Type VIII collagen is a product of vascular smooth-muscle cells in development and disease

Jonathan R. E. MacBEATH, Cay M. KIELTY and C. Adrian SHUTTLEWORTH*

Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, School of Biological Sciences, 2.205 Stopford Building, Oxford Road, Manchester M13 9PT, U.K.

Type VIII collagen is a short-chain collagen with considerable similarity to type X collagen. We have generated chain-specific antibodies to the α1 and α2 chains of type VIII collagen, and used them as probes to examine the synthesis of type VIII collagen by vascular smooth muscle cells (VSMC). In addition, chain-specific oligonucleotides have been used in reverse transcriptase–PCR (RT–PCR) reactions with RNA extracted from cultured smooth muscle cells in culture and from freshly isolated vascular tissues. Radiolabelling of VSMC in culture and immunoprecipitation with chain-specific antibodies showed that both chains were expressed. Lower levels of type VIII collagen were found in adult VSMC than in neonatal VSMC. RT–PCR showed that both chains were expressed in tissues as well as cells in culture. The results indicate that type VIII collagen is a product of VSMC of normal adult vessels and is expressed at high levels by VSMC in vascular lesions.

INTRODUCTION

Type VIII collagen is a non-fibrillar short-chain collagen originally identified as a biosynthetic product of endothelial cells from bovine aorta and rabbit cornea [1–3]. Subsequent studies have shown it to be synthesized by various epithelial, endothelial and mesenchymal cells, including microvascular endothelial cells, keratinocytes, glomerular cells, mast cells and tumour cells of diverse origin [4–10]. Type VIII collagen has been detected in various connective tissues, including sclera, choroid, optic nerve sheath, tendon, skin, periosteum, perichondrium and glomerulus [11–13], and it is a major structural component of Descemet's membrane of the eye [14,15]. Within the cardiovasculature, type VIII collagen is strongly expressed during cardiac morphogenesis [9], and is a key structural component of blood vessel walls [16].

Type VIII collagen has a short triple helix and contains α1(VIII) and α2(VIII) chains, which are highly similar to the α1(X) chain of type X collagen [17–19]. Sequence analysis revealed each α-chain to contain a collagenous domain of 454 amino acid residues with eight aligned imperfections within the Gly-Xaa-Yaa sequence, a short N-terminal non-triple-helical region (NC2), a longer C-terminal non-triple-helical domain (NC1), and a predicted overall M_r of 61000 [20–22]. The NC2 domain is basic in nature and has the potential to interact electrostatically with polyanionic molecules. The assembled triple-helical molecule comprises a collagenous domain with globular domains at either end, and pepsin treatment yields a major collagenous fragment of M_r 50000 [23]. However, tissue preparations of type VIII collagen with intact α-chains of widely differing molecular masses have been reported (M_r values 185000, 177000, 125000, 100000, 68000 and 61000) [3–5,13,14,24,25] These differences might reflect distinct post-translational modifications, the use of alternative mRNA transcripts resulting from the use of alternative promoter sequences [26], the formation of oligomers (dimeric β and trimeric γ forms) by covalent or strong non-covalent interactions, and/or association with other matrix components. Few details have emerged about the molecular composition or macromolecular organization of type VIII collagen. Available evidence suggests that, in Descemet's membrane at least, the molecule exists as a heterotrimer comprising two α1(VIII) chains and one α2(VIII) chain [27].

It is becoming increasingly clear that type VIII collagen is a key structural component of the vasculature, but its cellular and developmental origins, molecular composition and biological role in this tissue are not well established. So far, interest has been focused on its expression by vascular endothelial cells and its presence in subendothelium and capillary walls [16], but it has also been detected within the elastic fibre-rich tunica media of elastic arteries that is deposited by vascular smooth-muscle cells (VSMC) during development, and subsequently for maintenance of contractility and vascular tone [28]. During foetal and neonatal development, VSMC proliferate rapidly and synthesize abundant matrix, whereas in adult vessels medial VSMC are characteristically quiescent with emphasis on cytoskeletal components that underpin their contractility. More recently it has been shown that VSMC are present within the tunica intima, which accumulates inexorably with age and focally during development of vessel wall lesions.

As a first step towards determining the biological role of type VIII collagen in the vasculature in health and disease, we have performed a comprehensive assessment of its expression by VSMC in developing and adult aorta and arteries. We present evidence that this collagen is a substantial VSMC product predominantly expressed in abundance through gestation and perinatally, and that it is re-expressed by VSMC within vascular lesions.

EXPERIMENTAL

Reagents

Foetal calves were obtained from the local abattoir within 1 h of maternal death. Human aortic tissues were obtained after surgery, with ethical approval from the University Hospital of Wales. Pepsin (EC 3.4.23.1) from pig stomach mucosa, PMSF, *N*-

Abbreviations used: NEM, *N*-ethylmaleimide; RT–PCR, reverse transcriptase–PCR; SM-MHC, smooth-muscle cell myosin heavy chain; VSMC, vascular smooth-muscle cells.

* To whom correspondence should be addressed.

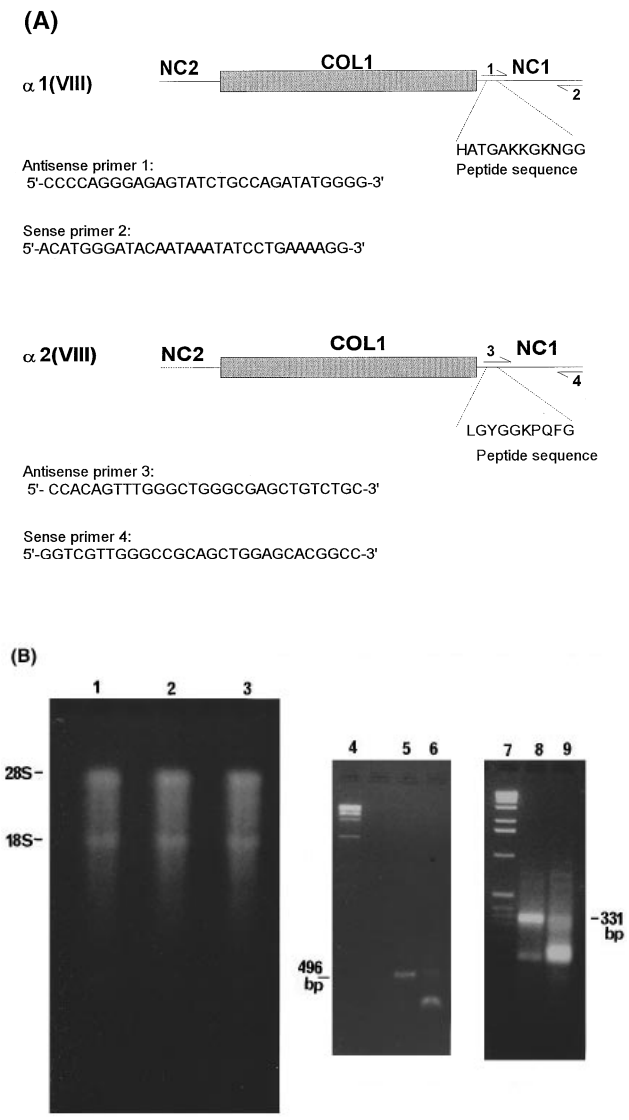

Figure 1 Peptide and oligonucleotide sequences of type VIII collagen, and demonstration of α_1(VIII) and α_2(VIII) chain products

(**A**) Schematic diagram of α1(VIII) and α2(VIII), showing the positions and sequences of chain-specific peptides and oligonucleotides. (**B**) Analysis of extracted RNA and RT–PCR products. Total RNA was revealed after ethidium bromide staining of electrophoresis on a 1% (w/v) agarose gel under denaturing conditions. The presence of 28 S and 18 S bands confirmed the intact state of the RNA. Lane 1, human neonatal VSMC RNA; lanes 2 and 3, two human adult VSMC RNAs; lanes 4 and 7, DNA markers (1 kb λ ladder); lane 5, α1(VIII) PCR product; lane 6, α1(VIII) PCR product after cleavage with *Acc*I; lane 8, α2(VIII) PCR product; lane 9, α2(VIII) product after cleavage with *Acc*I.

ethylmaleimide (NEM), Protein A–Sepharose, peroxidase-conjugated swine IgGs to rabbit immunoglobulins, normal rabbit serum, control mouse ascites fluid (clone NS-1), prestained molecular mass markers, monoclonal anti-(α smooth-muscle actin) (clone 1A4) and anti-(smooth-muscle cell myosin heavy chain) (SM-MHC) (clone hSM-V) were obtained from Sigma Chemical Company (Poole, Dorset, U.K.). Nitrocellulose membrane (0.45 μm pore size) was obtained from Schleier and Schuell (Dassel, Germany). Enhanced chemiluminescence kits were supplied by Amersham (Amersham, Bucks., U.K.). Reagents for cell culture were obtained from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). Unstained molecular mass standards were supplied by Pharmacia–LKB (Milton Keynes, Bucks., U.K.). [^{35}S]TransLabel (1097 Ci/mmol) and [^{14}C]formaldehyde (1.5 GBq/mmol) were purchased from ICN Biomedicals Ltd. (Thame, Oxon., U.K.). All other chemical reagents were from BDH/Merck (Poole, Dorset, U.K.). A monoclonal antibody specific for the α1 chain (50 kDa-B-peptide) of human, chicken, rabbit and bovine type VIII collagen [14,29] was obtained from AMS Biotechnology Ltd. (Witney, Oxon., U.K.).

Preparation of type VIII collagen antisera

Four polyclonal antisera to type VIII collagen antigens were raised in New Zealand White rabbits. Two were raised against type VIII collagen-enriched extracts of bovine Descemet's membrane, and two to synthetic peptides comprising unique sequences within the non-collagenous C-terminal regions of human α1(VIII) and α2(VIII) chains respectively (Figure 1A). Antibodies were partly purified from serum by precipitation with ammonium sulphate to 45% saturation. Subsequently, one antibody to Descemet's membrane type VIII collagen and both peptide antibodies were purified by affinity chromatography. Affinity columns were made by coupling the appropriate antigen to Sepharose CL-4B and passing the corresponding antisera over this column. The affinity-purified peptide antibodies were designated α1C and α2C respectively. The α1(VIII) peptide affinity-purified Descemet's membrane antibody was designated DM1, and the remaining Descemet's membrane antibody DM2.

Cells and cell culture

VSMC were established by explant from dissected tunica media of aortae obtained from second-trimester foetal calves, human aorta obtained *post mortem* from a cot-death infant, and adult aortae obtained at operation from Marfan syndrome individuals and patients with aortic aneurisms. VSMC were maintained in Dulbecco's modified Eagle's medium containing 20% (v/v) foetal calf serum, 400 i.u./ml penicillin, 50 μg/ml streptomycin, 200 μg/ml L-glutamine and non-essential amino acids. Low-passage cells (p1–p6) were used in all experiments. VSMC were monitored for expression of SMC-specific cytoskeletal elements (α-actin and SM-MHC), either by immunoprecipitation (as outlined below) or by indirect immunofluorescence.

Extraction of tissues and cell layers

Dissected foetal bovine aortae (approx 2 g wet weight) or confluent cell layers from 75 cm^2 flasks were washed in PBS containing 2 mM PMSF and 10 mM NEM, then homogenized and extracted in 1–2 ml of 4 M guanidinium isothiocyanate. Total solubilized protein was isolated from extracts after centrifugation at 10 000 ***g*** for 30 min at 20 °C, and concentrated by freeze-drying after exhaustive dialysis against distilled water. Total RNA was isolated by CsCl density-gradient centrifugation and phenol/chloroform extraction, as previously described [30], and its intact state confirmed by presence of 28 S and 18 S ethidium bromide-stained bands after denaturing agarose gel electrophoresis (Figure 1B).

SDS/PAGE and Western blotting

Protein samples (approx. 200 μg dry weight) were resuspended in Laemmli sample buffer with or without 3% (v/v) 2-

mercaptoethanol, boiled for 3 min and analysed by discontinuous SDS/PAGE on 8% (w/v) gels [31]. Gels were electroblotted on nitrocellulose filters with a Pharmacia-LKB semi-dry blotting apparatus [32]. Protein bands were revealed by Ponceau S staining [33]. Nitrocellulose filters were blocked with 5% (w/v) dried milk in TBS-T [20 mM Tris/HCl, pH 7.4, containing 137 mM NaCl and 0.2% (v/v) Tween-20] then incubated in rabbit polyclonal antiserum or non-immune rabbit serum (1:500 to 1:2000 in TBS-T). After three washes in TBS-T, filters were incubated with horseradish peroxidase-conjugated swine anti-rabbit IgG (1:5000 in TBS-T), then washed three times in TBS-T and once in 20 mM Tris/HCl, pH 7.4, containing 137 mM NaCl. Specific primary antibody immunoreactivity was detected by enhanced chemiluminescence with Kodak X-OMAT AR or Biomax film. Relative molecular masses of immunoreactive bands were determined by reference to pre-stained standards (lactate dehydrogenase, 39500; fumarase, 55000; pyruvate kinase, 67000; fructose-6-phosphate kinase, 84000; β-galactosidase, 108000; α2-macroglobulin, 190000).

Labelling and immunoprecipitation of newly synthesized type VIII collagen

Cells were labelled at confluency for 16–24 h with [^{35}S]TransLabel (50 μCi/ml). Medium was collected and proteinase inhibitors (5 mM EDTA, 10 mM NEM, 2 mM PMSF, final concentrations) and 0.1 vol of 10 × NET buffer (where NET buffer is 50 mM Tris/HCl, pH 7.5, containing 0.4 M NaCl, 1% Nonidet P40, 5 mM EDTA, 10 mM NEM and 2 mM PMSF) was added. Cell layers were lysed for 1 h at 4 °C in 1 ml of NET buffer, centrifuged (10000 ***g*** for 10 min) and supernatants stored at 4 °C. In some experiments, cell layers were sequentially extracted in 0.05 M Tris/HCl, pH 7.5, containing 0.4 M NaCl, and then in buffer containing 8 M urea. Both fractions were dialysed against NET buffer. Newly synthesized labelled type VIII collagen was immunoprecipitated from medium and cell layer compartments at 20 °C, essentially as previously described [34]. Briefly, fibronectin was first removed by incubation with 50 μl of gelatin–Sepharose [50% (v/v) suspension]. Samples were incubated for 1 h with appropriate antiserum or pre-immune controls (diluted 1:100 to 1:200), then for 3 h with 40 μl of Protein A–Sepharose [50% (v/v) suspension]. Immunoprecipitates were centrifuged (10000 ***g***, 1 min), then washed twice with 1 ml of NET buffer and once with 1 ml of 0.1 M Tris/HCl, pH 6.8, before resuspension in 30 μl of reducing Laemmli sample buffer. In some experiments, immunoprecipitates were taken up in 20 μl of 0.5 M acetic acid and incubated with pepsin (100 μg/ml) for 16 h at 4 °C before pH neutralization and addition of reducing Laemmli sample buffer. Digested and undigested samples were analysed by SDS/PAGE on 8% (w/v) gels and fluorography [35] with Kodak Biomax film. Relative molecular masses were determined by reference to prestained standards (see above) and to phosphorylase *b* (94000), BSA (67000), ovalbumin (43000) and carbonic anhydrase (30000), all radiolabelled by reductive alkylation with [^{14}C]formaldehyde and cyanoborohydride [36].

RNA analysis

The presence of mRNA species encoding α1(VIII) and α2(VIII) chains in developing and adult aortae and lesions was probed with reverse transcriptase–PCR (RT–PCR). For these experiments, unique sense and anti-sense primers specific for each human α-chain were designed (Figure 1A). Reverse transcription was for 2 h at 40 °C. PCR conditions for amplifications were: 94 °C for 1 min; 60 °C for 1 min; 72 °C for 2 min; 35 cycles. The specificity of each product was confirmed by specific restriction digestions with *Acc*I (Figure 1B), and by direct sequencing of PCR products. The α1(VIII) primer pair amplified bovine α1(VIII).

The oligonucleotide primers for human α1(VIII) were: anti-sense, 5′-CCC CAG GGA GAG TAT CTG CCA GAT ATG GGG-3′ (nt 1576–1605); sense, 5′-ACA TGG GAT ACA ATA AAT ATC CTG AAA AGG-3′ (nt 2042–2071). The predicted PCR product size was 496 nt; *Acc*I restriction fragments were 244 and 252 nt.

The oligonucleotide primers for human α2(VIII) were: anti-sense, 5′-CCACAGTTTGGGCTGGGCGAGCTGTCTGC-3′ (nt 1510–1538); sense, 5′-GGTCGTTGGGCCGCAGCTGGA-GCACGGCC-3′ (nt 1812–1840). The predicted product size was 331 nt; *Acc*I restriction fragments were 175 and 156 nt.

RESULTS

In this study we have investigated the presence of type VIII collagen in foetal, neonatal and adult aortae and in aortic and coronary arterial wall tissues, and its expression by freshly isolated cultured VSMC derived from these vessels. For immunochemical analyses we raised and utilized four new polyclonal antisera, two of which are α-chain-specific. For RT–PCR we designed oligonucleotide primers from unique C-terminal sequences for each α-chain.

Presence of type VIII collagen in developing aorta

The presence of type VIII collagen in aortae of second-trimester bovine foetuses was demonstrated by SDS/PAGE and Western blotting, with DM1 and DM2 (Figure 2). In all cases, under non-reducing conditions, bands (M_r values 95000–110000) and a lower-M_r diffuse component of 65000–70000 were identified, whereas under reducing conditions a single immunoreactive component of M_r 61000 was detected. The recognition of all of

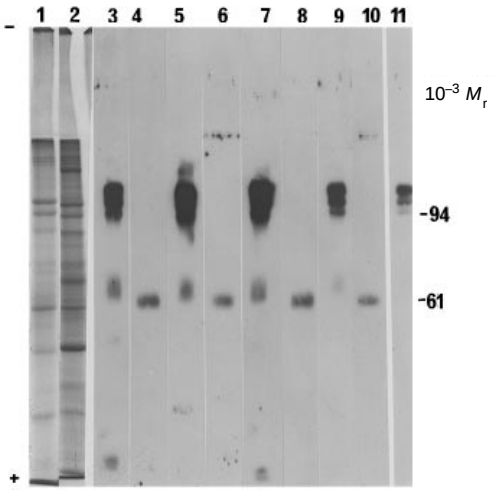

Figure 2 Immunoblot analysis of aortic tissue: SDS/PAGE and Western blotting of a foetal bovine aortic tissue extract, probed with a panel of polyclonal and monoclonal antisera to type VIII collagen

High-M_r type VIII collagen bands were detected under non-reducing conditions, but after reduction a single 61000-M_r component was apparent. Lanes 1, 3, 5, 7, 9 and 11, non-reduced; lanes 2, 4, 6, 8 and 10, reduced. Lanes 1 and 2, Coomassie Blue-stained; lanes 3 and 4, α1C antibody; lanes 5 and 6, α2C antibody; lanes 7 and 8, DM1 antibody; lanes 9 and 10, DM2 antibody; lane 11, monoclonal anti-α1(VIII) antibody.

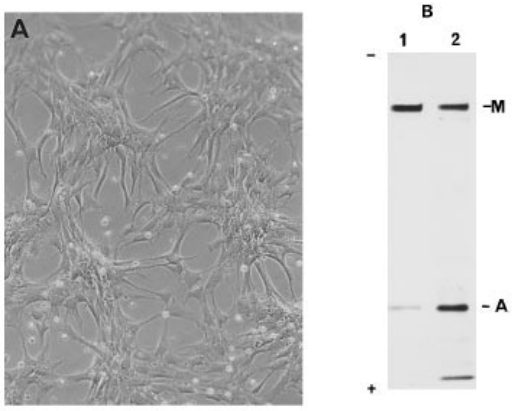

Figure 3 Characterization of VSMC

(**A**) The cells show a characteristic 'hills and valleys' appearance. (**B**) Smooth-muscle-specific myosin heavy chain (M) and smooth-muscle α-actin (A) were co-immunoprecipitated from neonatal (lane 1) and adult (lane 2) VSMC.

these bands by the peptide α-chain-specific antibodies (α1C and α2C), as well as a monoclonal anti-α1(VIII) antiserum, not only confirmed the presence of the two α-chains in developing aorta but also indicated that both chains were present as higher-M_r forms.

Expression of type VIII collagen by cultured VSMC

Cultured VSMC from foetal bovine aortae and human neonatal and adult (non-lesional) aortae were characterized in terms of their cellular morphology and pattern of growth and their expression of the smooth-muscle-specific cytoskeletal elements, α-actin and SM-MHC (Figure 3). All the VSMC cultures adopted, on confluence, a characteristic smooth muscle 'hills and valleys' appearance; this growth pattern was particularly marked in the adult VSMC cultures (Figure 3A). The cells concurrently expressed α-actin and SM-MHC, as judged both by immunoprecipitation from medium and cell layers after labelling with [^{35}S]TransLabel (Figure 3B).

The expression of type VIII collagen by cultured VSMC was confirmed by analysis of immunoprecipitated newly synthesized type VIII collagen by SDS/PAGE and fluorography (Figure 4a). Quantification of immunoprecipitated radioactive counts demonstrated that foetal bovine VSMC, and human neonatal VSMC and adult VSMC lines, synthesized and secreted type VIII collagen into medium and cell layers; less type VIII collagen was detected in the adult human VSMC cultures. Under reducing conditions, type VIII collagen resolved as a 61000-M_r band and a higher-M_r component with an M_r of 120000 (Figure 4). The electrophoretic pattern of immunoprecipitated type VIII collagen was similar in all VSMC and for all the antisera, except that the 61000-M_r component was more prominent in α1C and DM1 antisera immunoprecipitates. The identity of immunoprecipitated type VIII collagen was confirmed after pepsin digestion, which eliminated the 61000-M_r band and generated instead a 50000-M_r component.

In a separate approach, guanidine isothiocyanate-extracted cell layer proteins were examined directly for the presence of type VIII collagen by Western blotting with the synthetic peptide α-chain-specific α1C, α2C, DM1 and DM2 antibodies (Figure 4b). This approach confirmed that both α-chains of type VIII collagen were deposited within the cell layers.

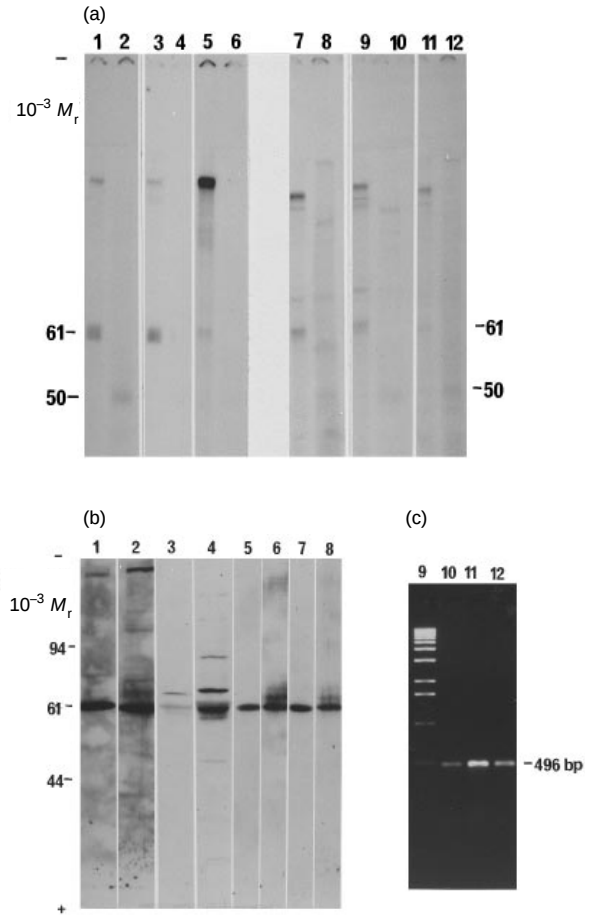

Figure 4 SDS/PAGE analysis of type VIII collagen immunoprecipitates

(**a**) SDS/PAGE analysis of type VIII collagen immunoprecipitated from medium and cell layer extracts of human neonatal VSMC. Prominent 61000-M_r and 120000-M_r components were immunoprecipitated with monoclonal anti α1(VIII) (lanes 1, 2, 7 and 8) and α1C (lanes 3, 4, 9 and 10); the 120000-M_r component was more abundant in the α2C immunoprecipitates (lanes 5, 6, 11 and 12). Electrophoresis was performed under reducing conditions. Lanes 1–6, medium immunoprecipitates; lanes 7–12, cell layer extract immunoprecipitates. Lanes 1, 3, 5, 7, 9 and 11, undigested immunoprecipitates; lanes 2, 4, 6, 8 and 10, pepsin-digested immunoprecipitates. (**b**) Immunoblot analysis of VSMC layers. SDS/PAGE and Western blotting of VSMC cell layers, probed with a panel of polyclonal and monoclonal antisera to type VIII collagen, are shown. Samples were subjected to electrophoresis under non-reducing (lanes 1, 3, 5 and 7) and reducing (lanes 2, 4, 6 and 8) conditions. A major 61000-M_r component was detected in all samples. High-M_r type VIII collagen bands were also apparent in the α1C and the DM1 immunoprecipitates. Lanes 1 and 2, α1C antibody; lanes 3 and 4, α2C antibody; lanes 5 and 6, DM1 antibody; lanes 7 and 8, DM2 antibody. (**c**) RT–PCR detection of mRNA encoding α1(VIII) in human cultured VSMC. Lane 9, DNA marker (1 kb λ ladder). Lanes 10–12, α1(VIII) PCR products: lane 10, foetal bovine VSMC; lane 11, human neonatal VSMC; lane 12, human adult VSMC.

RT–PCR analysis (Figure 4c) of the total RNA extracted from confluent cell layers of human neonatal and adult VSMC confirmed the presence of mRNA species encoding α1(VIII) and α2(VIII). The identities of the PCR products were confirmed by sequencing and restriction enzyme digestion patterns (results not shown).

Expression in normal and diseased human adult aorta and coronary artery

The expression of type VIII collagen in five atherosclerotic lesion specimens, two obtained from aortae by endarthrectomy and the

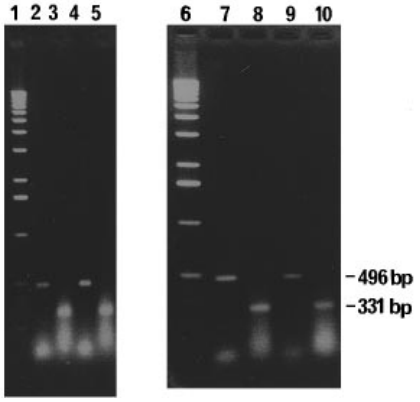

Figure 5 RT–PCR detection of mRNA species encoding α1(VIII) and α2(VIII) in human atherosclerotic lesion tissues

RT–PCR products were generated from diseased tissues derived from four individuals, and were subjected to electrophoresis as follows: lanes 2 and 3, patient A; lanes 4 and 5, patient B; lanes 7 and 8, patient C; lanes 9 and 10, patient D. Lanes 1 and 6, DNA markers (1 kb λ ladder); lanes 2, 4, 7, and 9, α1(VIII); lanes 3, 5, 8 and 10, α2(VIII).

remaining three from coronary arteries obtained by directional atherectomy, was confirmed by RT–PCR amplification of mRNA species encoding α1(VIII) and α2(VIII) (Figure 5).

DISCUSSION

Early studies of type VIII collagen have painted a somewhat confusing picture of its distribution and cellular origins in cardiovascular tissues. The dogma has arisen that the primary location of type VIII collagen is in the vascular subendothelium and its primary cellular source is vascular endothelial cells [16]. Indeed, it is clear that type VIII collagen is a key product of vascular endothelial cells of blood vessels and embryonic capillaries [13]. Interestingly, however, this collagen is also expressed by cardiac myoblasts and subsequently by endocardial mesenchymal cells during cardiac morphogenesis, and a short-chain collagen resembling type VIII collagen has been described as a product of pericytes [37]. Furthermore its presence in tunica media, at least in early development, is not disputed. There is far less documented information about the expression of type VIII collagen within developing and adult vasculature by VSMC. We have redressed this balance by conducting a comprehensive analysis demonstrating that type VIII collagen is an important matrix product of VSMC, particularly in foetal and neonatal aortae and in vessel wall lesions.

The expression of type VIII collagen by VSMC derived from both tunica intima and tunica media suggests not only that there is a role for this collagen as a component of the vascular matrix in both layers, but also that subendothelial type VIII collagen might be a product both of VSMC and endothelial cells. The expression of both α-chains by VSMC was confirmed after immunodetection of type VIII collagen by synthetic peptide α-chain-specific antisera and by RT–PCR experiments.

Virtually nothing is known about the molecular composition and macromolecular organization of type VIII collagen in the vasculature. Our Western blotting data demonstrated that both chains were apparently cross-linked in aorta. Synthetic studies on VSMC in culture showed that both chains could be immunoprecipitated by α-chain-specific antibodies. These experiments suggest that type VIII collagen heterotrimers occur in the vasculature. A number of studies have suggested that other forms of chain association are possible in type VIII collagen molecules, and the present results do not preclude the formation of some α1(VIII) and α2(VIII) homotrimers.

Type VIII collagen is the major component of the hexagonal lattice stucture identified in Descemet's membrane by electron microscopy [14]. It is likely that type VIII collagen forms a hexagonal lattice network in the vasculature, and its structural and supramolecular similarities to type X collagen [38] suggest that it might play a similar role in tissues. It is tempting to speculate that, as type X collagen is localized to the territorial matrix of hypertrophic chondrocytes in the growth plate, so type VIII collagen might localize to VSMC pericellular basement membranes. This network might form an interface between the VSMC basement membrane and surrounding matrix and play a central role in maintaining smooth-muscle cell phenotype.

Our analysis of human adult aortae and coronary arteries suggests that type VIII collagen is a constitutive product of contractile VSMC of normal mature vessels, and is expressed at high levels by VSMC in lesions. This observation has major implications for the role of type VIII collagen in vascular remodelling associated with the formation of lesions, and the use of this collagen as a marker for lesions and for 'dedifferentiated' VSMC. We are currently investigating the compositional and macromolecular forms of type VIII collagen in the vasculature and its role in maintaining smoooth muscle cell phenotype.

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REFERENCES

1 Sage, H., Pritzl, P. and Bornstein, P. (1980) Biochemistry **19**, 5747–5755
2 Benya, P. D. (1980) Renal Physiol. **3**, 30–35
3 Benya, P. D. and Padilla, S. R. (1986) J. Biol. Chem. **261**, 4160–4169
4 Alitalo, K., Bornstein, P., Vaheri, A. and Sage, H. (1983) J. Biol. Chem. **258**, 2653–2661
5 Sage, H., Balian, G., Vogel, A. M. and Bornstein, P. (1984) Lab. Invest. **50**, 219–231
6 Sage, H. and Iruela-Arispe, M. L. (1990) in Structure, Molecular Biology and Pathology of Collagen (Fleischmajer, R., Olsen, B. R. and Kuhn, K., eds.), pp. 17–31, New York Academy of Sciences, New York
7 Paulus, W., Sage, E. H., Liszka, U., Iruela-Arispe, M. L. and Jellinger, K. (1991) Br. J. Cancer **63**, 367–371
8 Salonen, J., Oda, D., Funk, S. E. and Sage, H. (1991) J. Periodont. Res. **26**, 355–360
9 Rosenblum, N. D., Briscoe, D. M., Karnovsky, M. J. and Olsen, B. R. (1990) Am. J. Physiol. **264**, F1003–F1010
10 Ruger, B., Dunbar, R., Hasan, Q., Sawada, H., Kittelberger, R., Greenhill, N. and Neale, T. J. (1994) Int. J. Exp. Path. **75**, 397–404
11 Kapoor, R., Sakai, L. H., Funk, S., Roux, E., Bornstein, P. and Sage, E. H. (1988) J. Cell Biol. **107**, 721–730
12 Kittelberger, R., Davis, P. F., Flynn, D. W. and Greenhill, N. S. (1990) Connect. Tiss. Res. **24**, 303–318
13 Iruela-Arispe, M. L. and Sage, H. (1991) Dev. Biol. **144**, 107–118
14 Sawada, H., Konomi, H. and Hirosawa, K. (1990) J. Cell Biol. **110**, 219–227
15 van der Rest, M. and Garrone, R. (1991) FASEB J. **5**, 2814–2823
16 Kittelberger, R., Davis, P. F. and Greenhill, N. S. (1989) Biochem. Biophys. Res. Commun. **159**, 414–419
17 Ninomiya, Y., Castagnola, P., Gerecke, D., Gordon, M. K., Jacenko, O., LuValle, P., McCarthy, M., Muragaki, Y., Nishimura, I., Oh, S., Rosenblum, N., Sato, N., Sugrue, S., Taylor, R., Vasios, G., Yamaguchi, N. and Olsen, B. R. (1990) in Extracellular Matrix Genes (Sandell, L. and Boyd, C., eds.), pp. 79–114, Academic Press, Orlando, FL
18 Kielty, C. M., Hopkinson, I. and Grant, M. E. (1993) in Connective Tissue and its Heritable Disorders: Molecular, Genetic and Medical Aspects (Royce, P. M. and Steinmann, B., eds.), pp. 103–147, Wiley–Liss, New York
19 Prockop, D. J. and Kivirikko, K. I. (1995) Annu. Rev. Biochem. **64**, 403–434
20 Yamaguchi, N., Mayne, R. and Ninomiya, Y. (1991) J. Biol. Chem. **266**, 4508–4513
21 Muragaki, Y., Jacenko, O., Apte, S., Mattei, M.-G., Ninomiya, Y. and Olsen, B. R. (1991) J. Biol. Chem. **266**, 7721–7727

22 Muragaki, Y., Mattei, M.-G., Yamaguchi, N., Olsen, B. R. and Ninomiya, Y. (1991) Eur. J. Biochem. **197**, 615–622
23 Sage, H., Trueb, B. and Bornstein, P. (1983) J. Biol. Chem. **258**, 13391–13401
24 Kapoor, R., Bornstein, P. and Sage, H. (1986) Biochemistry **25**, 3930–3937
25 Sage, H. and Bornstein, P. (1987) in Biology of the Extracellular Matrix: Structure and Function of Collagen Types (Mayne, R. and Burgeson, R., eds.), pp. 173–194, Academic Press, New York
26 Nishimura, I., Muragaki, Y. and Olsen, B. R. (1989) J. Biol. Chem. **264**, 20033–20041
27 Mann, K., Jander, R., Korsching, E., Kuhn, K. and Rauterberg, J. (1990) FEBS Lett. **273**, 168–172
28 Sawada, H. and Konomi, H. (1991) Cell Struct. Funct. **16**, 455–466
29 Tamura, Y., Konomi, H., Sawada, H., Takashima, S. and Nakajima, A. (1991) Invest. Opthalmol. Vis. Sci. **32**, 2636–2644
30 Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1983) Biochemistry (Tokyo) **18**, 5294–5299
31 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
32 Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354
33 Harlow, E. and Lane, D. (eds.) (1988) in Antibodies: A Laboratory pp. 492–494, Cold Spring Harbour Laboratory, NY
34 Kielty, C. M., Boot-Handford, R. P., Ayad, S., Shuttleworth, C. A. and Grant, M. E. (1990) Biochem. J. **272**, 787–795
35 Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. **46**, 83–88
36 Dottavio-Martin, D. and Ravell, J. M. (1978) Anal. Biochem. **87**, 562–565
37 Canfield, A. E. and Schor, A. M. (1991) FEBS Lett. **286**, 171–175
38 Kwan, A. P. L., Cummings, C. E., Chapman, J. A. and Grant, M. E. (1991) J. Cell Biol. **114**, 597–604

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