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Synthesis and assembly of fibrillin by fibroblasts and smooth muscle cells

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SUMMARY

The expression and assembly of the microfibrillar glycoprotein fibrillin has been investigated in cultures of nuchal ligament fibroblasts, skin fibroblasts and vascular smooth muscle cells. The level of fibrillin expression varied with the cell type and growth conditions. Higher levels of synthesis were recorded in quiescent post-confluent cells than in actively dividing subconfluent cultures. Nuchal ligament fibroblasts consistently synthesized the highest levels of fibrillin. Growth of cells in the presence of ascorbate resulted in an increased proportion of newly synthesized fibrillin retained within cell layers. Fibrillin was immunoprecipitated from medium and cell layer extracts in the form of monomers and high-M_r disulphide-bonded aggregates. Rotary shadowing electron microscopy of cell layer extracts and collagen gels provided direct evidence for the assembly of extensive intact microfibrils by smooth muscle cells and fibroblast cultures. Gel filtration chromatography of medium and cell layer extracts, in combination with immunoprecipitation of column fractions, provided a means of analysing the size distribution and assembly of newly synthesized fibrillin. This cell culture approach provides an opportunity to evaluate normal and aberrant synthesis and assembly of fibrillin in a wide range of cell types.

Key words: fibrillin synthesis, microfibril assembly

INTRODUCTION

The fibrillin-containing microfibrils are a class of connective tissue microfibrils with a widespread tissue distribution but whose composition, organization and molecular interactions remain cryptic. These microfibrils are particularly abundant in elastic tissues such as aorta, ligament and skin where they form integral components of the elastic fibres that generate elastic recoil (Cleary and Gibson, 1983; Mecham and Heuser, 1991). Ultrastructurally indistinguishable microfibrils are also present in many non-elastic tissues such as the ocular zonule, tendon and bone (Fleischmajer et al., 1991; Keene et al., 1991a,b; Wallace et al., 1991). These complex microfibrillar assemblies apparently play a central role in the process of elastic fibre formation (Mecham and Heusar, 1991), and they have also been shown to support cell adhesion in vitro (Kielty et al., 1992). However, their biological role in non-elastic tissues remains largely obscure. The recently described glycoprotein, fibrillin, has been identified as a major structural component of these microfibrils (Sakai et al., 1986, 1991). The importance of these microfibrillar assemblies as determinants of connective tissue architecture and integrity was recently brought into close focus by the identification of mutations in fibrillin as the primary lesions in the heritable connective tissue disorder, Marfan syndrome, which is characterized by cardiovascular, skeletal and ocular abnormalities (Dietz et al., 1991; Lee et al., 1991; Peltonen and Kainulainen, 1992).

Fibrillin is a large non-collagenous glycoprotein, which has recently been partially cloned and sequenced (Lee et al., 1991; Maslen et al., 1991). It has a multi-domain structure characterized by multiple epidermal growth factor (EGF)-like motifs interspersed with 8-cysteine repeats with homology to the TGF-β1 binding protein. It has a particularly high cysteine content (14% of amino acids), and the formation of multiple intra- and intermolecular disulphide bonds apparently both determines the secondary structure of fibrillin monomers and contributes to stabilizing the assembled microfibrils. Molecular details of the process of microfibril assembly, however, have not been elucidated.

Rotary shadowing electron microscopic analyses have revealed that the fibrillin-containing microfibrils have a complex ultrastructure with a diameter of 10-14 nm, a distinctive beaded morphology and an average, but variable, periodicity of 50 nm (Wright and Mayne, 1988; Fleischmajer et al., 1991; Keene et al., 1991b; Kielty et al., 1991). In skin, these assemblies form a unique and extensive dermal network that stretches as a continuum from the periphery of elastic fibres to thin microfibril bundles close to the dermal-epithelial junction (Cotta-Periera et al., 1976; Smith et al., 1982; Dahlback et al., 1990). This distribution suggests a primary role for fibrillin microfibrils in anchoring the dermal elastic fibres in the extracellular matrix and

to the lamina densa, and correlates well with its proposed role in directing the deposition of elastin during elastic fibrillogenesis. In the vasculature the fibrillin-containing microfibrils form integral components of the concentric layers of elastic fibres within the medial layer and they also occur as discrete loose microfibril bundles devoid of elastin, in the subendothelium (Cleary and Gibson, 1983). The bovine nuchal ligament is a highly elastic tissue that contains abundant elastic fibres, and in this tissue the fibrillin-containing microfibrils are primarily found in association with elastin.

The composition and organization of the fibrillin-containing microfibrils remain poorly defined, in part because these structures have proved particularly difficult to extract as intact high-M_r aggregates from tissues. A cell culture approach has previously been used to highlight differences in fibrillin in Marfan cell lines (Milewitz et al., 1992). However, little is known about the molecular mechanisms involved in the processes of fibrillin secretion, processing, deposition and assembly. In this study, we have developed a cell culture system that provides an opportunity to define these processes in a range of normal cell types. Application of this methodology to cells derived from patients with connective tissue diseases associated with microfibrillar abnormalities will provide a means of correlating clinical phenotypes with mutations causing specific defects in fibrillin expression and organization.

MATERIALS AND METHODS

Materials

Foetal calves were obtained from the local abattoir within 1 h of maternal death. Bacterial collagenase (type 1A), phenylmethanesulphonyl fluoride (PMSF), *N*-ethylmaleimide (NEM), diaminobenzidine, dithiothreitol, anti-human fibronectin serum, Tween-20 and prestained molecular mass markers were obtained from the Sigma Chemical Company, Poole, Dorset, UK. Sepharose CL-2B, Protein A-Sepharose and an electrophoresis calibration kit containing high-M_r proteins were supplied by Pharmacia-LKB, Milton Keynes, Bucks, UK. Mica sheets were obtained from TAAB Laboratory Equipment, Ltd., Reading, Berks, UK. Tissue culture media and plastics were obtained from Gibco BRL, Paisley, Scotland, UK. The radioisotopes [^{14}C]amino acid hydrolysate and [^{35}S]cysteine were obtained from ICN Biomedicals Ltd., High Wycombe, Bucks, UK.

Cells and cell culture

Human smooth muscle cell (SMC) cultures were established by explant from the medial layers of aortae from neonatal victims of sudden infant death. Normal human skin fibroblast cultures were established by explant from skin biopsies. Bovine fibroblast and SMC cultures were established from second trimester foetuses using standard collagenase digestion and explant protocols, respectively. Cells from passages 2 to 7 were used in the experiments reported here. Fibroblasts were routinely maintained in minimum essential medium supplemented with 10% foetal calf serum, penicillin (400 units/ml), streptomycin (50 μg/ml) and glutamine (200 μg/ml). SMC were maintained in the same medium, except that it was supplemented with 20% foetal calf serum and included non-essential amino acids. Cells were either grown to confluency or maintained for six weeks in a post-confluent, quiescent state. In some experiments, cells were grown at high cell density in three-dimensional collagen gels for up to four weeks.

Cells were labelled for 18 h with [^{14}C]amino acid hydrolysate or [^{35}S]cysteine in medium containing 1% foetal calf serum. In some experiments, cells were pulse-labelled for 30 min, then chased with unlabelled medium for 1 h, 2 h, 6 h and 24 h. Medium was fractionated by addition of solid $(NH_4)_2SO_4$ to 30% saturation at 4°C in the presence of 5 mM *N*-ethyl maleimide (NEM), 2 mM phenylmethanesulphonyl fluoride (PMSF) and 10 mM EDTA. Cell layers were sequentially extracted in 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl, 0.005 M EDTA and 1% Nonidet P40 (NET buffer), and 0.05 M Tris-HCl, pH 7.4, containing 4.0 M $GuCl_2$. Collagen gels were incubated in 1 ml of 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl and 0.005 M $CaCl_2$ and subjected to digestion with 0.1 mg bacterial collagenase (type 1A), overnight at 4°C in the presence of 2 mM PMSF and 5 mM NEM. The digestions were terminated by addition of EDTA to a final concentration of 20 mM, and the soluble extracts clarified by centrifugation for 15 min on a bench microfuge at 7,500 ***g***.

Immunoprecipitation

Immunoprecipitations were carried out as described by Kielty et al. (1990). Briefly, 30% $(NH_4)_2SO_4$ precipitates of cell culture medium were taken up in 1 ml NET buffer. Cell layer NET buffer extracts and corresponding column fractions were used directly for immunoprecipitation. Cell layer $GuCl_2$ extracts and corresponding column fractions were dialysed extensively against 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl and 0.005 M EDTA, and Nonidet P40 was added prior to immunoprecipitation. In view of the similar electrophoretic mobilities on SDS-PAGE of fibronectin, the α3(VI) chain of type VI collagen and fibrillin, a sequential immunoprecipitation regime was followed in all cases. The immunoprecipitation of fibrillin was preceded by three initial anti-fibronectin and two anti-collagen VI immunoprecipitations in order to eliminate the possibility of non-specific immunoprecipitation of these molecules. A 1:100 dilution of antiserum was used in each immunoprecipitation step, and this was adsorbed out with 60 μl/ml of a 1:1 (v/v) solution of Protein A-Sepharose in NET buffer. The following antisera were used in these immunoprecipitations: a commercially available polyclonal antiserum to human fibronectin, two polyclonal antisera to type VI collagen that recognize specifically the α3(VI) chain (Ayad et al., 1989) and the α1(VI)/α2(VI) component (Kielty et al., 1991), respectively, and a polyclonal antiserum raised in rabbits to fibrillin (Kielty et al., 1992; Shuttleworth et al., 1992; Waggett et al., 1993).

Size fractionation of medium and cell layer macromolecules

Unconcentrated medium, ammonium sulphate precipitates or cell layer NET buffer extracts from 18 h labelling experiments and pulse-chases were chromatographed directly without concentration under non-reducing, non-denaturing conditions on an analytical gel filtration column (1.5 cm × 25 cm) of Sepharose CL-2B. The column was equilibrated and eluted at room temperature with 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl. The cell layer $GuCl_2$ extracts were chromatographed under non-reducing conditions on a Sepharose CL-2B column of similar dimensions equilibrated in 0.05 M Tris-HCl, pH 7.4, containing 4.0 M $GuCl_2$. In each run, 60 × 1 ml fractions were collected. Column runs were constantly monitored at 280 nm and the incorporated cpm present in each fraction determined from a 50 μl aliquot of each fraction.

The size-distribution of total (labelled and unlabelled) fractionated fibrillin present in each medium or cell layer extract was determined by binding an aliquot (100 μl) of each fraction onto nitrocellulose membrane using a vacuum slot-blot apparatus, followed by western blotting detection of fibrillin (Kielty et al., 1990). The elution position of newly synthesized metabolically

labelled fibrillin was determined in each case by immunoprecipitating fibrillin from appropriately pooled fractions, followed by SDS-PAGE and fluorography.

Electrophoresis

Immunoprecipitates were analysed by discontinuous SDS-PAGE on 6% or 8% gels (Laemmli, 1970) in the presence or absence of 10 mM dithiothreitol, and by fluorography. Relative molecular masses were determined by reference to non-collagenous standards, which were fumarase (M_r 48,500), pyruvate kinase (M_r 58,000), bovine serum albumin (M_r 67,000), fructose 6-phosphate (M_r 84,000), β-galactosidase (M_r 116,000), lactate dehydrogenase (M_r 140,000), α2-macroglobulin (M_r 180,000), catalase (M_r 232,000), ferritin (M_r 440,000) and thyroglobulin (M_r 669,000).

Rotary shadowing electron microscopy

Cell layer extracts from post-confluent cultures and material solubilized from collagen gels were visualized for their microfibrillar content by rotary shadowing electron microscopy using a modification of the mica sandwich technique (Kielty et al., 1991; Waggett et al., 1993). Briefly, extracts equilibrated either in NET buffer or in 0.05 M Tris-HCl, pH 7.4, containing 4.0 M $GuCl_2$ were dialysed into 0.2 M ammonium acetate, pH 6.0, to a concentration of approximately 100 μg/ml. In some experiments, void volume material from extracts fractionated on Sepharose CL-2B was used directly. Aliquots (5 μl) were sandwiched between two sheets of mica, and allowed to adsorb for 5 min. The mica sandwiches were washed in 0.2 M ammonium acetate and then plunged into liquid nitrogen. The sandwiches were split open under nitrogen, dried in vacuo, rotary shadowed with platinum wire on a tungsten filament at an angle of 4°C and then coated with carbon. The carbon replicas were floated off onto distilled water, and picked up on uncoated 200 mesh copper grids. Specimens were examined in a JEOL 1200 EX electron microscope at 120 kV.

RESULTS

Synthesis and assembly of fibrillin in SMC and fibroblast cultures

The results presented in this paper were obtained from low-passage cultures of bovine nuchal ligament fibroblasts, skin fibroblasts and aortic SMC derived from second trimester foetuses, and human perinatal aortic SMC and skin fibroblasts. The synthesis and assembly of fibrillin by fibroblasts or SMC cultures grown to confluency or maintained for six weeks in a post-confluent, quiescent state, were compared biochemically and ultrastructurally. In some experiments, microfibril assembly by skin fibroblasts grown at high cell density for four weeks in three-dimensional collagen gels was assessed.

Distribution of counts incorporated into newly synthesized fibrillin

Metabolically labelled fibrillin was immunoprecipitated from the 30% $(NH_4)_2SO_4$ precipitates of culture medium and from sequential extracts of cell layers. Analysis of the distribution in medium and cell layer compartments of those counts specifically incorporated into fibrillin provided a means of comparing the expression and deposition of fibrillin by these cultures (Table 1). Nuchal ligament fibroblasts grown to confluency synthesized higher levels of fibrillin than the skin fibroblasts and SMC. The distribution of metabolically labelled fibrillin in medium and cell layers of confluent fibroblast and SMC cultures was, however, broadly comparable, with 46-59% secreted directly into the medium. Post-confluent fibroblasts and SMC synthesized higher levels of fibrillin than those recorded for the same cells grown to confluency. Maintenance of cells at post-confluence and growth of cells in the presence of ascorbate affected the distribution of newly synthesized fibrillin. Both culture conditions led to a marked increase in the proportion of metabolically labelled fibrillin within the cell layer compartments.

Secretion and deposition of newly synthesized fibrillin

Metabolically labelled fibrillin was immunoprecipitated

Table 1. Distribution of counts incorporated into fibrillin by cultured foetal bovine cells

	Medium		Cell layers: NP40		Cell layers: $GuCl_2$		Total fibrillin counts
Cell culture	cpm/l per 10^6 cells	%	cpm/l per 10^6 cells	%	cpm/l per 10^6 cells	%	cpm/l per 10^6 cells
Confluent							
SMC	3392	46	3245	44	736	10	7373
Nuchal ligament fibroblasts	8429	59	5286	37	571	4	14286
Skin fibroblasts	4182	46	4364	48	546	6	9092
Confluent plus ascorbate							
SMC	1836	30	3733	62	518	8	6487
Nuchal ligament fibroblasts	3488	29	6616	55	1925	16	12029
Skin fibroblasts	2246	26	5787	67	605	7	8638
Post-confluent							
SMC	2271	24	5772	61	1419	15	9462
Nuchal ligament fibroblasts	3491	18	13964	72	1940	10	19395
Skin fibroblasts	2693	20	9693	72	1077	8	13463

Cells were labelled for 18 h with [^{35}S]cysteine in the presence or absence of 50 μg/ml ascorbate. Fibrillin was immunoprecipitated from 30% $(NH_4)_2SO_4$ precipitates of cell culture medium and from sequential detergent (NET buffer) and denaturing ($GuCl_2$) cell layer extracts. The amount and distribution of newly synthesized fibrillin in the different cell cultures was compared by determining for each cell type the total immunoprecipitable fibrillin counts present in the medium and cell layer extracts. The values represent the averages obtained from duplicate labelling experiments on each of two cell lines derived foetal bovine aorta, nuchal ligament and skin.

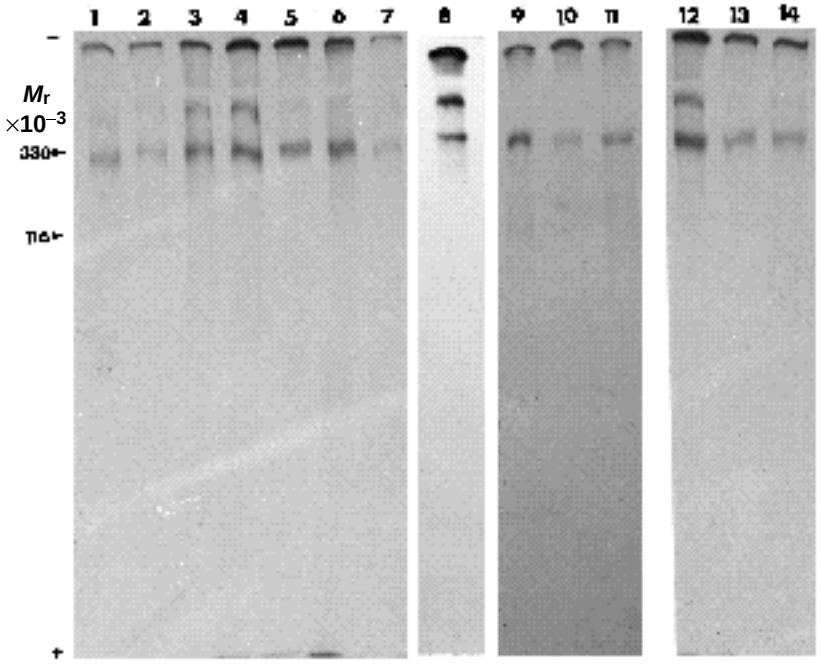

Fig. 1. Fluorograms of [^{35}S]cysteine-labelled newly synthesized fibrillin present in culture medium and NET buffer cell layer extracts of cultures of human and bovine aortic SMC and foetal bovine skin fibroblasts and nuchal ligament fibroblasts. In tracks 1-7 and 9-11 medium was fractionated with $(NH_4)_2SO_4$ to 30% saturation prior to fibrillin immunoprecipitation. Fibrillin immunoprecipitated directly from unconcentrated medium is shown in track 8. In tracks 12-14, fibrillin was immunoprecipitated from NET buffer cell layer extracts. Immunoprecipitates were electrophoresed on 8% SDS-PAGE gels under non-reducing and reducing conditions. Tracks 1-8 and 12-14, non-reducing conditions; tracks 9-11, reducing conditions. Tracks 1, 2, 10 and 13, foetal bovine aortic SMC; tracks 3, 4, 9 and 12, foetal bovine nuchal ligament fibroblasts; tracks 5, 6, 8, 11 and 14, foetal bovine skin fibroblasts; track 7, human aortic SMC.

from the 30% $(NH_4)_2SO_4$ precipitates of confluent and post-confluent cultures and analysed by SDS-PAGE on 6% or 8% gels under reducing or non-reducing conditions, and by fluorography (Fig. 1). The anti-fibrillin serum immunoprecipitated a major component of M_r 300,000, which in some cases was accompanied by a second higher-M_r band at the top of the running gel that was apparent when immunoprecipitates were run under non-reducing conditions. Extrapolation of M_r values from standards suggests that this larger component is of the order of 600,000 to 700,000, although its electrophoretic position precludes more precise M_r estimates. All of the immunoprecipitates contained some aggregated labelled fibrillin that did not enter the gel even when reducing agent was present. When newly synthesized fibrillin was immunoprecipitated from unconcentrated medium, a similar electrophoretic pattern was observed (Fig. 1, track 8).

Confluent and post-confluent cell layers were extracted sequentially in detergent (NET) and denaturing ($GuCl_2$) buffers, and the solubilized fibrillin immunoprecipitated and analysed by SDS-PAGE on 6% or 8% gels under reducing or non-reducing conditions and fluorography (Fig. 1B). It became apparent that the bulk of the newly synthesized fibrillin (77-91%) present in the cell layers of the three cell types could be solubilized by detergent action (Table 1). Fibrillin immunoprecipitates of these NET extracts contained both the major band of M_r 300,000 and the higher-M_r band that was particularly prominent in nuchal ligament extracts (Fig. 1). In addition, non-reducible high-M_r material was always present.

The time-course of secretion of fibrillin by confluent cultures of human SMC and skin fibroblasts was determined in pulse-chase experiments with an initial 30 min pulse-label period (Fig. 2). In both cell types, fibrillin was first detected in the medium after 2 h chase and in abundance by 6 h chase. The major processing of these molecules appeared to be their early assembly into high-M_r disulphide-bonded aggregates.

Size fractionation of newly synthesized fibrillin

Unconcentrated medium and NET buffer cell layer extracts of confluent and post-confluent SMC that had been grown in the presence of [^{35}S]cysteine for 18 h were chromatographed on an analytical column of Sepharose CL-2B under non-reducing conditions (Fig. 3). Western blotting analysis of aliquots of the fractions demonstrated the presence of fibrillin (labelled and unlabelled) in the void volume and within the included volume of chromatographed medium and cell layer extracts (results not shown).

Fibrillin was immunoprecipitated from the pooled column fractions of chromatographed post-confluent SMC medium and cell layer NET buffer extracts. The fibrillin counts in each pooled fraction were determined to establish the elution positions and size distribution of the newly synthesized fibrillin in these extracts (Fig. 3). This protocol demonstrated that the newly synthesized metabolically labelled fibrillin was present as a spectrum of molecular masses from monomers to high-M_r aggregates in medium and cell layer extracts. These results highlight variations in levels of fibrillin aggregates in medium and cell layer compartments.

The chromatography profiles of newly synthesized fibrillin extracted from confluent and post-confluent cell layer extracts also reflected differences in the relative amounts of labelled fibrillin incorporated into high-M_r assemblies. The NET buffer extracts of confluent cultures contained a lower proportion of metabolically labelled fibrillin in high-M_r aggregates than did the corresponding post-confluent cell layers. The subsequent $GuCl_2$ cell layer extracts contained few incorporated fibrillin counts and virtually no high-M_r material containing labelled fibrillin was recovered from these extracts.

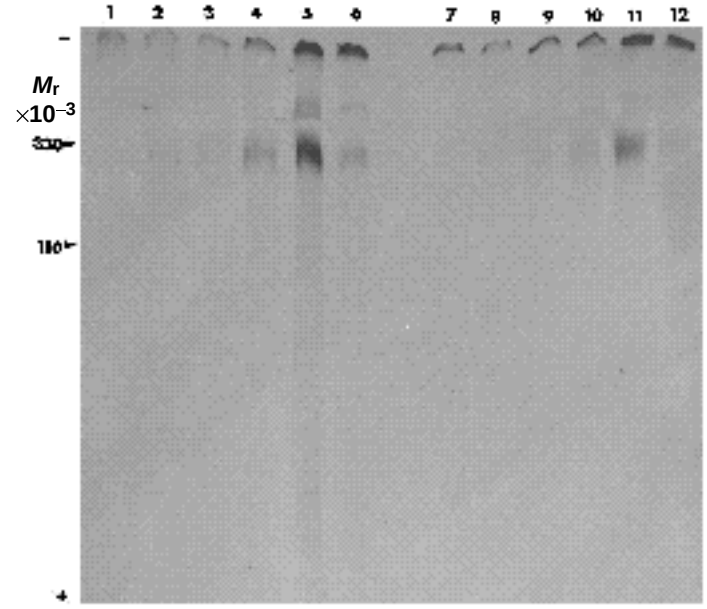

Fig. 2. Time-course of secretion of fibrillin from human SMC and fibroblasts. Post-confluent cells were pulse-labelled for 30 min with [^{35}S]cysteine, then chased with unlabelled medium for up to 24 h. Fibrillin was immunoprecipitated from culture medium and electrophoresed on 8% SDS-PAGE gels under non-reducing conditions. Tracks 1-6, bovine SMC; tracks 7-12, bovine skin fibroblasts. Tracks 1 and 7, pulse; tracks 2 and 8, 30 min chase; tracks 3 and 9, 1 h chase; tracks 4 and 10, 2 h chase; tracks 5 and 11, 6 h chase; tracks 6 and 12, 24 h chase.

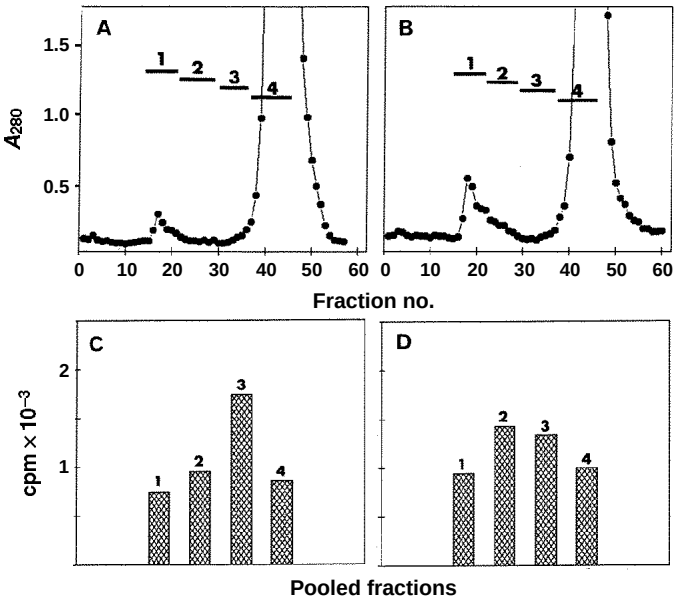

Fig. 3. Size-fractionation of newly synthesized metabolically labelled fibrillin present in unconcentrated medium (A and C) and cell layer NET extracts (B and D) of confluent foetal bovine SMC after cells were labelled for 18 h with [^{35}S]cysteine. Samples were chromatographed directly without concentration under non-reducing, non-denaturing conditions on an analytical column of Sepharose CL-2B (1.5 cm × 25 cm). The column was equilibrated and eluted at room temperature with 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl. The elution positions of the labelled fibrillin were determined by immunoprecipitation of fibrillin from pooled column fractions. (A) and (B) show the gel filtration chromatography profiles; (C) and (D), histograms comparing the immunoprecipitated fibrillin counts present in each pooled column fraction.

A similar pattern of fibrillin assembly was seen when skin fibroblasts were examined, but the rate of fibrillin assembly appeared to be slower than in SMC cultures.

Ultrastructural analyses

Direct confirmation that all of these cell types were capable of assembling fibrillin-containing microfibrils in culture was obtained by rotary shadowing electron microscopic visualization of post-confluent cell layers solubilized directly into $GuCl_2$-containing buffer and after bacterial collagenase digestion of cultures grown in collagen gels (Fig. 4). In this way, both SMC and fibroblasts were shown to have assembled characteristic beaded fibrillin-containing microfibrils that had an average, but variable, periodicity of 50-55 nm. The interbead domains of these macromolecules frequently occurred in a relatively loose conformation. The SMC cultures contained extensive microfibrillar arrays in excess of 5 μm in length, in comparison to the short microfibrillar sections that were extracted from skin fibroblast cultures grown on plastic and within collagen gels.

DISCUSSION

Cell culture has been used to investigate the synthesis, deposition and assembly of the microfibrillar glycoprotein, fibrillin, using a combined biochemical, immunochemical and ultrastructural approach. We present evidence for the synthesis, secretion and assembly of fibrillin by cells derived from various elastic tissues. These studies highlight differences between the cells in terms of amounts of fibrillin synthesized, secreted and assembled. These variations in fibrillin expression in vitro correlate with the relative contribution of the fibrillin-containing microfibrils to the structural and functional integrity of each of these tissues in vivo. Thus, the high level of fibrillin expression recorded in the foetal bovine nuchal ligament fibroblasts reflects the major role of this cell type in elastic fibre formation and the proposed role of the fibrillin-containing microfibrils in directing this process. Lower, but significant, levels of fibrillin were also expressed by skin fibroblasts and SMC, and this highlights the importance of fibrillin in the normal development and function of the skin and vasculature. The

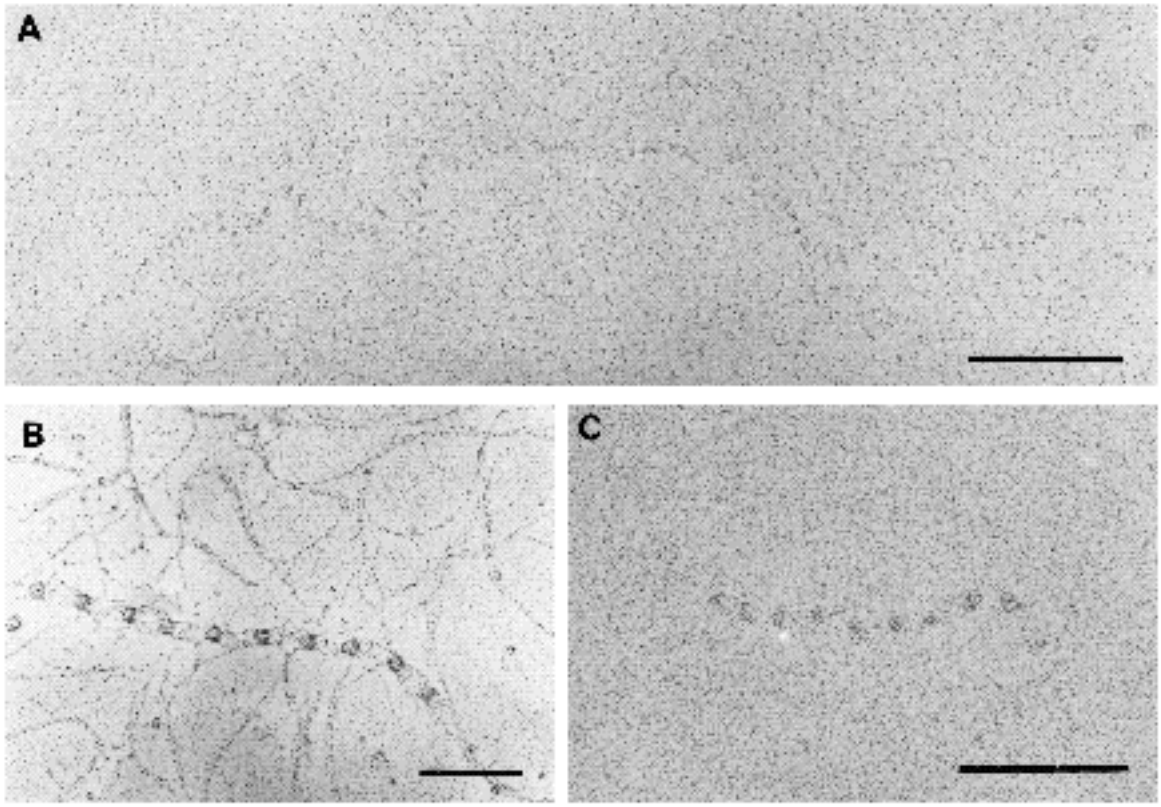

Fig. 4. Rotary shadowing electron micrographs of intact fibrillin-containing microfibrils extracted from the post-confluent cell layers of human SMC and skin fibroblast cultures. Post-confluent SMC and skin fibroblast cell layers were extracted directly into 0.05 M Tris-HCl, pH 7.4, containing 4.0 M $GuCl_2$. In some experiments, skin fibroblasts were grown for four weeks at high cell density in three-dimensional collagen gels, prior to bacterial collagenase digestion of the collagen gels and fractionation of soluble extracts on a Sepharose CL-2B column (1.5 cm × 25 cm) equilibrated in 0.05 M Tris-HCl, pH 7.4, containing 0.4 M NaCl. The $GuCl_2$ extracts and void volume fractions were dialysed into 0.2 M ammonium acetate prior to rotary shadowing. (A) SMC $GuCl_2$ extract; (B) skin fibroblasts, $GuCl_2$ extract; (C) skin fibroblasts, collagen gel extract, void volume fraction. Bars: (A) 500 nm; (B, C), 100 nm.

observation that ascorbate affects the distribution of fibrillin between medium and cell layer compartments may reflect increased elaboration of microfibrillar elements within the cell layers of post-confluent cultures. The molecular mechanisms involved in this response are likely to be complex and suggest a regulatory system that may operate to coordinate the expression of fibrillin and elastin during elastic fibre formation in vivo (Faris et al., 1984; Kielty et al., 1993).

The involvement of fibrillin-containing microfibrils in elastic fibre formation is well founded. However, the molecular basis of the mechanism of fibrillin assembly remains ill-defined, and the possibility that other molecules may participate in the formation of microfibrils has not been resolved. Our experiments demonstrate that newly synthesized fibrillin monomers form aggregates in medium and cell layers. Size fractionation and SDS-PAGE analysis clearly identify a variety of molecular aggregates of fibrillin, and while it is difficult to assess the molecular masses of macromolecules that barely enter the gels, the results strongly suggest that dimer formation is a critical step in microfibril formation. Ultrastructural analyses have identified assembled fibrillin-containing microfibrils in these cultures similar to those that we have previously extracted intact from elastic and non-elastic tissues (Kielty et al., 1991). These observations confirm the suitability of this culture system as a means of studying the mechanism of microfibril formation and an opportunity to investigate immunologically the potential role of other putative microfibril-associated molecules in this assembly process.

Mutations in fibrillin have been linked to the Marfan syndrome, a heritable connective tissue disorder with cardiovascular, skeletal and ocular abnormalities. Fibrillin also represents a candidate protein for the primary lesion in several other heritable disorders associated with microfibrillar abnormalities, including pseudoxanthoma elasticum and cutis laxa. An obvious application of the methodologies reported here is in evaluating fibrillin synthesis and microfibril assembly by skin fibroblasts derived from patients with these connective tissue disorders. In addition, this system may provide clues to the factors regulating normal fibrillin synthesis in processes such as development and wound healing, and those that lead to abnormal fibrillin expression such as occurs in several major acquired disorders such as systemic sclerosis, atherosclerosis and fibrosis.

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