Journal of Cellular Biochemistry 80:146–155 (2000)

Type V Collagen Regulates the Assembly of Collagen Fibrils in Cultures of Bovine Vascular Smooth Muscle Cells

Kyriakos E. Kypreos,[1] David Birk,[2] Vickery Trinkaus-Randall,[1] Daniel J. Hartmann,[3] and Gail E. Sonenshein[1]*

[1]Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118
[2]Department of Pathology, Anatomy, and Cell Biology, Jefferson Medical College, Philadelphia, Pennsylvania, 19107
[3]Laboratoire des Biomatériaux (EA 3090), Faculté de Pharmacie- ISPB, F-69375 Lyon, France

Abstract Vascular smooth muscle cells (SMCs), the major cellular constituent of the medial layer of an artery, synthesize the majority of connective tissue proteins, including fibrillar collagen types I, III, and V/XI. Proper collagen synthesis and deposition, which are important for the integrity of the arterial wall, require the antioxidant vitamin C. Vitamin C serves as cofactor for the enzymes prolyl and lysyl hydroxylase, which are responsible for the proper hydroxylation of collagen. Here, the role of type V collagen in the assembly of collagen fibrils in the extracellular matrix (ECM) of cultured vascular SMCs was investigated. Treatment of SMCs with vitamin C resulted in a dramatic induction in the levels of the cell-layer associated pepsin-resistant type V collagen, whereas only a minor induction in the levels of types I and III collagen was detected. Of note, the deposition of type V collagen was accompanied by the formation of striated collagen fibrils in the ECM. Immunohistochemistry demonstrated that type V collagen, but not type I collagen, became masked as collagen fibrils matured. Furthermore, the relative ratio of type V to type I collagen decreased as the ECM matured as a function of days in culture, and this decrease was accompanied by an increase in the diameter of collagen fibrils. Together these results suggest that the masking of type V collagen is caused by its internalization on continuous deposition of type I collagen on the exterior of the fibril. Furthermore, they suggest that type V collagen acts as framework for the initial assembly of collagen molecules into heterotypic fibrils, regulating the diameter and architecture of these fibrils. J. Cell. Biochem. 80:146–155, 2000.

Type V collagen, and the highly related type XI collagen, belong to the group I fibrillar collagens. Type V collagen was originally discovered in human placenta [Chung et al., 1976] and in skin [Sage and Bornstein, 1979]. Type V/XI collagen is also expressed in a variety of other tissues including smooth muscle tissue [Liau and Chan, 1989; Brown et al., 1991]. Atherosclerotic lesions were found to contain increased levels of collagen type V compared to the normal vessel wall [Ooshima, 1981]. Type V/XI collagen contains a long triple helical region, a small globular carboxy-terminal domain, and a large globular amino-terminal domain. Between the major triple helical region and the amino-terminal domain, there is a "hinge region" that introduces a bend to the molecule, and three short triple helical domains [Fessler and Fessler, 1987]. Previous studies showed that in the avian corneal stroma, collagen type V participates, along with collagen type I, in heterotypic fibrils [Linsenmayer et al., 1993]. It was speculated that the hinge region in type V molecule allowed the amino-terminal domain to lie on the surface of the heterotypic fibril. Immunohistochemical studies on chick cornea showed that in heterotypic fibrils formed with collagen types I and V, the collagen molecules were arranged in such a way that collagen type I hinders type V

Abbreviations used: ddH_2O, double distilled water; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PHMB, *p*-hydroxymercurybenzoate; PMSF, phenylmethylsulfonyl fluoride; SMCs, smooth muscle cells.

Grant sponsor: National Institutes of Health; Grant numbers: EY05129, EY06000, and HL13262.

*Correspondence to: Gail E. Sonenshein, Department of Biochemistry, Boston University School of Medicine, 715 Albany Street, Boston MA 02118. E-mail: gsonensh@bu.edu

epitopes in the normal fibrillar structure [Linsenmayer et al., 1985; Birk et al., 1988; Linsenmayer et al., 1993]. Other studies, originally in vitro and later in vivo, showed that fibril diameter is related to the amount of type V collagen within heterotypic type I/V fibrils, and it is the amino-terminal globular domain that confers this regulatory property [Birk et al., 1990; Marchant et al., 1996]. Additional data obtained from transgenic animal studies showed that collagen type V plays a very important role in the architecture of collagen fibrils during matrix assembly [Andrikopoulos et al., 1995].

Collagen synthesis and correct assembly into mature fibrils is a multistep process that requires the antioxidant vitamin C [Linsenmayer, 1991]. Vitamin C acts as a cofactor for two important enzymes: prolyl- and lysyl-hydroxylase, which are responsible for the proper hydroxylation of collagen. Properly hydroxylated collagen α chains assemble into a triple helical structure with increased stability against thermal denaturation [Rosenbloom et al., 1973; Berg and Prockop, 1973; Jimenez et al., 1973a]. However, even in the absence of vitamin C, collagen α chains can assemble intracellularly into triple helices with normal periodicity, although the rate of secretion of these trimers and their thermal stability are reduced [Jimenez et al., 1973b; Peterkofsky, 1991].

SMCs are the major cellular constituent of the medial layer of an artery. During the development of an artery, SMCs are in a synthetic stage, where they produce the bulk of the proteins of the ECM, including collagen types I, III, and V/XI [Ross, 1993, 1999]. In a developed artery, SMCs differentiate into a contractile state, and are primarily responsible for maintaining the vascular tone [Chamley-Campbell et al., 1979]. In response to vascular injury, SMCs migrate from the medial layer of the artery into the intimal layer, where they revert to synthetic phenotype [Chung et al., 1976; Chamley-Campbell et al., 1979; Ross, 1993]. Initial stages of proliferation are followed by the synthesis of the collagenous proteins, which constitute the atherosclerotic plaque.

SMCs in culture similarly revert to synthetic state, synthesizing and depositing fibrillar collagen protein types I, III, and V/XI [Beldekas et al., 1982; Brown et al., 1991]. Cultured vascular SMCs have been used extensively to characterize the regulation of collagen synthesis. Work from several laboratories, including our own, has shown that the level of collagen synthesis varies inversely with the rate of proliferation of the vascular SMC [Beldekas et al., 1982; Kindy et al., 1988; Liau and Chan, 1989; Ang et al., 1990; Brown et al., 1991; Chang and Sonenshein, 1991]. For example, SMCs plated at low density grew exponentially but synthesized only low levels of type I, III, or V/XI collagen, whereas, as cells approached confluence, the levels of synthesis of these collagen species increased dramatically [Beldekas et al., 1982; Brown et al., 1991]. Here we examine the synthesis and assembly of collagen fibrils by the vascular SMCs, and the role of vitamin C. We show that treatment of bovine aortic SMCs with vitamin C results in a selective induction in the protein levels of type V collagen. This induction is accompanied by enhanced deposition of type V collagen in the ECM. Importantly, in the developing collagen fibril type V, collagen is localized internally, forming a framework that appears to regulate heterotypic fibril diameter.

MATERIALS AND METHODS

Cell Culture and Treatment Conditions

Tissue culture reagents were purchased from Life Technologies, Inc., except for DMEM, which was purchased from JRH Biosciences. SMC explants were derived from the aortic arches of female calves, as we have described previously [Beldekas et al., 1982]. Cultures were fed every 2–3 days. For the effects of cell density on collagen protein levels, cells were cultured in the presence of 20 μg/ml vitamin C (in ddH_2O), for the indicated amount of time. For the effects of vitamin C on collagen synthesis and deposition in the ECM, cells were plated at an initial density of 5.0×10^5 cells/P150 dish (0.35×10^4 cells/cm^2) and grown for 9 days (postconfluent cultures), in medium supplemented with 10% FBS (10% FBS-DMEM), in the absence of vitamin C. Postconfluent cultures were incubated then treated with 20 μg/ml vitamin C or the equivalent volume of ddH_2O as control, for an additional 24 h.

Immunohistochemistry

The procedure for preparing cells for indirect immunohistochemistry was described previously [Grushkin-Lerner et al., 1991; Wu et al.,

1994]. Bovine aortic SMCs were seeded in four-well glass slides at a density of 7×10^4 cells/well (0.35×10^4 cells/cm^2) in 10% FBS-DMEM, supplemented daily with 20 μg/ml vitamin C, and allowed to grow for the indicated amount of time. Then, some of the cultures were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, and then washed once more with PBS. The rest of the cultures were incubated with 0.15M acetic acid for 1 min, washed with PBS, and then fixed, as described above. For the immunohistochemical analysis, cells were rinsed with PBS containing 1% bovine serum albumin (BSA), incubated with antibodies to either type I (1:500 dilution) or type V collagen (1:500 dilution) (Novotec, Lyon, France) at 4°C for 1 h, rinsed with PBS, and washed for 10 min in 3% BSA in PBS. Then, cells were incubated with goat anti-rabbit fluorescein isothiocyanate immunoglobulin G (IgG) (1:40 dilution) (Vector Labs, Burlington, CA) at 4°C for 1 h. Negative controls were incubated with rabbit IgG instead of the primary antibody.

Cells were imaged on a Leica upright confocal laser microscope equipped with an argon ion laser with an output power of 2–50 mV, two photomultiplier tubes, and a narrow-band filter. Controls were always examined first, and the aperture and voltage were set to obtain background levels. All experimental cultures were then examined under these conditions. A series of 8 μm was taken for each sample, and each slide was scanned in three random areas. Representative basal sections were taken from each scan. All images were processed identically.

Pepsin Digestion of Conditioned Media and ECM Proteins

Cultures of aortic SMCs were labeled with 25 mCi/ml [^{3}H]-proline, as described previously [Lawrence et al., 1994]. The medium was removed, and the cell layers were washed three times in ice-cold PBS. The cell layer was scraped in pepsin digestion buffer, transferred to a 50-ml Falcon conical tube, and digested at 4°C for 16 h. The pepsin-digested, cell-layer-associated matrix was dialyzed exhaustively against 0.5 M acetic acid, lyophilized, and reconstituted to 1 ml distilled H_2O supplemented with protease inhibitors (2 mM PMSF and 2 mM PHMB). Samples were normalized for DNA content as described previously [Kypreos and Sonenshein, 1998] and resolved by electrophoresis in a 7.5 or 5–15% polyacrylamide gradient–sodium dodecyl sulfate (SDS) gel. To resolve α1(III) and α1(I) collagen chains by polyacrylamide gel electrophoresis (PAGE)–SDS, a modification of a delayed reduction protocol of Nakamura et al. [1989] was used. The radioactive signal was enhanced using fluorography with 2,5-diphenyloxazole/dimethyl sulfoxide, as described [Laskey and Mills, 1975]. Molecular mass markers used included species at 200, 97.4, 69, 46, 30, 21.5, and 14.3 kDa (Rainbow molecular mass markers, Amersham Corp.). The molecular mass of the species was estimated based on their migration relative to the markers.

Conventional Transmission Electron Microscopy

Cells were fixed in 4% paraformaldehyde/ 2.5% glutaraldehyde/ 0.1 M sodium cacodylate, pH 7.4 with 8.0 mM $CaCl_2$ for 2 h on ice followed by postfixation with 1% osmium tetroxide for 1 h. After dehydration in a graded ethanol series, the cells were infiltrated and embedded in a mixture of Polybed 812, nadic methyl anhydride, dodecenylsuccinic anhydride, and DMP-30 (Polysciences, Warrington, PA). Thin sections were prepared using a Reichert Ultracut E ultramicrotome and a diamond knife. Staining was with 2% aqueous uranyl acetate followed by 1% phosphotungstic acid pH 3.2. Sections were examined and photographed at 80 kV using a Philips CM-10 transmission electron microscope. The microscope was calibrated using a line grating. Fibril diameter measurements were done using calibrated micrographs. The micrographs were digitized and diameters were measured using an RM Biometrics-Bioquant Image Analysis System (Memphis, TN).

Immunoelectron Microscopy

Preembedding immunoelectron microscopy was done essentially as described previously [Doane et al., 1992a]. Briefly, cells were fixed with 4% paraformaldehyde in PBS (pH 7.3) for 30 min on ice followed by treatment with sodium borohydride (50 mg/100 ml PBS) for 15 min at room temperature, and nonspecific binding sites were blocked by incubation in 5% normal goat serum. Cells were then incubated in primary antibody against type I collagen. This was followed by secondary goat anti-

rabbit gold-conjugated antibody [1:20]. Negative controls were incubated identically, but without the addition of primary antibody.

RESULTS

Treatment of Bovine Vascular SMCs with Vitamin C Results in Formation of Striated Collagen Fibrils in the ECM

Previously, it had been noted that vitamin C was critical for correct formation of fibrils [Linsenmayer, 1991]. Thus, first the effects of vitamin C on fibril assembly by bovine aortic SMCs in culture were monitored. To this end, bovine aortic SMCs were seeded at a low cell density (0.35×10^4 cells/cm^2) in 10% FBS-DMEM, and allowed to grow for 9 days (postconfluent cultures), when collagen synthesis and deposition is maximal [Brown et al., 1991; Beldekas et al., 1982]. Cultures were then either treated daily with 20 μg/ml vitamin C (dissolved in ddH_2O), or the equivalent volume of ddH_2O, as control, and analyzed for collagen fibril formation using transmission electron microscopy. In the absence of vitamin C, only very thin nonstriated filaments were detected (Fig. 1). Addition of vitamin C resulted in the formation of striated collagen fibrils with 67-nm periodicity (Fig. 1). Thus, striated collagen fibril formation occurs in the ECM in cultures only on incubation in the presence of vitamin C.

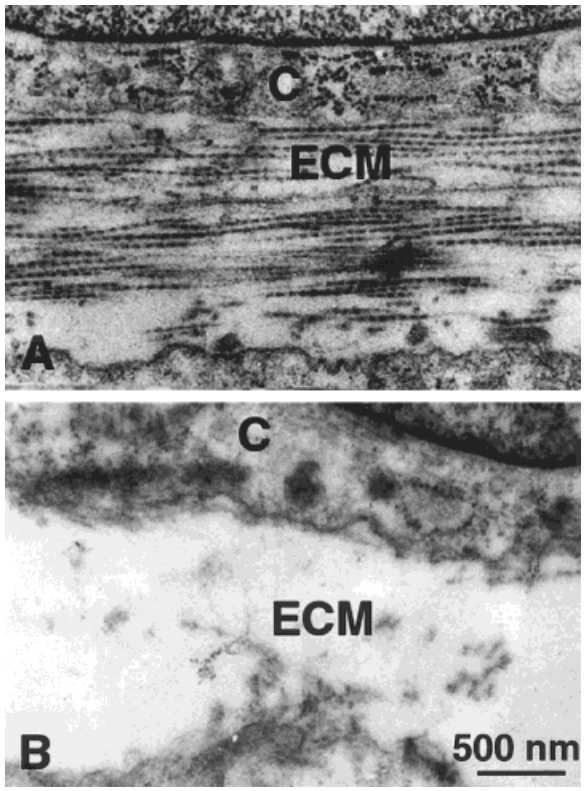

Fig. 1. Collagen fibril formation and deposition in the ECM in the presence of vitamin C. Transmission electron micrographs of the ECM in cultures of bovine aortic SMCs. Cells were seeded in four-well glass slides at a density of 7×10^4 cells/well (0.35×10^4 cells/cm^2), in normal culture media, and were allowed to grow for 9 days. Cultures were either supplemented daily with 20 μg/ml fresh vitamin C **(A)** or the equivalent volume of ddH_2O, as control **(B)**. At the end of this period, cultures were analyzed by transmission electron microscopy for the formation of striated collagen fibrils in the extracellular matrix. In the presence of ascorbate, striated fibrils were detected in the ECM. C, SMC. Scale bar = 500 nm.

Treatment of Bovine Vascular SMCs with Vitamin C Increases the Levels of Pepsin-Resistant Type V Collagen in the Cell-Layer-Associated Matrix

Because type V collagen has been implicated in control of fibril architecture, we sought to determine whether treatment with vitamin C affected the deposition of type V collagen. The levels of type V collagen in the cell-layer-associated matrix of cultures incubated in the presence versus absence of vitamin C were compared. Bovine aortic SMCs were seeded at a low density (0.35×10^4 cells/cm^2). Cultures allowed to grow for 9 days (postconfluent cultures) in normal medium (10% FBS-DMEM) in the absence of vitamin C. Then, either 20 μg/ml vitamin C or 20 μl/ml ddH_2O, as control, was added to the respective cultures. After incubation for 24 h, cultures were metabolically labeled with 25 μCi/ml [^{3}H]-proline for 8 h, and pepsin-resistant collagen was isolated from the cell-layer-associated material. Samples for electrophoresis were normalized for the amount of DNA present in the cultures. The collagen types present in these samples were separated by delayed reduction analysis, and the resulting gel was subjected to fluorography. In the absence of vitamin C (control cultures), types I and III collagen were the major species present in the sample (Fig. 2). Only very low levels of pepsin-resistant type V collagen were detected in the absence of vitamin C. Addition of vitamin C resulted in a significant induction in the levels of the cell-layer-associated type V collagen protein compared to the untreated cultures. In contrast, only minor changes in the levels of types I and III collagen were detected. Thus, treatment of bovine vascular SMCs with

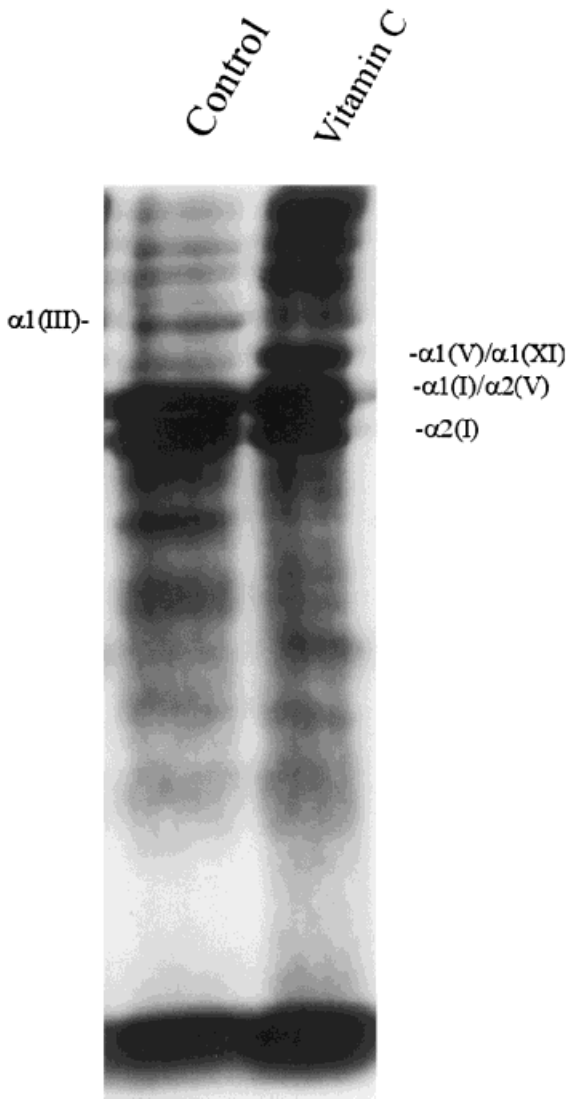

Fig. 2. Treatment with vitamin C increases the levels of pepsin-resistant type V collagen in the cell-layer-associated matrix. SMCs were plated at a density of 5×10^5 cells/P150 (0.35×10^4 cells/cm^2) dish in normal culture medium (10% FBS-DMEM), and were allowed to grow for 9 days (postconfluent cultures) in the absence of vitamin C. Then, cells were treated with 20 μg/ml vitamin C or the equivalent volume of ddH_2O, as control. After a 24-h incubation period, cultures were metabolically labeled with 25 μCi/ml [^{3}H]-proline for 8 h, and cell-layer-associated, pepsin-resistant material was extracted. Samples, normalized for DNA content, were analyzed by delayed reduction in a 7.5% polyacrylamide gel electrophoresis–sodium dodecyl sulfate gel and proteins were visualized by autoradiography. Positions of the types I, III, and V collagen protein chains were determined on the basis of comparison to molecular weight markers and purified collagen protein standards [Lawrence et al., 1994, and data not shown].

vitamin C results in a selective increase in the deposition of type V collagen protein in the ECM. Furthermore, formation of fibrils correlates with deposition of type V collagen.

Type V Collagen Lies Inside Heterotypic Collagen Fibrils, Surrounded by Type I Collagen

Type V collagen acted as a framework for the deposition of type I collagen into striated collagen fibrils produced in cornea [Birk et al., 1990]. To begin to assess the localization of types I and V collagen within fibrils produced by the vascular SMC, immunohistochemistry was done on untreated and acetic acid treated cultures. Minimal acetic acid treatment has been shown to disrupt the normal triple helical collagenous structure, and make internal collagen epitopes more accessible to antibodies [Doane et al., 1992b]. Bovine aortic SMCs were seeded at a low cell density (0.35×10^4 cells/cm^2) in 10% FBS-DMEM. Cultures, supplemented daily with 20 μg/ml vitamin C, were allowed to grow for either 2 days (subconfluent), 6 days (approaching confluence), or 9 days (postconfluent). Half of the cultures were extracted with 0.15 M acetic acid for 1 min. Cultures were fixed with 4% paraformaldehyde and analyzed by immunohistochemistry using antibodies specific for either type I or type V collagen or normal rabbit antiserum (IgG) and confocal microscopy (Fig. 3).

In the subconfluent cultures (day 2), a low level of type V collagen staining was easily detected in untreated as well as in acetic acid–treated cultures (Fig. 3, D2, upper panel). Disruption of the fibrillar structure by acetic acid treatment increased the signal only slightly (Fig. 3, D2, lower panel). In contrast, no staining was detected in acetic acid–extracted or not extracted cultures, which were incubated with the normal rabbit IgG (data not shown). Thus, at this early stage of collagen fibril assembly, epitopes of type V collagen are unmasked and accessible to the antibodies.

In cultures approaching confluence at day 6, type V collagen immunoreactivity was still easily detectable even without acid treatment (Fig. 3, D6, upper panel). However, acid disruption significantly increased the intensity of the signal (Fig. 3, D6, lower panel), suggesting that in this stage of collagen fibril assembly, type V collagen epitopes are partially masked within the fibrillar structure. On day 9, type V collagen was not detectable in these postconfluent

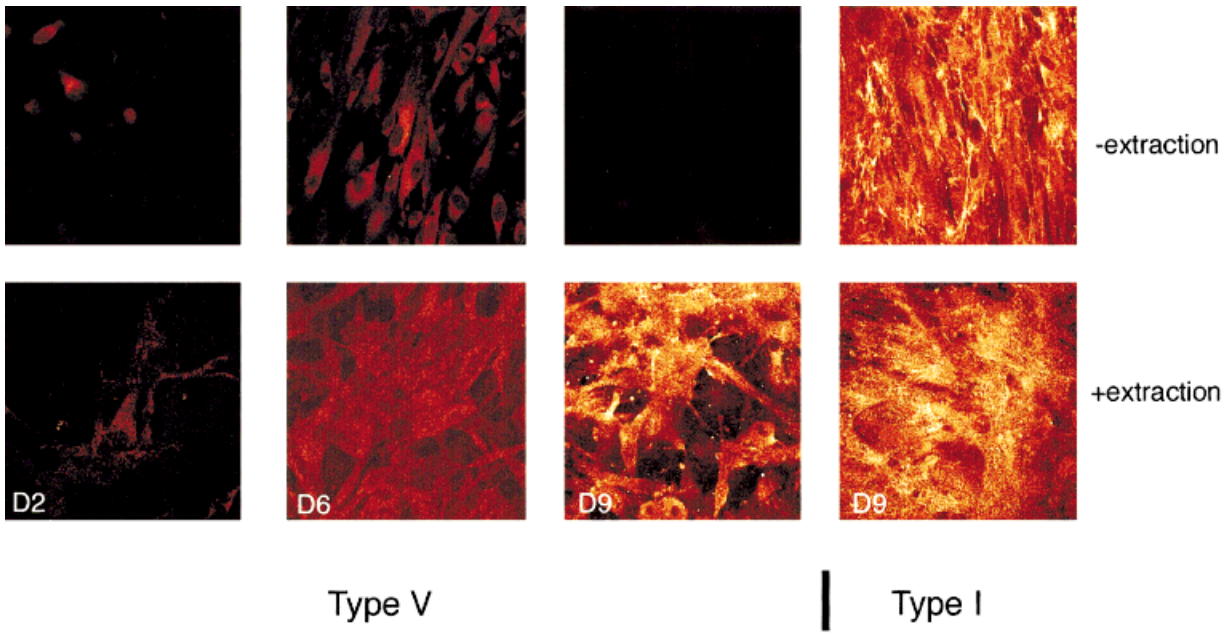

Fig. 3. Confocal localization of type I and V collagens in the developing collagen fibril. Bovine aortic SMCs were seeded in four-well glass slides at a density of 7×10^4 cells/well (0.35×10^4 cells/cm^2) in 10% FBS-DMEM, supplemented daily with 20 μg/ml vitamin C, and allowed to grow for 2, 6, or 9 days (upper panels) (−extraction). Where indicated, cultures were treated with 0.15 M acetic acid for 1 min, before fixation (lower panels) (+extraction), to disrupt the normal triple-helical collagenous structure, and allow antibody access. Cultures were then washed twice in PBS, fixed with 4% paraformaldehyde, and subjected to immunohistochemistry using either antibodies specific for type I (right panels) or type V (left panels) collagen. Samples were analyzed using a Leica upright confocal microscope, and all images correspond to the basal view of the cells. D2, day 2; D6, day 6; D9, day 9. Upper panels: No acid extraction; Lower panels: with acid extraction.

cultures before treatment with acetic acid (Fig. 3, D9, upper panel). Disruption of the triple helical structure of the fibril by acetic acid extraction resulted in a dramatic induction in the signal (Fig. 3, D9, lower panel). In contrast, type I collagen was detectable at all time points, including day 9, even without acid extraction (Fig. 3 and data not shown). No increase in the intensity of staining for this collagen type was seen on treatment with acetic acid (Fig. 3), suggesting that the epitopes for this collagen type lie on the external portion of the fibril. Furthermore, no staining was detected when normal rabbit IgG was used instead of primary antibody, as expected (data not shown). Taken together, these results suggest that type V collagen is incorporated early, and becomes internalized when type I collagen is laid down on the external portion of the heterotypic fibril.

Diameter of Collagen Fibrils Increases as a Function of Time in Culture

Previous work on the structure of in vitro synthesized type I/V collagen heterotypic fibrils suggested that the relative amount of type V collagen present in these fibrils correlates inversely with their diameter [Birk et al., 1990]. Because type V collagen appeared to be masked as the fibril matured, the effects of aging on collagen fibril diameter were next assessed. SMCs were seeded at a density of 0.35×10^2 cells/cm^2 in normal culture medium supplemented with 20 μg/ml fresh vitamin C, and allowed to grow for 6 or 9 days. Then, the diameter of collagen fibrils was determined using transmission electron microscopy. On day 6, thin filamentous structures with no apparent periodicity were observed (Fig. 4A,B). The diameter of these filaments was in the range of 8–10 nm. On day 9, collagen fibrils with normal periodicity were the most prominent matrix structures (Fig. 4E,F). These fibrils were significantly thicker than the nonperiodic filaments seen on day 6 and readily detectable, with a 67-nm normal periodicity. The diameter of these fibrils was in the range of 25–35 nm. To verify that the filaments were collagenous, cultures on days 6 (Fig. 4C,D) and 9 (data not shown) were analyzed by immunoelectron mi-

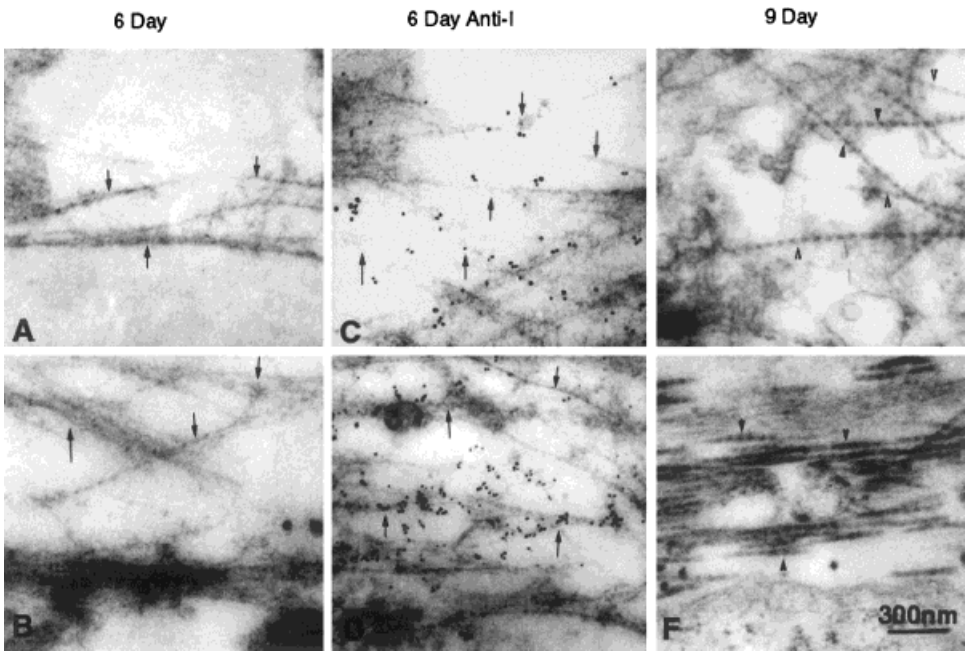

Fig. 4. The diameter of collagen fibrils increases as a function of days in culture. Bovine aortic SMCs were seeded in four-well glass slides at a density of 7×10^4 cell/well (0.35×10^4 cells/cm^2), and grown in 10% FBS-DMEM supplemented daily with 20 μg/ml fresh vitamin C for 6 days **(A–D)** or 9 days **(E and F)**. Cultures were analyzed by electron microscopy for the formation of striated collagen fibrils in the ECM. C and D: A collagen type I specific antibody and colloidal gold conjugated secondary antibodies were used for analysis of day 6 cultures by immunoelectron microscopy (6 Day Anti-I). Dots represent staining for type I collagen. Scale bar = 300 nm.

croscopy with a type I collagen-specific antibody, and visualized using a secondary antibody coupled to 5-nm gold particles. Gold particles were detected along these filamentous structures, indicating that they are made of collagen. Thus, the diameter of collagen fibrils increases as a function of time in culture.

Ratio of Type V to Type I Collagen Decreases as a Function of Time in Culture

The observed order of collagen deposition, as well as the increase in the diameter of the fibrils with fiber maturation, suggested that the ratio of type V to type I collagen might be higher in the initial stages of fibril assembly, and decrease as collagen fibers become mature. To determine the relative ratio of type V to type I collagen in the ECM of cultured SMCs as a function of maturation, cells were plated at a low cell density and incorporated newly synthesized collagen proteins monitored as a function of time. Cultures, maintained in 10% FBS-DMEM supplemented daily with fresh 20 μg/ml vitamin C, were incubated for 1 h with 25 μCi/ml [^{3}H]-proline at 6 h (day 0), or 2 days after plating. Pepsin resistant collagen was isolated from the cell-layer-associated material. Samples were normalized for DNA content, and analyzed by delayed-reduction gel electrophoresis (Fig. 5A). Positions of the various bands were determined by migration of collagen and molecular weight standards. The relative intensity of the bands corresponding to α1(V)/αI(XI) and α2(I) collagen chains was measured by densitometric analysis of the resulting autoradiograph. On day 0 (6 h after plating), this ratio was 0.69. On day 2, it dropped to 0.35. In a separate experiment, the relative ratio of type V to type I collagen in the pepsin-resistant fraction was similarly determined after 6 and 9 days (Fig. 5B). On day 6, a ratio of 0.12 was obtained, whereas on day 9, the value was only 0.06. Thus, the relative amount of type V compared to type I collagen deposited in the ECM varies inversely as a function of days in cultures; it is very high during the initial stages of fibril formation and decreases dramatically as fibril structure becomes more mature. Thus, the relative amount of type V collagen within these collagen fibrils

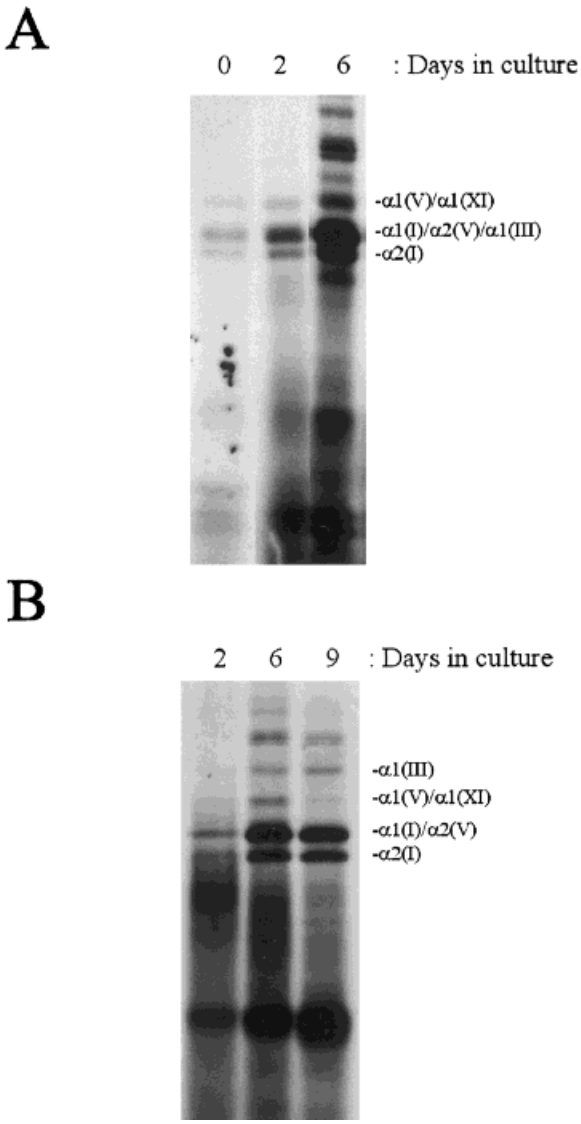

Fig. 5. The ratio of type V to type I collagen decreases as a function of days in culture. **A:** Bovine aortic SMCs were plated at a density of 5×10^5 cells/P150 dish (0.35×10^4 cells/cm^2) and maintained in 10% FBS-DMEM, supplemented daily with fresh 20 μg/ml vitamin C. Six hours (day 0), 2 days (day 2), or 6 days (day 6) after plating, cultures were incubated in 25 μCi/ml [^{3}H]-proline for 1 h. Pepsin-resistant collagen was isolated from the cell-layer-associated material, and samples were normalized for DNA content and analyzed in a 7.5% PAGE-SDS gel. **B:** Bovine aortic SMCs were plated as described above. Two days (day 2), 6 days (day 6), or 9 days (day 9) after plating, cultures were incubated in 25 μCi/ml [^{3}H]-proline for 8 h. After isolation of pepsin-resistant collagen from the cell-layer-associated material, samples were normalized for DNA content, analyzed by delayed reduction in a 7.5% PAGE-SDS gel, and subjected to autoradiography. Positions of the types I, III, and V/XI collagen protein chains were made on the basis of comparison to molecular weight markers and purified collagen protein standards [Lawrence et al., 1994, and data not shown].

decreases with fibril maturation, correlating inversely with fibril diameter.

DISCUSSION

Vitamin C plays a critical role in deposition of type V collagen into the ECM of vascular SMCs. The dramatic increase in the level of type V collagen was accompanied by the formation of striated collagen fibrils with a 67-nm normal periodicity. Using immunohistochemistry, type V collagen appeared internal as collagen fibrils matured. In contrast, type I collagen was always on the external portion of the fibril. Furthermore, the relative amount of type V collagen present in the ECM varied inversely with time in culture and the diameter of collagen fibrils. These findings suggest that type V collagen is an important structural component of the ECM produced by the vascular SMC, similar to its role in the developing cornea. Type V collagen appears to act as a framework for the assembly of collagen monomers into mature fibrils, and regulates the diameter of these fibrils.

Previous work by many groups indicated that collagen fibril formation and deposition in the ECM requires vitamin C [Rosenbloom et al., 1973; Berg et al., 1973; Jimenez et al., 1973b; Faris et al., 1984]. This effect was attributed to the high thermal stability of properly hydroxylated type I collagen trimers. In the absence of vitamin C, type I collagen molecules are underhydroxylated [Hutton et al., 1966; Kivirikko and Prockop, 1967; Pinnell, 1987]. Thus, the triple-helical structure is less stable against thermal denaturation, with a melting temperature of approximately 25°C vs. 42°C for properly hydroxylated collagens [Rosenbloom et al., 1973; Berg and Prockop, 1973; Jimenez et al., 1973b]. Here, we show that in the absence of vitamin C, essentially no type V collagen was deposited in the ECM, although significant levels of types I and III collagen were detectable. Furthermore, no significant reactivity for type V collagen was detected in acid-extracted cultures that were cultured in the absence of vitamin C (data not shown), consistent with the absence of type V collagen from the ECM of vitamin C deprived cultures. Addition of vitamin C to the cultures resulted in a selective induction in the levels of type V protein. This induction occurred concomitantly with formation of striated fibrils with 67-nm normal periodicity. Thus, in addi-

tion to the previously reported underhydroxylation of collagen monomers, the lack of type V collagen in the ECM may also be responsible for the lack of formation of striated fibrils in the absence of vitamin C. Consistent with this hypothesis, our findings suggest that type V collagen is laid down early, during the initial stages of collagen fibril assembly, and becomes internal as collagen fibrils develop. Type V collagen was readily detectable on day 2 even in the absence of acetic acid treatment. As cultures became older and fibrils became mature, detection of type V collagen required disruption of the triple-helical structure. In contrast, type I collagen was always readily detectable even without acetic acid treatment, consistent with an external position in the fibril. Taken together, these results suggest that type V collagen is important in the initiation of collagen fibril formation, and may act as a framework for the deposition of type I collagen molecules into the developing fibrils.

Fibril diameter varied inversely with the relative amount of type V collagen present in the ECM of cultured bovine aortic SMCs. A similar inverse relationship between fibril diameter and the amount of type V collagen present within a fibril has been observed in collagen fibrillogenesis experiments in vitro. Recent in vivo studies also showed that type V collagen regulates fibril diameter in the chicken cornea [Marchant et al., 1996], where types I and V collagen coassemble within heterotypic fibrils [Birk et al., 1988]. In addition, data obtained from transgenic animal studies showed that collagen type V plays a very important role in the regulation of collagen fibril diameter [Andrikopoulos et al., 1995]. More specifically, mice that express a structurally abnormal $\alpha 2$(V) collagen chain, which lacks the hinge region, exhibited skin fragility and collapse of the corneal stroma. These abnormalities correlated with type I collagen fibrils of unusually large diameters.

Work by Ooshima [1981], Morton and Barnes [1982], and Murata and coworkers [1986] has shown that type V collagen is increased by as much as twofold relative to types I and III collagen in human atherosclerotic lesions. The data presented here indicate that the relative increase in the levels of type V collagen might result in a decrease in fibril diameter. Consistent with this hypothesis, Merrilees et al. [1987] found a decrease in the average fibril diameter within the intimal lesion versus the normal medial layer. Thus, our findings suggest that the physiologic consequences of the increased expression of type V collagen in the diseased artery is the altered fibril architecture in the plaque compared to the normal artery. The factors regulating expression of type V collagen are currently under investigation.

ACKNOWLEDGMENTS

We thank D. Sloneker for assistance in preparation of this article. This work was supported by National Institutes of Health grants EY05129 (D.B.), EY06000 (V.T-R.), and HL13262 (G.E.S.).

REFERENCES

Andrikopoulos K, Liu X, Keene DR, Jaenisch R, Ramirez F. 1995. Targeted mutation in the col5a2 gene reveals a regulatory role for type V collagen during matrix assembly. Nature Genetics 9:31–36.

Ang AH, Tachas G, Campbell JH, Bateman JF, Campbell GR. 1990. Collagen synthesis by cultured rabbit aortic smooth-muscle cells. Alteration with phenotype. Biochem J 265:461–469.

Beldekas J, Gerstenfeld L, Sonenshein GE, Franzblau C. 1982. Cell density and estradiol modulation of procollagen type III in cultured calf smooth muscle cells. J Biol Chem 257:12252–12256.

Berg RA, Prockop DJ. 1973. The thermal transition of a non-hydroxylated form of collagen. Evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen. Biochem Biophys Res Commun 52:115–120.

Birk DE, Fitch JM, Babiarz JP, Linsenmayer TF. 1988. Collagen type I and type V are present in the same fibril in the avian corneal stroma. J Cell Biol 106:999–1008.

Birk DE, Fitch JM, Babiarz JP, Doane KJ, Linsenmayer TF. 1990. Collagen fibrillogenesis in vitro: interaction of types I and V collagen regulates fibril diameter. J Cell Sci 95:649–657.

Brown KE, Lawrence R, Sonenshein GE. 1991. Concerted modulation of $\alpha 1$(XI) and $\alpha 2$(V) collagen mRNAs in bovine vascular smooth muscle cells. J Biol Chem 266: 23268–23273.

Chamley-Campbell J, Campbell GR, Ross R. 1979. The smooth muscle cell in culture. Physiol Rev 59:1–61.

Chang CJ, Sonenshein GE. 1991. Increased collagen gene expression in vascular smooth muscle cells cultured in serum or isoleucine deprived medium. Matrix 11:242–251.

Chung E, Rhodes K, Miller EJ. 1976. Isolation of three collagenous components of probable basement membrane origin from several tissues. Biochem Biophys Res Commun 71:1167–1174.

Doane KJ, Yang G, Birk DE. 1992a. Corneal cell-matrix interactions: type VI collagen promotes adhesion and spreading of corneal fibroblasts. Exp Cell Res 200:490–499.

Doane KJ, Babiarz JP, Fitch JM, Linsenmayer TF, Birk DE. 1992b. Collagen fibril assembly by corneal fibroblasts in three-dimensional collagen gel cultures: small-diameter heterotypic fibrils are deposited in the absence of keratan sulfate proteoglycan. Exp Cell Res 202:113–124.

Faris B, Ferrera R, Toselli P, Nambu J, Gonnerman WA, Franzblau C. 1984. Effect of varying amounts of ascorbate on collagen, elastin and lysyl oxidase in aortic smooth muscle cell cultures. Biochim Biophys Acta 797: 71–75.

Fessler JH, Fessler LI. 1987. Type V collagen. New York: Academic Press.

Grushkin-Lerner LS, Trinkaus-Randall V. 1991. Localization of integrin and syndecan in vivo in a corneal epithelial abrasion and keratectomy. Curr Eye Res 10:75–85.

Hutton JJ Jr, Trappel AL, Udenfriend S. 1966. Requirements for alpha-ketoglutarate, ferrous ion and ascorbate by collagen proline hydroxylase. Biochem Biophys Res Commun 24:179–184.

Jimenez SA, Dehm P, Olsen BR, Prockop DJ. 1973a. Intracellular collagen and procollagen from embryonic tendon cells. J Biol Chem 248:720–729.

Jimenez SA, Harsch M, Rosenbloom J. 1973b. Hydroxyproline stabilizes the triple helix of chick tendon collagen. Biochem Biophys Res Commun 52:106–114.

Kindy MS, Chang C-J, Sonenshein GE. 1988. Serum deprivation of vascular smooth muscle cells enhances collagen gene expression. J Biol Chem 263:11426–11480.

Kivirikko KI, Prockop DJ. 1967. Hydroxylation of proline in synthetic polypeptides with purified protocollagen hydroxylase. J Biol Chem 242:4007–4012.

Kypreos KE, Sonenshein GE. 1998. Basic fibroblast growth factor decreases type V/XI collagen expression in cultured bovine aortic smooth muscle cells. J Cell Biochem 68:247–258.

Laskey RA, Mills AD. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur J Biochem 56:335–341.

Lawrence R, Hartmann DJ, Sonenshein GE. 1994. Transforming growth factor beta 1 stimulates type V collagen expression in bovine vascular smooth muscle cells. J Biol Chem 269:9603–9609.

Liau G, Chan, LM. 1989. Regulation of extracellular matrix RNA levels in cultured smooth muscle cells. Relationship to cellular quiescence. J Biol Chem 264:10315–10320.

Linsenmayer TF. 1991. Collagen. New York: Plenum Press.

Linsenmayer TF, Fitch JM, Gross J, Mayne R. 1985. Are collagen fibrils in the developing avian cornea composed of two different collagen types? Evidence from monoclonal antibody studies. Ann NY Acad Sci 460:232–245.

Linsenmayer TF, Gibney E, Igoe F, Gordon MK, Fitch JM, Fessler LI, Birk DE. 1993. Type V collagen: molecular structure and fibrillar organization of the chicken alpha 1(V) NH2- terminal domain, a putative regulator of corneal fibrillogenesis. J Cell Biol 21:1181–1189.

Marchant JK, Hahn RA, Linsenmayer TF, Birk DE. 1996. Reduction of type V collagen using a dominant-negative strategy alters the regulation of fibrillogenesis and results in the loss of corneal-specific fibril morphology. J Cell Biochem 135:1415–1426.

Merrilees MJ, Tiang KM, Scott L. 1987. Changes in collagen fibril diameters across artery walls including a correlation with glycosaminoglycan content. Connect Tissue Res 16:237–257.

Morton LF, Barnes MJ. 1982. Collagen polymorphism in the normal and diseased blood vessel wall. Investigation of collagens types I, III and V. Atherosclerosis 42:41–51.

Murata K, Motayama T, Kotake C. 1986. Collagen types in various layers of the human aorta and their changes with the atherosclerotic process. Atherosclerosis 60:251–262.

Nakamura K, Inoue S, Abiko S, Aoki H, Takeo K. 1989. Improved separation of alpha chains of collagen type I, type III, and type V by noninterrupted electrophoresis using thioglycolic acid as a negatively charged reducer. Electrophoresis 10:29–33.

Ooshima A. 1981. Collagen alpha B chain: increased proportion in human atherosclerosis. Science 213:666–668.

Peterkofsky B. 1991. Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy. Am J Clin Nutr 4:1135S–1140S.

Pinnell SR. 1987. The skin in Ehlers-Danlos syndrome [letter]. J Am Acad Dermatol 16:399–400.

Rosenbloom J, Harsch M, Jimenez S. 1973. Hydroxyproline content determines the denaturation temperature of chick tendon collagen. Arch Biochem Biophys 158:478–484.

Ross R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 362:801–809.

Ross R. 1999. Atherosclerosis—an inflammatory disease. N Engl J Med 340:115–126.

Sage H, Bornstein P. 1979. Characterization of a novel collagen chain in human placenta and its relation to AB collagen. Biochemistry 18:3815–3822.

Wu XY, Cornell-Bell A, Davies TA, Simons ER, Trinkaus-Randall V. 1994. Expression of integrin and organization of F-actin in epithelial cells depends on the underlying surface. Invest Ophthalmol Vis Sci 35:878–890.