

# Expression of Extra Domain A Fibronectin Sequence in Vascular Smooth Muscle Cells Is Phenotype Dependent

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**Abstract.** Different fibronectin (FN) variants arise from the single gene transcript alternatively spliced in a tissue-specific manner (Hynes, R. O. 1985. *Annu. Rev. Cell Biol.* 1:67-90; Owens, R. J., A. R. Kornblihtt, and F. E. Baralle. 1986. *Oxf. Surv. Eurcaryotic Genes.* 3:141-160). We used mAb IST-9, specific for extra domain A (ED-A) FN sequence, and cDNA probe to ED-A exon to determine whether ED-A is present in FN synthesized by vascular smooth muscle cells (SMCs) and, if so, whether expression of ED-A is SMC phenotype dependent. ED-A-containing FN (A-FN) was not revealed in tunica media of human arteries and normal rat aorta by immunofluorescence and immunoblotting techniques. A cDNA probe to ED-A exon did not hybridize with RNA isolated from human aortic media. A positive reaction with IST-9 was observed in (a) diffuse intimal thickening and atherosclerotic plaque from human arteries; (b) ex-

perimentally induced intimal thickening in rat aorta; and (c) cultured vascular SMCs. A-FN mRNA was present in the RNA preparation from human aortic intima as judged by hybridization with cDNA probe to ED-A.

On the other hand, an mAb interacting with an epitope common for all FN variants revealed FN in both intima and media of human arteries and in the normal rat aorta. A cDNA probe to a sequence shared by all FN variants hybridized with RNA from both intima and media of human aorta, though the level of expression was higher in intima. The data suggest that ED-A exon is omitted during splicing of the FN mRNA precursor in medial SMCs while the expression of A-FN is characteristic of "modulated" SMCs—those of intimal thickenings, of atherosclerotic lesions, and growing in culture.

**F**IBRONECTINS (FNs)<sup>1</sup> are high molecular weight glycoproteins playing an important role in different cellular phenomena—e.g., adhesion, migration, and differentiation—and, thereby, in embryogenesis, wound healing, hemostasis, and thrombosis (26, 35, 61). The FN molecule is a dimer with a subunit mass of ~250,000 D. The primary structure of FN has been studied both on protein (human and bovine FN) and cDNA (chicken, rat, and human FN) levels (7, 16-18, 32, 38, 42, 45, 50, 53, 55). The FN subunit is composed of three different kinds of repeating sequences (32, 45, 53). Two fibrin-binding domains, NH<sub>2</sub> and COOH terminal, and a collagen-binding domain, NH<sub>2</sub> terminal, are made up of 45-50-amino acid-long repeats types I and II, while a 90-amino acid-long repeat type III composes the central 150,000-180,000-D part of the FN subunit (45, 53). Subtle alterations in subunits give rise to various FN forms. Cir-

culating form, or plasma FN (pFN), is synthesized by hepatocytes; fibroblasts, endothelial cells, and some other cells produce cellular FN (cFN), which is accumulated in the insoluble extracellular matrix (6, 43, 52, 54, 57). pFN was also shown to penetrate from circulation into the tissue and to be incorporated in the extracellular matrix (39).

The differences in the primary structures of FN synthesized by various cells are the results of alternative splicing of the RNA transcript of the single FN gene (25, 41). Sequence variations can occur in three points of the FN subunit: extra domains A and B (ED-A and ED-B, respectively) and III<sub>CS</sub> in human FN; and EIIIA, EIIIB, and V in the rat protein (23, 25, 41, 51, 62). The two former sequences are either omitted or included, while the latter one varies in length, if present. ED-A and ED-B exons were not detected in liver mRNA, and the corresponding peptides are absent in pFN; however, ED-A and ED-B were identified in mRNA from fibroblasts and several other cell lines (6, 13, 30-32, 43, 51, 54, 57). Thus, ED-A and ED-B sequences code for unique domains of cFN.

The microheterogeneity of FN synthesized by different

1. *Abbreviations used in this paper:* A-FN, fibronectin containing extra domain A sequence; cFN, cellular fibronectin; ED-A, extra domain A; ED-A<sup>-</sup>FN, fibronectin that lacks ED-A; FN, fibronectin; pFN, plasma fibronectin; SM, smooth muscle; SMC, smooth muscle cell.

cells is intensively studied now not only with specific cDNA probes but also with antibodies recognizing certain FN sequences (6, 8–10, 44, 57). mAb IST-9, described earlier, recognizes the ED-A fragment of human FN and, therefore, reacts with cFN rather than pFN (6, 8). The ED-A sequence corresponds to a 270-nucleotide exon coding for one unit of repeat type III (58). We used IST-9 and the cDNA probe for the ED-A sequence to reveal ED-A-containing FN (A-FN) and the corresponding mRNA in vascular smooth muscle cells (SMCs).

The mature mammalian artery wall consists of three layers: tunica intima, tunica media, and tunica adventitia (46). In the infant human aorta, as well as in the normal adult aorta of the majority of the mammals, tunica intima appears to be rather thin, consists of only endothelial monolayer and underlying basement membrane, and does not contain SMCs. On the contrary, in the grossly normal segments of the adult human arteries (aorta, carotid, iliac arteries, etc.) a diffuse intimal thickening is always found. The major cell type in this layer as well as in tunica media is smooth muscle (SM) (46). Atherosclerotic lesions basically involve only the intimal layer of the arteries.

SMCs both in situ and in culture are known to express phenotypes that represent various differentiation states (11, 14, 36, 49). The cytoplasm of differentiated or "contractile" SMCs is packed with myofilaments and contains very little mitochondria, rough endoplasmic reticulum, free ribosome, etc. In "synthetic" or "modulated" SMCs, the organelles are quite numerous while contractile elements are reduced. Synthetic cells produce and secrete a lot of extracellular matrix material. SMCs from atherosclerotic lesions of human arteries, as well as those from experimental intimal thickening in animal arteries, express the synthetic or fibroblast-like phenotype (14, 37, 48, 49). In the tunica media of mature arteries, SMCs are in the contractile state, but they undergo a spontaneous change in phenotype (phenotypic modulation) if they are isolated from the tissue by enzyme treatment and seeded into culture. Here we use the term modulated to de-

scribe an SMC phenotype altered, at least partly, from that of differentiated medial SMCs. It is well known that modulated SMCs may retain some characteristics of the contractile phenotype. For instance, alpha-SM-actin is expressed in SMCs proliferating in culture (40); and subendothelial intimal cells, which were shown to contain decreased amounts of the SM markers gamma-vinculin, meta-vinculin, and 150-kD caldesmon have an isoactin pattern typical of contractile vascular SMCs (4, 20).

Both the level of FN gene transcription and the pattern of FN mRNA splicing were shown to be altered in response to physiological stimuli—exposure to growth factors—and on transformation (1, 5, 6, 10, 27, 51). Therefore, we have suggested that the changes expressed in the spectrum of FN variants may be associated with SMC phenotypic modulation.

The aim of this study was to determine whether the ED-A sequence specific for cFN is present in FN synthesized by vascular SMCs and, if so, whether the expression of ED-A is SMC phenotype dependent. We were able to demonstrate that A-FN is synthesized by modulated rather than contractile vascular SMCs.

## Materials and Methods

### mAbs

mAbs IST-9 and IST-4 to human FN (6, 8, 52) were a generous gift of Dr. L. Zardi (Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy). IST-9 recognizes the ED-A sequence present only in cFN; IST-4 interacts with an epitope that is located within the first four type III homology repeats and shared by different FN subunit variants (52). Undiluted culture supernatants were used for both immunofluorescence and immunoblotting. Mouse mAb 4.29.AB raised against human pFN and cross reacting with different mammalian FN was kindly provided by Dr. M. Chernousov (Institute of Experimental Cardiology, Cardiology Research Center, Moscow, USSR). For staining of rat aorta sections ascites fluid was diluted 1:100.

mAb IIG10, raised against cultured human aortic SMCs, recognizes a surface antigen of SMCs and fibroblasts; endothelial cells, macrophages/monocytes, peripheral blood lymphocytes, etc., were shown to be IIG10 negative (19). The IgM fraction purified from ascites fluid was diluted to 50 µg/ml for immunostaining.

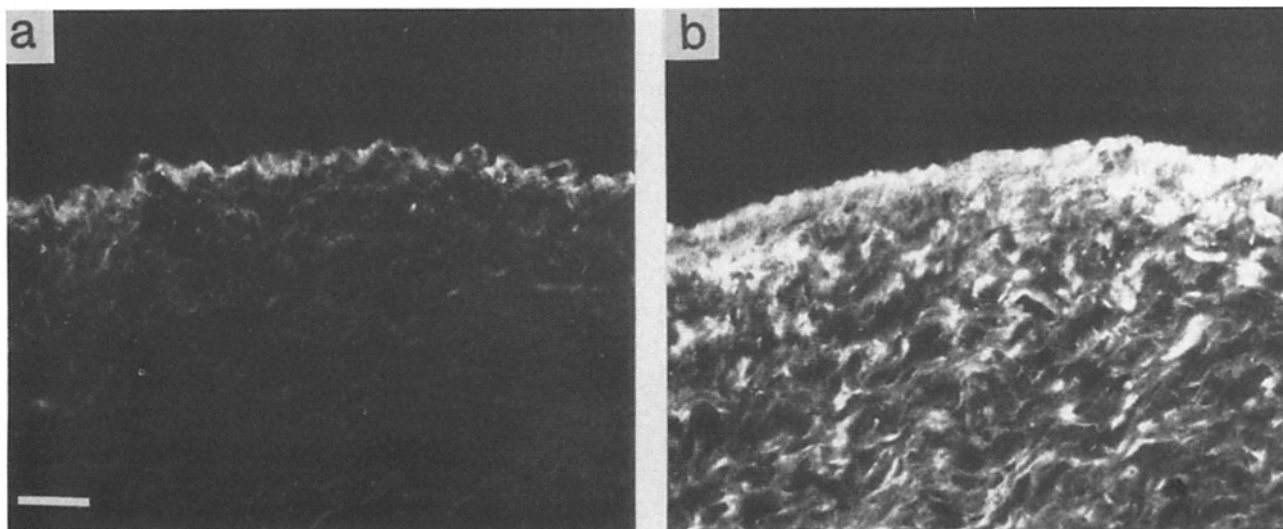
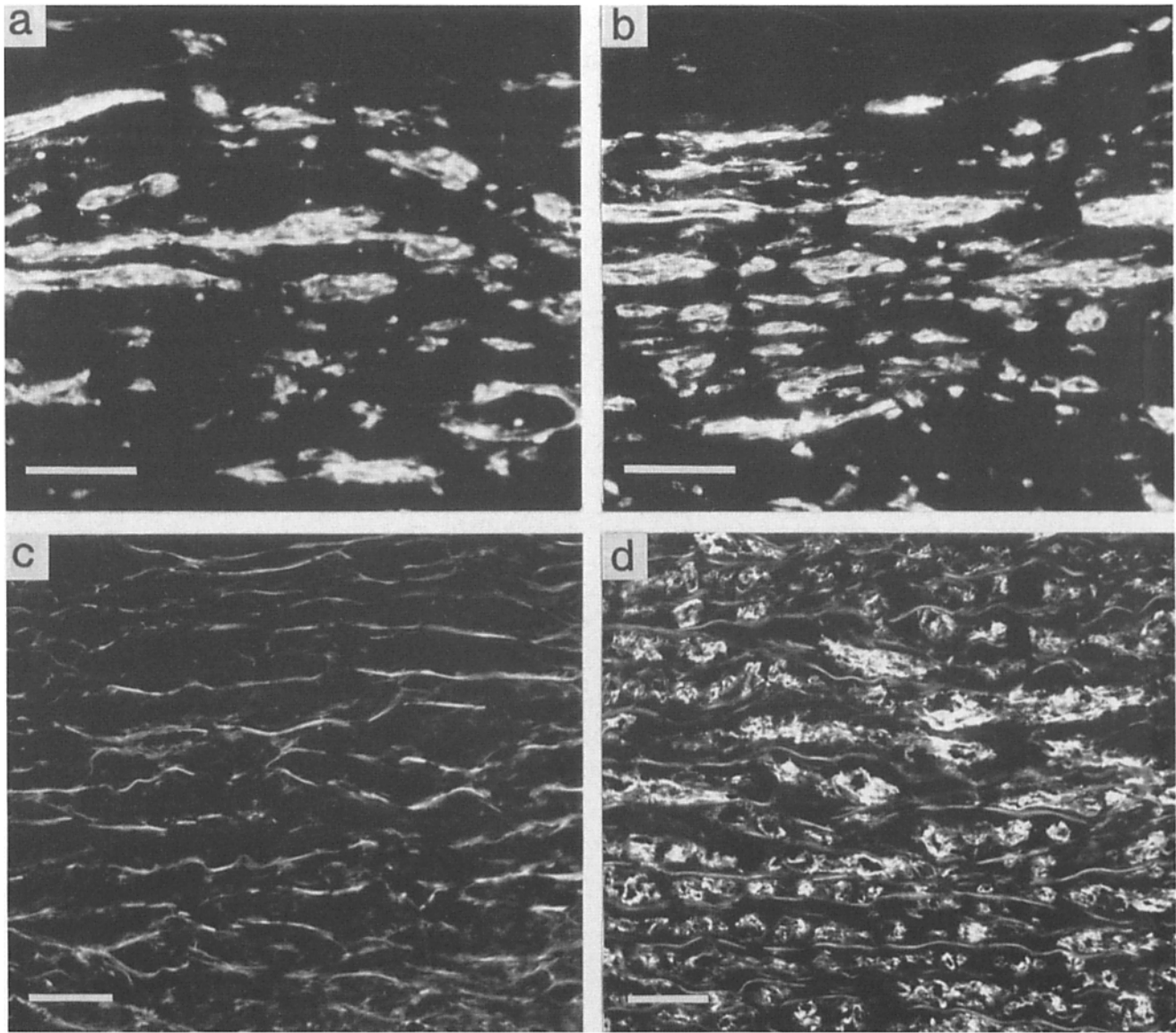


Figure 1. Indirect immunofluorescence staining of 3-mo-old human child thoracic aorta serial sections with anti-FN mAb. (a) mAb IST-9 (A-FN); (b) mAb IST-4 (total FN). Bar, 10 µm.



**Figure 2.** Indirect immunofluorescence staining of human adult aortic intima and media sections with anti-FN mAb. (a and b) Diffuse intimal thickening; (c and d) media. (a and c) mAb IST-9 (A-FN); (b and d) mAb IST-4 (total FN). Bars, 20  $\mu\text{m}$ .

### Human Tissue Samples

Human artery samples were obtained at autopsies taken within 4–6 h of death. For immunoblotting analysis, arteries were opened; adventitia was discarded; endothelium was removed mechanically; and intima and media were separated, frozen in liquid nitrogen, minced, and dispersed in Laemmli sample buffer (33). For immunofluorescence, segments of the arteries were dropped into freezing isopentane, and 5- $\mu\text{m}$  cryostat sections were cut.

### Balloon Injury Model

Male Wistar rats (350–400 g) were used in the experiments. Balloon injury of the aorta was performed via left carotid artery according to a modification (22) of the method originally developed by Baumgartner (3). The animals were killed on day 14, and thoracic aortas were dissected, washed in DME, frozen, and cut into 5- $\mu\text{m}$  cryostat sections.

### Cell Culture

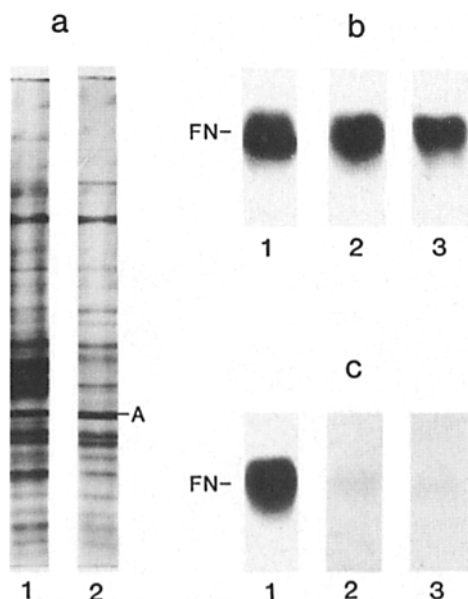
SMCs from media and intima of human aorta were isolated by collagenase-elastase digestion of the tissue (11), and cultured in DME (Flow Laboratories, Inc., McLean, VA) supplemented with 10 mM Hepes, 100

$\mu\text{g/ml}$  sodium pyruvate, 50  $\mu\text{g/ml}$  ascorbic acid, 0.6 mg/ml L-glutamine, 50  $\mu\text{g/ml}$  gentamicin sulfate, and 10% heat-inactivated human serum. In the primary culture >90% of intimal and medial cells were stained with anti-SM-actin antibody, CGA-7, kindly provided by Dr. Allen M. Gown (Department of Pathology, University of Washington, Seattle, WA).

### Immunofluorescence and Immunoblotting

For immunofluorescence, frozen sections were fixed in absolute acetone for 5 min at room temperature. SMCs growing on the coverslips were washed in PBS, fixed with 4% paraformaldehyde, and again washed in PBS. Fixed sections or cells were incubated with primary antibodies for 1 h at room temperature, washed in PBS for 30 min, incubated with FITC-rabbit anti-mouse IgG (Miles Scientific Div., Naperville, IL) diluted 1:40 for 45 min, thoroughly washed, air dried, and mounted in UV-inert embedding medium (Serva Fine Biochemicals Inc., Garden City Park, NY). Specimens were observed with a microscope (E. Leitz, Inc., Wetzlar, FRG) using 25- and 16 $\times$  objectives. Photographs were taken with Tri-X film (Eastman Kodak Co., Rochester, NY).

Immunoblotting was performed according to the method of Towbin et al. (56).  $^{125}\text{I}$ -Labeled rabbit anti-mouse IgG (2  $\mu\text{g/ml}$ , sp act  $0.5 \times 10^6$  cpm/ $\mu\text{g}$ ) was used as a secondary antibody.



**Figure 3.** Immunoblotting analysis of FN variants present in human aortic intima and media. (a) SDS-PAGE (5–15%) of the human aortic intimal and medial extracts, Coomassie blue-stained gel. (b and c) Autoradiographs of the immunoblots done with mAb IST-4 (b), recognizing total FN, and IST-9 (c), recognizing A-FN. (Lane 1) Intimal extract; (lane 2) medial extract; (lane 3) purified pFN. A, actin; FN, fibronectin. The loading for immunoblotting for intimal extract (b and c, lanes 1) was the same as in a, for medial extract: five times as much as in a, lane 2.

### RNA Extraction and Hybridization

Separated intima and media of human aorta were homogenized in guanidine thiocyanate lysis buffer (6 M guanidine thiocyanate, 0.2 M Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM  $\beta$ -mercaptoethanol, 1% *N*-laurylsarkosin) followed by phenol extraction (12). For dot hybridization, denatured RNA was diluted in 10 mM Tris-HCl, pH 7.6, to achieve the desired concentration, and immobilized on a Z-probe blotting membrane (Bio-Rad Laboratories, Richmond, CA) using Minifold apparatus (Schleicher & Schuell, Inc., Keene, NH). The membranes were baked and prehybridized for at least 4 h in buffer containing 50% deionized formamide, 1% SDS, 10% Dextran sulfate, 0.25 M Na-phosphate, pH 7.5, 0.25 M NaCl, 5 mM EDTA, 0.125% pirophosphate, 1 mg/ml heparin, and 0.25 mg/ml of sonicated chicken erythrocyte DNA. Hybridization was performed at 50°C for 18–24 h in the same buffer containing the appropriate  $^{32}$ P-labeled DNA probe ( $10^6$  cpm/ml). The filters were washed, dried, and exposed to XAR-5 film (Eastman Kodak Co.) at  $-70^{\circ}\text{C}$ .

pFH1 probe (29) was kindly provided by Dr. F. E. Baralle and contained a 1,700-bp sequence coding for human FN, inserted in pATI53/Pvu II/8 plasmid vector. Plasmid DNA was isolated, nick translated (47), denatured, and used in hybridization assay. A 173-bp probe to ED-A FN sequence was kindly provided by Dr. L. Zardi and labeled by nick translation.

## Results

### Localization of A-FN in the Human Artery Wall

Transverse sections of 3-mo-old human child aorta, which had no diffuse intimal thickening, were stained with mAbs IST-9, specific for the ED-A region of FN, and IST-4, recognizing an epitope shared by all FN variants. Data presented in Fig. 1 a show that only the luminal surface of the aorta wall was stained with IST-9. The pattern of the staining was practically identical to that obtained with the antibody to factor VIII, a marker of the endothelial cells (data not shown); however, an SM layer of the wall (the whole media) was

negative and, thus, did not contain A-FN. On the other hand, in the serial section, IST-4 revealed a significant amount of FN (Fig. 1 b). The most intensive fluorescence was concentrated in the juxtaluminal region and, although moderate, it was quite obvious in the rest of the wall. The data show that only FN that did not contain ED-A (ED-A<sup>-</sup> FN) was present in the SM layer of the 3-mo-old human child aorta.

To determine whether A-FN is present in the adult human aorta, the transverse sections of the grossly normal segments of the aorta (from 45–65-yr-old donors) were stained with IST-9. A positive reaction was observed only in the intima (Fig. 2); media did not stain with antibody, and only elastic fibers were visible in Fig. 2 c because of their intensive autofluorescence.

The antigenic determinant recognized by IST-9 could be masked in the media by the tissue components. Therefore, SDS extracts of separated media and intima were analyzed by immunoblotting (Fig. 3). The content of FN in the intimal extract was obviously higher than in the medial; therefore, to make total FN loading in both extracts similar, the amount of medial extract applied to the gel was approximately five times greater than that of intimal, as judged by actin content (see Fig. 3 a). Nevertheless, A-FN was found only in the intima, while the media contained only traces of IST-9-positive material, if any (Fig. 3 c).

In atherosclerotic fibrous plaque from the human iliac artery, media also did not contain A-FN while intima stained with mAb IST-9; both of the layers contained FN, as was revealed by mAb IST-4 (Fig. 4). SMCs in the intima and media were stained with mAb IIG10.

### Identification of ED-A Sequence in the mRNA from Human Aorta

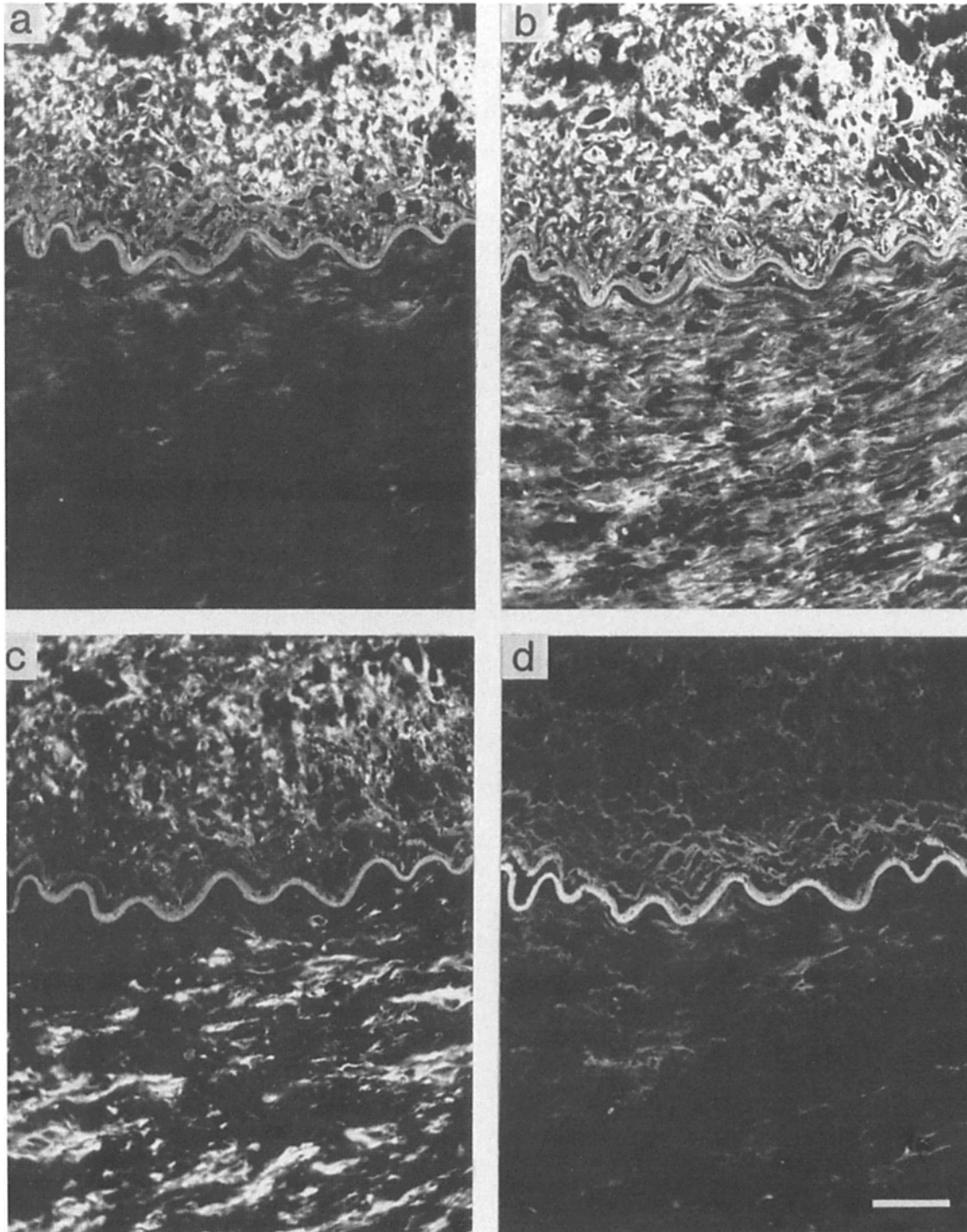
To determine whether A-FN mRNA species were represented in mRNA from human aorta, a cDNA probe to ED-A sequence was hybridized with total RNA prepared from the media and subendothelial intima of human adult aorta. To reveal total FN mRNA, a cDNA probe to the sequence present in all FN subunit types was used. The data shown in Fig. 5 demonstrate that FN mRNA was present in RNA preparations from both intima and media, and that the amount of ED-A-containing species was almost negligible in medial RNA and rather high in intimal RNA. Notably, total FN mRNA content in intimal RNA preparations was three- to fivefold higher compared with medial RNA.

### A-FN in the Extracellular Matrix of Cultured SMCs from Intima and Media of Human Adult Aorta

SMCs were isolated by enzyme digestion of the separated media and intima of human adult aorta, seeded into culture, and on days 5 and 14 stained with mAb IST-9 to reveal A-FN. Medial as well as intimal SMCs secreted A-FN, which was already visible on the cell surface on the fifth day of cultivation, being incorporated in the characteristic extracellular matrix network (Fig. 6). Northern blot analysis demonstrated the presence of ED-A sequence in RNA preparation from cultured medial SMCs (data not shown).

### Identification of A-FN in the Experimental Intimal Thickening in the Rat Aorta

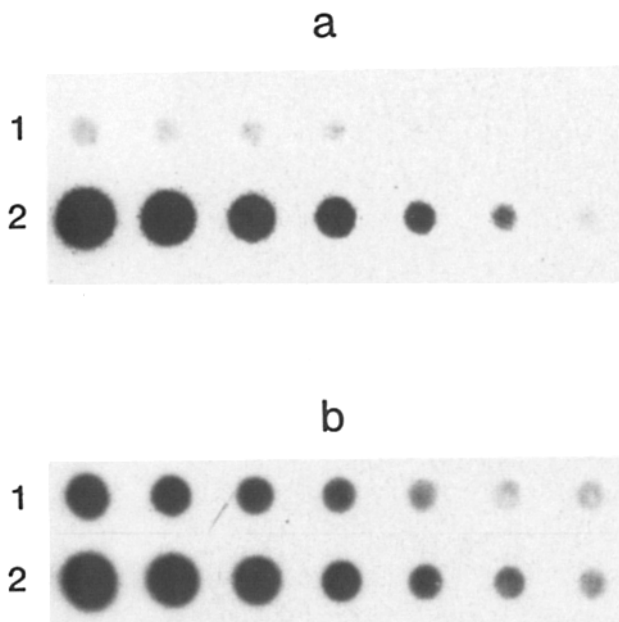
In the normal rat aorta wall only the endothelial lining was



**Figure 4.** Serial sections showing indirect immunofluorescence staining of atherosclerotic plaque from human iliac artery. (a) mAb IST-9 (A-FN); (b) mAb IST-4 (total FN); (c) mAb IIG10 (SMC surface antigen); (d) negative control (section was incubated with PBS instead of the primary antibody). Bar, 20  $\mu\text{m}$ .

stained with IST-9; the SM layer was negative (Fig. 7). The whole media was stained with mAb 4.29.AB, which interacts with an epitope present in all FN types. mAb IST-4 could not be used in these experiments since it did not cross react with rat FN. In the neointima, which was formed in the aorta as

a response to endothelial injury with a balloon catheter, mAb IST-9 revealed A-FN; the underlying media remained IST-9 negative. Thus, only ED-A-negative FN (ED-A<sup>-</sup> FN) was present in the SM layer of the normal rat aorta, while in the experimental intimal thickening A-FN was found.



**Figure 5.** Dot hybridization assay identifying FN mRNA in RNA preparation from human aortic intima and media. cDNA probe to ED-A exon of human FN (*a*) and pFH 1 probe to human FN (*b*) were hybridized with total RNA isolated from media (1) and intima (2) of human aorta. RNA amount in the initial point, 10  $\mu$ g; dilution factor, 2.

## Discussion

### *Expression of A-FN Is Characteristic of Modulated Rather Than Contractile Vascular SMCs*

We have shown that tunica media of the arteries did not contain A-FN and that ED-A-containing mRNA species were practically absent in the RNA samples from media. On the contrary, in the diffuse intimal thickening of the adult human aorta, as well as in atherosclerotic fibrous plaque, the content of A-FN was rather high and the ED-A sequence was revealed in the RNA prepared from human adult aortic intima.

It is well established that the expression of differentiated SMC phenotype characteristics is altered in the intimal thickenings of human and animal arteries and in atherosclerotic lesions (2, 4, 15, 20, 28, 34, 36, 59, 60). At birth, sub-endothelial intima of human aorta usually consists of only a thin connective tissue layer which does not contain SMCs. With aging, intima becomes progressively thickened as SMCs of the subjacent layers migrate to the intima, proliferate, synthesize, and secrete extracellular matrix components. In the 3-mo-old human child aorta, A-FN was found only at the luminal surface rather than in SM layer. On the contrary, by analyzing an 11-yr-old human child aorta we were able to identify minor intimal thickenings which were all IST-9 positive and, thus, contained A-FN (not shown). In the arteries of the adult donors (45–60 yr old), A-FN was always found in the thickened intima regardless of the presence of atherosclerotic lesions. Also, cultured medial, as well as intimal, SMCs secrete A-FN which is incorporated into extracellular matrix network. Therefore, we conclude that the expression of A-FN is associated with the synthetic or modulated rather than contractile SMC phenotype.

It should be mentioned that besides SMCs, some blood-borne cells, particularly monocytes/macrophages, might be present in intima, especially in its preluminal part. However, even if present, these cells would not contribute appreciably to the production of A-FN in the intima because of their extremely low density. Recently we have shown that isoactin pattern of the cells from diffuse intimal thickening was identical to that of medial cells (4, 20) and, therefore, it can be concluded that the vast majority of intimal cells are of SM origin. On the other hand Gown et al. have demonstrated that SMCs are a predominant cell type present in the atherosclerotic fibrous plaque (21).

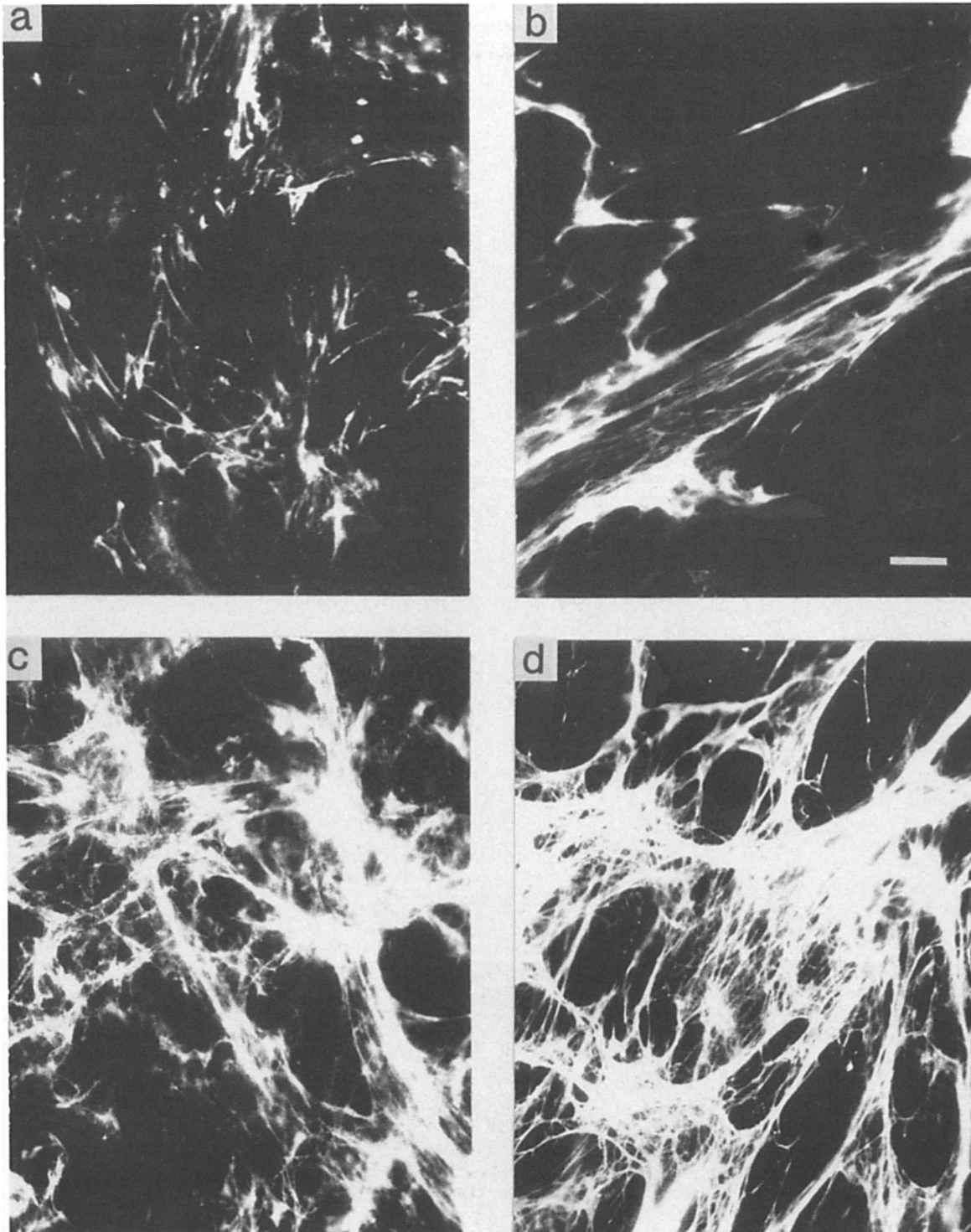
### *Phenotypic Changes of SMCs Are Accompanied by the Changes of FN mRNA Splicing Pattern*

Medial SMCs are known to undergo a phenotypic transition in primary culture. In the animal arteries in vivo, phenotypic modulation occurs when endothelial integrity is disturbed by a balloon catheter—contractile medial SMCs become activated, migrate to the intima, proliferate, and form intimal thickening. We were able to demonstrate that in both cases the transition from a contractile to a synthetic state was accompanied by the A-FN accumulation and, since the RNA preparation from the human aortic media did not contain the ED-A sequence, by the alteration of the FN mRNA splicing pattern. The data suggest that the FN mRNA splicing pattern is controlled not only in the tissue-specific manner, but can be altered in the SMCs in response to physiological stimuli that cause phenotypic modulation. Recently, the cell type-specific fashion of alternative splicing of the rat and human FN gene transcript was shown to be a subject for modulation also during transformation (1, 6, 10, 51).

SMC phenotype modulation, observed as a response to the endothelium injury, is associated with cell migration—i.e., with the changes of the cell–matrix interaction involving neccitins and integrins. Phenotypic modulation of the SMC isolated by the enzyme digestion of media begins only when the cell is well adhered to the surface and spread. Hedin et al. have shown that SMCs lose contractile phenotype very rapidly if seeded onto a FN-coated surface, but rather slowly if laminin is used as a coating protein (24). FN provides better adhesion and spreading for SMCs than laminin does. In the process of adhesion, cell–extracellular matrix linkages are formed that may dramatically differ from those in the tissue. Thus, not just the exposure to the growth factors but also the alteration of the cell–matrix interrelationship might serve as a signal to the cascade events that lead to phenotypic modulation: (a) reduction and even loss of SM-characteristic contractile and cytoskeletal proteins; (b) an increase of extracellular matrix component production and secretion and (c), as we demonstrate here, the expression and accumulation of A-FN (i.e., the alteration of FN mRNA splicing pattern).

### *Only ED-A<sup>-</sup> FN Is Expressed in Contractile Vascular SMCs*

The presence of mRNA encoding FN in the RNA preparation from media, as well as a significant amount of FN found in the media of the aorta and iliac artery, prove that the local synthesis of FN exists and, since A-FN was not found in the media, only ED-A<sup>-</sup> species are synthesized. Earlier, hepa-



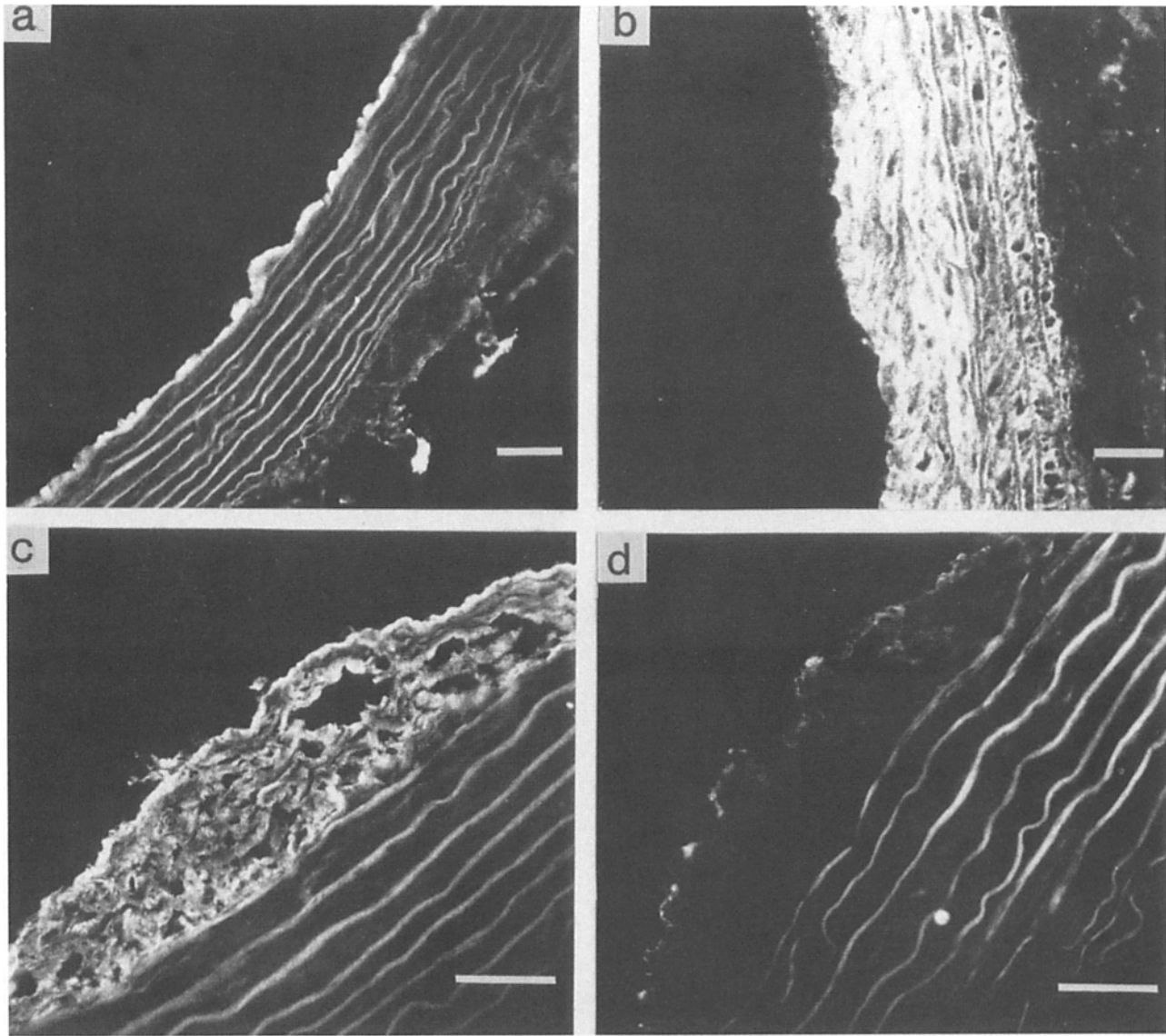
**Figure 6.** Indirect immunofluorescence staining of cultured SMCs from the intima and media of human aorta with mAb IST-9. (*a* and *b*) 5 d of cultivation; (*c* and *d*) 14 d of cultivation. (*a* and *c*) Medial cells; (*b* and *d*) intimal cells. Bar, 3  $\mu$ m.

toocytes were shown to produce exclusively ED-A<sup>-</sup> FN and, therefore, to be a source of pFN. Our data suggest that at least a part of the circulating ED-A<sup>-</sup> FN might originate from vascular SMCs.

It should be mentioned that the absence of A-FN in the media may be a result of (*a*) a decreased stability of the protein and/or corresponding mRNA; or (*b*) an alteration of the FN

mRNA splicing pattern. The first possibility seems unlikely since ED-A<sup>-</sup> containing mRNA was not detected in RNA preparation from media.

Although A-FN is present in the intima, it is not yet clear whether it is the only FN variant synthesized locally since ED-A<sup>-</sup> FN might be expressed in the intimal layer as well. It seems important to study the pattern of FN variants synthe-



**Figure 7.** Indirect immunofluorescence staining of rat aorta sections with anti-FN mAb. (*a* and *b*) Normal aorta; (*c* and *d*) segments with thickened intima. (*a* and *c*) mAb IST-9 (A-FN); (*b*) mAb 4.29.AB (total FN); (*d*) negative control (section was incubated with PBS instead of the primary antibody). Bars, 50  $\mu$ m.

sized by intimal and medial SMCs that express different phenotypes. Besides the antibodies recognizing certain domains of the FN molecule, cDNA probes to variable regions of FN might be a useful tool in such a study, and in situ hybridization seems to be a most promising approach.

In conclusion, we demonstrate here that (*a*) the FN gene is expressed in both medial and intimal SMCs of the human arteries, though the level of expression is higher in intimal SMCs; (*b*) the ED-A exon is always omitted during splicing of FN mRNA precursor in medial SMC and, therefore, only ED-A<sup>-</sup> FN is produced by these cells; and (*c*) the expression of A-FN is characteristic of modulated vascular SMCs—those of intimal thickenings, atherosclerotic lesions, and growing in culture.

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