal-ligament fibroblasts and skin fibroblasts was subjected to digestion before analysis by SDS/PAGE as described in the Experimental section. Fluorograms were scanned with an LKB laser densitometer at 632.8 nm. Scans 1 and 2 depict pepsinized collagen VI from confluent cultures, and scans 3 and 4 depict pepsinized collagen VI from 10-day post-confluent cultures. Scans 1 and 3, nuchal-ligament fibroblasts; scans 2 and 4, skin fibroblasts.

preparation of undegraded RNA but that also provides a total protein fraction from each tissue for further specific analyses. This method utilizes the potent protein denaturant guanidinium isothiocyanate in the presence of 2-mercaptoethanol (Chirgwin *et al.*, 1983).

The Laemmli sample buffer extracts and the guanidinium isothiocyanate extracts of each tissue exhibited broadly similar total protein profiles with only minor variations. Type I fibrillar collagen (α and β) chains could be identified in all the extracts, especially in those of skin (Fig. 4*a*). Specific information on the abundance and chain composition of the collagen VI solubilized in the tissue extracts was obtained after analysis by SDS/PAGE in combination with Western blotting with two anti-(type VI collagen) sera with distinct specificities for the α3(VI) chain (Ayad *et al.*, 1989) and the α1(VI) and α2(VI) chains (King *et al.*, 1989) respectively. The extracts were shown to contain solubilized collagen VI (Fig. 4*b*). However, remarkable variations were apparent in its chain composition and relative abundance in the two tissues examined. In the nuchal-ligament extracts, immunostaining with the anti-α3(VI) serum revealed a heterogeneous component comprising several closely spaced bands in the M_r range of 260000–280000. When the anti-[α1(VI)+α2(VI)] serum was used as probe, a single broad band with an M_r of 140000–150000 was detected. These extracts exhibited (α1+α2)/α3 chain ratios of 2:1 by scanning of Western blots. In contrast, the skin extracts apparently contained an abundance of the (α1+α2) component but only traces of the α3(VI) component, and in all these extracts chain ratios of above 4:1 were recorded. The identity of the collagen VI bands was further corroborated by their susceptibility to bacterial-collagenase digestion under reducing conditions and glycoprotein staining by the periodic acid/Schiff reaction (results not shown). Solubilization of the tissues was virtually complete, and Western blotting failed to detect any collagen VI in the residues. Type VI collagen was

Table 1. Densitometric analysis of type VI collagen synthesized by confluent and post-confluent cultures of nuchal-ligament fibroblasts and skin fibroblasts

The results of a typical experiment are shown, and they represent consistent observations. (*a*) Protein values were obtained by densitometric scanning of fluorograms. Arbitrary densitometric units corresponding to the relative abundance of the three type VI collagen chains in each experiment are represented as proportions, with the α1(VI) value in each case set to 1. All protein values were adjusted to take account of the methionine content of the triple-helical sequences of each chain (Chu *et al.*, 1988). (*b*) mRNA values were obtained by densitometric scanning of slot-blot hybridizations. Arbitrary densitometric units corresponding to the relative abundance of the three type VI collagen mRNAs are represented as proportions, with the α1(VI) mRNA value in each case set to 1.

	Confluent	Post-confluent
	α1 : α2 : α3	α1 : α2 : α3
(*a*) Collagen protein		
Nuchal ligament	1 : 1.09 : 1.21	1 : 1.13 : 0.24
Skin	1 : 1.14 : 1.24	1 : 1.15 : 0.12
(*b*) Collagen mRNA		
Nuchal ligament	1 : 1.05 : 0.94	1 : 1.10 : 0.69
Skin	1 : 0.97 : 0.92	1 : 1.07 : 0.63

present in much greater abundance (up to 4-fold) in the nuchal-ligament extracts than in the corresponding skin extracts.

Expression of type VI collagen mRNAs in fibroblast cultures and in corresponding tissues

Steady-state concentrations of the mRNA species encoding the three individual chains of type VI collagen were determined by Northern-blot and slot-blot hybridization of specific human cDNA probes to total RNA extracted from cell layers of the various bovine fibroblast cultures, both at confluence and post-confluence, and from the corresponding tissues. RNA extracted from human and bovine placenta and from human skin fibroblasts was included as controls to establish the specificity of each of the probes (Fig. 5). The human RNA exhibited hybridization signals corresponding to the known pattern of type VI collagen mRNAs, with bands at 4.2 kb for α1(VI), 3.5 kb for α2(VI) and 8.5 kb for α3(VI) (Chu *et al.*, 1988). The α1(VI) and α2(VI) cDNA probes were of comparable size and specific radioactivity, and the relative abundance of these mRNAs could therefore be determined directly by scanning densitometry. The α3(VI) cDNA probe was significantly smaller at 1.2 kb, and the lower specific radioactivity associated with this probe was distributed among the three or more closely spaced mRNA species known to code for this chain (Chu *et al.*, 1987). Thus the hybridization signal corresponding to the α3(VI) mRNA was more difficult to detect than those of the α1(VI) and α2(VI) mRNAs. However, by using a combination of Northern-blot and slot-blot hybridization analyses, the relative abundance of the three type VI collagen mRNA species present in the confluent and post-confluent cultures could be determined (Fig. 6*a* and Table 1 part *b*). Highest steady-state concentrations of α1(VI) and α2(VI) mRNAs were recorded in the nuchal-ligament fibroblast cultures at both confluence and post-confluence. Approximately equimolar amounts of the three mRNA species were exhibited by nuchal-ligament and skin fibroblast cultures at confluence, but by 10 days post-confluence a 1.4–1.6-fold increase in the concentrations of both the α1(VI) and α2(VI) mRNAs was apparent in both cell types. The abundance of the α3(VI) mRNA in these

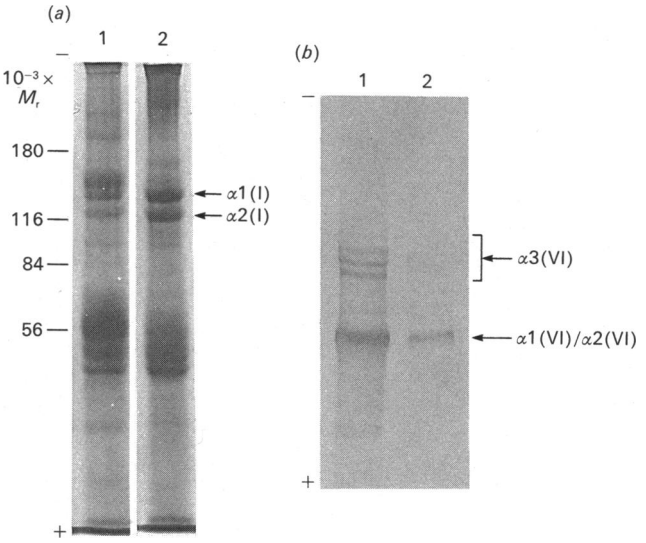

Fig. 4. Electrophoretic analysis of the intact type VI collagen present in foetal bovine nuchal ligament and skin

Tissues from 140-day bovine foetuses were extracted in guanidinium isothiocyanate in the presence of 2-mercaptoethanol before analysis by SDS/PAGE on 6.5% (w/v) gels under reducing conditions. Extracted proteins were detected by staining with Coomassie Brilliant Blue, and intact type VI collagen was identified by Western blotting with a combination of antisera to both bovine and pig type VI collagen, which together recognize all three α-chains of collagen VI. (*a*) Total protein extracts of 140-day foetal nuchal ligament (track 1) and skin (track 2). (*b*) Western blots of equal loadings of total extracted protein from 140-day foetal tissues, showing the chain composition and relative abundance of the collagen VI present in nuchal ligament (track 1) and skin (track 2). The migration positions of the α-chains of types I and VI collagens and of the non-collagenous proteins of known M_r are indicated.

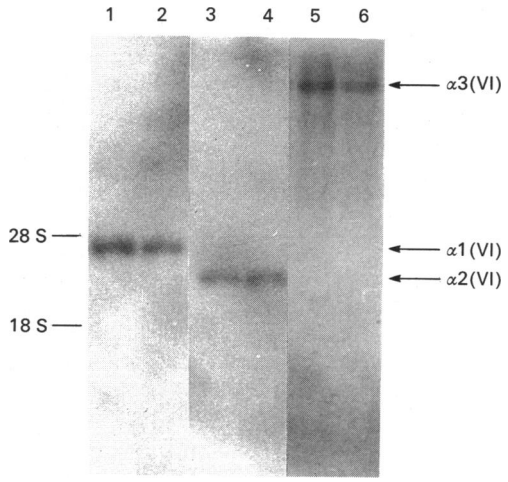

Fig. 5. Northern-blot analysis of human and bovine mRNA

mRNA (5μg) was electrophoresed in a 1%-formaldehyde-containing agarose gel. The mRNA was transferred to nitrocellulose filters, and parallel lanes were hybridized with α1(VI), α2(VI) and α3(VI) cDNA probes respectively as described in the text. The positions of 28 S and 18 S rRNA are indicated as size markers. Tracks 1, 3 and 5 were loaded with human placental mRNA. Tracks 2, 4 and 6 were loaded with bovine placental mRNA. Tracks 1 and 2, α1(VI) mRNA; tracks 3 and 4, α2(VI) mRNA; tracks 5 and 6, α3(VI) mRNA.

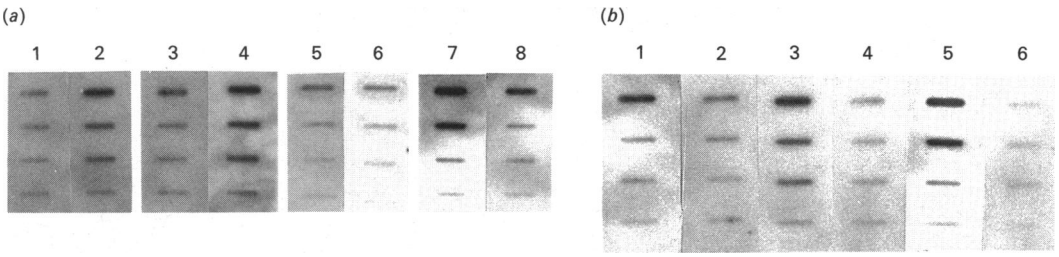

Fig. 6. Examples of slot-blot quantification of total RNA obtained from foetal bovine cultured cells at confluence and at post-confluence, and from foetal tissues

Various amounts of total RNA (1.25–10 μg) were loaded on to nitrocellulose filters under vacuum, and hybridized with α1(VI), α2(VI), α3(VI) or fibronectin cDNA probes. (*a*) Slot-blot analysis of total RNA from nuchal-ligament fibroblast cultures at confluence and at post-confluence. Tracks 1, 3, 5 and 7 contain RNA from confluent cultures, and tracks 2, 4, 6 and 8 contain RNA from post-confluent cultures. Tracks 1 and 2, α1(VI) mRNA; tracks 3 and 4, α2(VI) mRNA; tracks 5 and 6, α3(VI) mRNA; tracks 7 and 8, fibronectin mRNA. (*b*) Slot-blot analysis of total RNA extracted from foetal bovine nuchal ligament (tracks 1–3) and skin (tracks 4–6). Tracks 1 and 4 were hybridized with α1(VI) cDNA, tracks 2 and 5 with α2(VI) cDNA, and tracks 3 and 6 with α3(VI) cDNA.

Table 2. Densitometric analysis of tissue type VI collagen mRNA concentrations in nuchal ligament and skin of 140 bovine foetuses

Values are averages obtained from triplicate slot-blot hybridization experiments analysing the RNA extracted from three separate foetuses. Arbitrary densitometric units corresponding to the relative abundance of the three type VI collagen mRNAs are represented as proportions, with the α1(VI) mRNA value in each case set to 1.

	Tissue collagen mRNAs
	α1(VI) : α2(VI) : α3(VI)
Nuchal ligament	1 : 1.08 : 1.17
Skin	1 : 1.13 : 0.49

fibroblasts was unaffected by the proliferative state of the cells. Fibronectin mRNA was also measured and shown to be present in diminished abundance in the post-confluent cultures.

Extracts of nuchal ligament and skin contained equimolar ratios of the α1(VI) and α2(VI) mRNA species (Table 2). In the case of nuchal ligament, all three mRNAs encoding collagen VI were present at similar concentrations, and this pattern was comparable with the equimolar proportions demonstrated in the corresponding confluent cultures. In skin, however, the α1(VI) and α2(VI) mRNAs were present in greater abundance than the α3(VI) mRNA, a similar situation to that existing in the post-confluent skin fibroblast cultures. Total concentrations of type VI collagen mRNAs were 40% higher in the nuchal ligament than in the skin (Fig. 6*b* and Table 2). The relative abundance of the collagen VI mRNAs extracted from tissues was generally lower than that present in the corresponding cell cultures.

DISCUSSION

The essential contribution that type VI collagen microfibrils make to the structure and integrity of connective tissues has only recently begun to emerge. A detailed structural and functional characterization of type VI collagen has historically proved difficult to establish, since extraction of this glycoprotein from tissues generally requires highly dissociative conditions (Trueb *et al.*, 1987). However, the elucidation of the primary sequence of human and chick collagen VI from cDNA nucleotide sequence data has led to major advances in our understanding of the biological role of this species (Chu *et al.*, 1988, 1989, 1990; Bonaldo & Colombatti, 1989; Koller *et al.*, 1989; Trueb *et al.*, 1989). Type VI collagen has been revealed as a mosaic structure with a potentially multifunctional character, capable of providing not only mechanical support through its collagenous sequences but also an additional range of functions, the most significant among which appear to be cell adhesion and collagen binding. These functions are consistent with its proposed central role as a flexible link between cells and matrix. Each constituent chain of collagen VI contains putative functional domains that contribute to the overall function of the molecule. Significant motifs include 11 Arg-Gly-Asp sequences within the triple-helical domain, at least some of which appear to be involved in cell adhesion (Aumailley *et al.*, 1989; Bonaldo *et al.*, 1990), and 17 repetitive domains within the globular regions of the three chains that exhibit extensive sequence similarity to the A domains of von Willebrand factor. Numerous cysteine residues are distributed within the globular sequences, but only a single cysteine residue is present in the helical domain of each chain. Interruptions in the Gly–Xaa–Yaa sequences can be aligned to accommodate the predicted supercoiling between anti-parallel triple-helical domains in the formation of dimers (Chu *et al.*, 1988). The α3(VI) chain is the largest collagen chain so far sequenced, with an M_r of 340000, which is substantially higher than that estimated from electrophoretic analyses (Bonaldo & Colombatti, 1989; Chu *et al.*, 1990). A number of functional characteristics have been ascribed to the *C*-terminal domain of this chain, but their significance *in vivo* is unclear because recent data suggest that processing of a substantial portion of this domain may occur (Chu *et al.*, 1990). In addition, spliced α3(VI) chain variants have been demonstrated in a number of human cDNA clones (Chu *et al.*, 1990), and may account for the heterogeneity of the α3(VI) chain previously observed at the protein level (Engvall *et al.*, 1986; Colombatti *et al.*, 1989). In order to establish the biological role(s) of the assembled collagen VI microfibrillar network *in vivo*, it is necessary to establish the precise chain composition and molecular assembly of the monomers from which these microfibrils are assembled. We initially adopted a biosynthetic approach to analyse this aspect of the structure of type VI collagen during early foetal development, with particular emphasis on the phenotypic changes induced by altering the proliferative state of the cells. The scope of the study was subsequently broadened to include a parallel analysis of the chain composition of the collagen VI present in foetal tissues.

We have been able to demonstrate by immunoprecipitating intact native type VI collagen aggregates from culture medium

and cell-layer extracts that foetal bovine fibroblasts grown to confluency synthesize, assemble and secrete type VI collagen molecules apparently containing the three constituent α-chains [α1(VI)α2(VI)α3(VI)]. However, significant deviations from these heterotrimeric chain proportions can be induced by maintenance of these cells in a post-confluent state of diminished proliferation, for the increased amounts of type VI collagen immunoprecipitable from the culture medium under these conditions were found to comprise predominantly α1(VI) and α2(VI) chain assemblies. This modification was particularly pronounced in the skin fibroblast cultures, where assemblies apparently composed of α1(VI) and α2(VI) chains predominated and (α1+α2)/α3 ratios of as high as 7:1 were recorded (Fig. 2 and Table 1). Such assemblies were not representative of the total type VI collagen synthesized by these post-confluent cultures, since all the cell layers contained type VI collagen molecules with a chain composition of [α1(VI)α2(VI)α3(VI)]. This observation implicates the α3(VI) chain as a rate-limiting component in the laying down of stable type VI collagen microfibrils in the cell layer, as has been previously suggested (Hatamochi *et al.*, 1989). Confirmation of the unique chain composition and triple-helical conformation of the intact molecules secreted into the medium at post-confluence was provided by pepsinization of immunoprecipitated native type VI collagen. Since data on the methionine content of the triple-helical domain of bovine type VI collagen are not available, it was not possible to calculate the exact proportions of chains. However, assuming that the bovine chains have the same methionine content as the human sequences (Chu *et al.*, 1988, 1989, 1990), then it would appear that the α1(VI) and α2(VI) chains are present in approximately equimolar amounts in these cultures. It is not clear what chain composition the molecular assemblies lacking the α3(VI) chain may be able to assume, but possibilities include $[\alpha1(VI)]_2\alpha2(VI)$, $\alpha1(VI)[\alpha2(VI)]_2$ or homotrimers. When the chain compositions of intact collagen VI from extracts of foetal nuchal ligament were analysed, the [α1(VI)+α2(VI)]/α3(VI) chain ratios obtained were directly comparable with those synthesized by the corresponding confluent cultures, suggesting that in these tissues a heterotrimeric chain assembly predominates. The skin extracts, however, contained an abundance of the [α1(VI)+α2(VI)] component (Fig. 4), which closely reflects the response of the skin fibroblasts to conditions of diminished proliferation. Type VI collagen was particularly abundant both in the foetal nuchal ligament and in the corresponding fibroblast cultures, suggesting that the collagen VI microfibrillar network plays a crucial role in the development of this highly elastic tissue.

Analysis of the steady-state concentrations of mRNAs for the three α-chains of type VI collagen in cultures at confluence and post-confluence revealed variations both in proportions and in relative abundance that were consistent with those observed at the protein level. A comparison of these data with tissue collagen VI mRNA concentrations indicates that the chain composition of the collagen VI synthesized by the confluent nuchal-ligament fibroblasts accurately reflects that in the corresponding foetal tissue. However, the elevated [α1(VI)+α2(VI)]/α3(VI) mRNA ratio detected in foetal skin (up to 4:1) reflects that observed in the post-confluent skin fibroblast cultures, suggesting that the biosynthetic status of these cells more closely corresponds to that existing *in vivo*. The expression of α1(VI) and α2(VI) chains appeared to be co-ordinately regulated in all the cells by similar mechanisms under these culture conditions. In contrast, there was clear evidence for independent regulatory mechanisms controlling the expression of the α3(VI) chain. These combined protein and mRNA data showing that changes in the concentrations of the mRNAs for the type VI collagen chains may be transmitted to the protein level strongly suggest that the primary controls over type VI collagen gene expression occur at the level of transcription, although differential mRNA stabilities cannot be precluded.

As with all collagens, the expression of type VI collagen is likely to be under the regulation of a complexity of mechanisms responding both to the developmental programme of the organism and to external factors. Such regulation is particularly in evidence at the transcription level, with a number of recorded examples both of co-ordinated regulation of the three α-chains (Chu *et al.*, 1987) as well as un-co-ordinated regulation of the α3 chain relative to the α1 and α2 chains (Hatamochi *et al.*, 1989; Heckmann *et al.*, 1989), which, it is shown here both in cell cultures and tissues, can be transmitted to the protein level. These data indicate that the type VI collagen microfibrillar system is particularly sensitive to modulation at the levels both of the total amount of collagen synthesized and of its chain composition. We now have evidence not only for developmental alterations in the chain composition of collagen VI in skin, with the elevated α1(VI) and α2(VI) concentrations of early gestation giving way to chain ratios consistent with a heterotrimeric assembly during the third trimester of gestation, but also for tissue-specific variations in the heterogeneous α3(VI) component (C. M. Kielty, unpublished work). It can be envisaged that alterations in the chain composition of collagen VI in response to developmental or disease-related changes may represent an elegant mechanism for adjusting the function of the collagen VI microfibrils of connective tissues. Thus, although the highly stable heterotrimer-based microfibrillar network undoubtedly predominates in most adult connective tissues (Jander *et al.*, 1983; Ayad *et al.*, 1984, 1989; Trueb *et al.*, 1987), alternative disulphide-bonded type VI collagen assemblies composed of α1(VI) and α2(VI) chains apparently occur in some developing tissues such as skin. The particular biological function of these assemblies is unknown, but any collagen-binding capacity might be expected to be of reduced significance since they lack the major contribution that the α3(VI) chain makes in this respect. Since these structures may be more easily degraded than heterotrimeric structures, an involvement in the development of certain pathological disorders as well as in some inherited diseases of connective tissues remains a possibility. The tissue-specific expression of the α3(VI) chain variants may represent a further level of functional fine tuning to this versatile microfibrillar network.

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