Diverse Effects of Fibronectin and Laminin on Phenotypic Properties of Cultured Arterial Smooth Muscle Cells

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Abstract. Plasma fibronectin promotes modulation of rat arterial smooth muscle cells from a contractile to a synthetic phenotype during the first few days in primary culture. This process includes cell adhesion and spreading, loss of myofilaments, and formation of a widespread rough endoplasmic reticulum and a prominent Golgi complex. The structural reorganization is accompanied by activation of overall RNA and protein synthesis. Moreover, the cells gain the ability to replicate their DNA and divide in response to platelet-derived growth factor. Here, it is demonstrated that the power of fibronectin to bring about this change in the differentiated properties of the smooth muscle cells resides in a 105-kD cell-binding fragment, whereas a 70-kD collagen-binding fragment and a 31-kD heparin-binding fragment are inactive in this respect. Laminin, another adhesive glycoprotein and a component of the basement membrane that normally surrounds arterial smooth muscle, was contrarily found to maintain the cells in a contractile phenotype. However, with increasing time more and more cells went through the modulation into a synthetic phenotype. This "catch-up" was counteracted by a peptide that contained the cell-attachment sequence of fibronectin (Arg-Gly-Asp-Ser). Hence, it is possible that the delayed modulation on laminin was due to production of fibronectin by the cells themselves. In support of this notion, fibronectin isolated from smooth muscle cultures was found to be as effective as plasma fibronectin in stimulating the phenotypic modulation. Moreover, using a combination of chemical, immunochemical, and immunocytochemical methods, it was demonstrated that the cells secreted fibronectin as well as laminin at an increasing rate during the first 4 d in primary culture and, notably, cells cultured on laminin produced more fibronectin than cells cultured on fibronectin. Newly synthesized fibronectin was incorporated into a network of pericellular and intercellular fibrils, whereas laminin formed a more diffuse layer covering the cells in a basement membrane–like manner. Taken together, the findings suggest diverse roles for fibronectin and laminin in the control of the differentiated properties of arterial smooth muscle cells. They further indicate that the ability of arterial smooth muscle cells to produce fibronectin and laminin early in primary culture is not directly related to the phenotypic state as determined morphologically and by measurement of overall rates of RNA and protein synthesis. This may be due to the cells being able to sense the macromolecular composition of the pericellular matrix and to modify their secretory activity accordingly. In the intact organism, fibronectin and laminin may fulfill important functions during development and growth of the vascular system, as well as in atherogenesis.

F IBRONECTIN is a 500-kD glycoprotein found in blood plasma and extracellular matrices. It consists of two subunit chains linked by disulfide bonds close to their carboxy-terminal ends. Each subunit is divided into domains with binding specificities for cell surface components, collagen, heparin, and fibrinogen/fibrin. By means of these domains, fibronectin mediates the attachment and spreading of cells on a variety of substrates, and influences their migration, growth, and differentiation (Hynes, 1985; Ruoslahti et al., 1985; Yamada et al., 1985; Dufour et al., 1986). Recently, we reported that plasma fibronectin promotes modulation of arterial smooth muscle cells (SMCs)[1] from a con-

tractile to a synthetic phenotype early in primary culture (Hedin and Thyberg, 1987). This process, which includes marked alterations in cell morphology with loss of myofilaments and formation of an extensive rough endoplasmic reticulum (RER) and a large Golgi complex, is a prerequisite for the onset of cellular proliferation (Chamley-Campbell et al., 1979; Thyberg et al., 1983). In vivo, a similar change in the differentiated properties of the SMCs appears to be one of the initial events in the development of an atherosclerotic lesion (Ross, 1986; Schwartz et al., 1986).

In the present investigation, we have studied the role of plasma fibronectin in SMC phenotypic modulation in more detail. The cells were seeded on substrates of intact fibronectin, a 105-kD cell-binding fragment, a 70-kD collagen-binding fragment, or a 31-kD heparin-binding fragment (Woods

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1. *Abbreviations used in this paper:* PDGF, platelet-derived growth factor; RER, rough endoplasmic reticulum; SMC, smooth muscle cell.

et al., 1986; Perris and Johansson, 1987), and cultured under serum-free conditions. They were examined by light and electron microscopy, as well as biochemically, to follow the structural transformation, the activation of overall RNA and protein synthesis, and the ability to synthesize DNA in response to platelet-derived growth factor (PDGF), a powerful mitogen for cells of mesenchymal origin (Heldin et al., 1985; Ross et al., 1986). For comparative purposes and to further define the specificity of the action of fibronectin, the cells were also cultured on substrates of laminin or type IV collagen, two major components of the basement membrane that normally surrounds smooth muscle (Chamley-Campbell et al., 1979; Timpl and Dziadek, 1986). In an attempt to distinguish the possible effect of fibronectin produced by the SMCs themselves, cells were grown on a substrate of laminin in the presence of the peptide GRGDS (Gly-Arg-Gly-Asp-Ser), which contains the cell-attachment sequence (RGDS) of fibronectin (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984), or the control peptide GRGES (Gly-Arg-Gly-Glu-Ser), both distinct from the cell-attachment sequence of laminin (Graf et al., 1987). The secretion and extracellular deposition of fibronectin and laminin by the SMCs when cultured on different substrates was studied by double indirect immunofluorescence microscopy, biosynthetic labeling and immunoprecipitation in combination with SDS-PAGE and fluorography, and an ELISA.

The results indicate that extracellular matrix components play an important role in the control of the phenotypic properties of cultured arterial smooth muscle. Adhesion to a substrate of fibronectin promotes modulation of the cells into a synthetic, PDGF-responsive phenotype, whereas adhesion to a substrate of laminin or type IV collagen holds the cells back in a contractile phenotype. The rates of secretion of fibronectin and laminin increased day by day early in primary culture, partly depending on the composition of the substrate on which the cells were seeded, but not in strict correspondence to the rate of modulation of the cells from a contractile to a synthetic phenotype. Fibronectin was incorporated into a pericellular and intercellular network of fibrils, whereas laminin was arranged in a basement membrane–like layer.

Materials and Methods

Cell Culture

SMCs were isolated from the aortic media of 300–400-g male Sprague-Dawley rats by digestion with 0.1% collagenase in Ham's medium F-12 supplemented with 10 mM each of the organic buffers Hepes and Tes (pH 7.3), 50 μg/ml of L-ascorbic acid, 50 μg/ml of gentamycin sulfate, and 0.1% BSA (medium F-12/0.1% BSA; for further details, see Palmberg et al., 1985; Hedin and Thyberg, 1987). After rinsing and counting, the cells were suspended in medium F-12/0.1% BSA, seeded on appropriate substrates at a density of 5×10^4 cells/cm², and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air (medium changed every second day). To initiate DNA synthesis, 20 ng/ml of pure human PDGF (Heldin et al., 1987) was added to the medium. Light microscopic examination of the cultures was done with an inverted microscope (model CK2; Olympus Corporation of America, New Hyde Park, NY) using the phase–contrast mode.

Preparation of Substrates

Human plasma fibronectin was isolated by adsorptive chromatography on gelatin-Sepharose 4B (Ruoslahti et al., 1982; Hedin and Thyberg, 1987). The following fragments of human plasma fibronectin were isolated as de-

scribed: a 105-kD cell-binding fragment (Woods et al., 1986), a 70-kD collagen-binding fragment (Perris and Johansson, 1987), and a 31-kD heparin-binding fragment (Woods et al., 1986; see Fig. 1). Type IV collagen (human placenta) was purchased from Sigma Chemical Co. (St. Louis, MO). Laminin was extracted from the murine Engelbreth-Holm swarm sarcoma and purified by DEAE–cellulose chromatography and agarose gel filtration (Timpl et al., 1979). For preparation of substrates, the proteins were dissolved at 20 μg/ml in Dulbecco's PBS (pH 7.4), added to the culture dishes (3–4 μg/cm²), and allowed to adsorb at 25°C for 60 min. The vessels were then rinsed twice with PBS and left in medium F-12/0.1% BSA for 5 min before use.

The peptides GRGDS (Gly-Arg-Gly-Asp-Ser) and GRGES (Gly-Arg-Gly-Glu-Ser) were produced using an automated synthesizer and further purified by reverse-phase HPLC (Åke Engström, Department of Immunology, Uppsala University, Uppsala, Sweden).

Biochemical Assays of RNA and Protein Synthesis

Cells were seeded in 12-well multidishes (4 cm²/well) and cultured in medium F-12/0.1% BSA. To assay RNA and protein synthesis (days 2, 4, and 6), they were exposed to 2 μCi/ml of [³H]uridine (38 Ci/mmol; Amersham International, Amersham, UK) for 6 h and 2 μCi/ml of [³H](4,5)-L-leucine (155 Ci/mmol; Amersham International) for 6 h, respectively. The cultures were then rinsed three times and macromolecular material was precipitated with cold 5% TCA. After three rinses with cold 5% TCA, the cells were dissolved in 0.1 M KOH. Aliquots of the lysates were mixed with Safeflour (Lumac, Schaesberg, Netherlands) and radioactivity was determined in a liquid scintillation spectrometer (Packard Instruments Co., Inc., Downs Grove, IL). The protein content of the lysates was analyzed by the dye-binding method of Bradford (1976), using BSA as the standard. Parallel cultures were treated with 0.25% trypsin and 0.02% EDTA in calcium- and magnesium-free PBS (pH 7.4), and the cells counted in a hemocytometer.

Autoradiographic Assay of DNA Synthesis

Cells were seeded on plastic coverslips in 24-well multidishes (2 cm²/well), cultured in medium F-12/0.1% BSA, exposed to 2 μCi/ml of [³H]-thymidine (5 Ci/mmol; Amersham International) for 24 h (days 2, 4, and 6), and fixed in 3% cacodylate-buffered glutaraldehyde. After dehydration in ethanol, the coverslips were mounted on glass slides, dipped in Kodak NTB 2 emulsion, exposed at 4°C for 2 d, developed in Kodak D-19, and stained with 1% methylene blue (Eastman Kodak Co., Rochester, NY). The labeling index was determined by counting at least 500 cells in each specimen.

Preparation of Cellular Fibronectin

SMCs were grown to confluence (2 wk) in medium F-12 with 10% newborn calf serum (Gibco, Paisley, Scotland) that had been depleted of fibronectin by prior chromatography on a column of gelatin-Sepharose 4B (Hedin and Thyberg, 1987). The cultures were rinsed and incubated in medium F-12/0.1% BSA with 5 μCi/ml of [³H](4,5)-L-leucine (155 Ci/mmol; Amersham International) for 24 h. After collection of the media, the cell layers were extracted with 1.0 M deionized urea in PBS (pH 7.4) for 2 h at 37°C on a gyratory shaker (Yamada et al., 1975). The media and cell layer extracts were centrifuged at 10,000 g for 10 min, dialyzed against 0.05 M Tris-HCl buffer (pH 7.5) with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.02% NaN₃, combined, and subjected to affinity chromatography on gelatin-Sepharose 4B (Hedin and Thyberg, 1987). The isolated cellular fibronectin was visualized by SDS-PAGE and fluorography (see below) and its protein concentration was determined by the methods of Lowry et al. (1951) and Bradford (1976), using BSA as the standard.

Immunological Reagents

Rabbit antiserum against rat fibronectin was prepared as described (Johansson and Höök, 1984). Antibodies against a 31-kD heparin-binding fragment and against a 65-kD cell-binding fragment of fibronectin were raised in hens and purified from egg yolks (Woods et al., 1986, 1988). Rabbit antiserum to purified mouse laminin was purchased from E. Y. Laboratories, Inc. (San Mateo, CA), rhodamine-conjugated goat anti–rabbit IgG from Cappel Laboratories (Malverne, PA), and FITC-conjugated goat anti–chicken IgG from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD).

Biosynthetic Labeling and Immunoprecipitation

Primary SMC cultures were set up in 60-mm plastic petri dishes and labeled for 24-h intervals with 50 μCi/ml of [^{35}S]L-methionine (>800 Ci/mmol; New England Nuclear, Braunschweig, FRG) in MEM without L-methionine. Parallel dishes were treated with 0.25% trypsin and 0.02% EDTA in calcium- and magnesium-free PBS (pH 7.4), and the cells counted in a hemocytometer. The media of the labeled cultures were collected in Eppendorf tubes and made 0.1 mM in PMSF. The cell layers were extracted for 20 min with 1.5 ml PBS (pH 7.3) containing 2.0 M urea, 0.5% NP-40, 0.1% SDS, 0.1% BSA, and 0.1 mM PMSF (Holderbaum and Ehrhart, 1986) and then transferred to Eppendorf tubes (scraping with a rubber policeman). The petri dishes were rinsed with another 0.5 ml of the same buffer and the combined cell layer fractions were drawn repeatedly through a 0.4-mm (inner diameter) needle. Thereafter, 60 μl of protein A-Sepharose slurry (50% packed beads in 0.15 M NaCl, 20 mM Tris, 0.2% Triton X-100, pH 7.3; Pharmacia Fine Chemicals, Uppsala, Sweden) were added and the samples were incubated for 60 min at 4°C. After centrifugation at 10,000 g for 5 min at 4°C and transfer of the supernatants to fresh Eppendorf tubes, the samples were incubated overnight at 4°C with 10 μl of the appropriate immune serum (i.e., rabbit anti–rat fibronectin or rabbit anti–laminin); to the control samples, 10 μl of rabbit preimmune serum was added instead of the specific antisera. To recover the immune complexes, 60 μl of protein A-Sepharose slurry was added, and the incubation was prolonged for 60 min at 4°C. The beads were then sedimented by centrifugation at 10,000 g for 5 min and washed three times with PBS/0.1% BSA (pH 7.3), all at 4°C. To detach the adsorbed material, 80 μl of SDS sample buffer (Laemmli, 1970) was added and the beads were heated to 95°C for 4 min.

SDS-PAGE and Fluorography

After reduction in SDS sample buffer, the samples were subjected to electrophoresis for 8–10 h at constant current in polyacrylamide gradient gels (Laemmli, 1970). Fixation and staining in 40% methanol/10% acetic acid with Coomassie Brilliant Blue R-250 were followed by destaining in 40% methanol/10% acetic acid. Gels destined for fluorography were subsequently dehydrated in glacial acetic acid, impregnated with 18% 2,5-diphenyloxazole in glacial acetic acid, and rehydrated in 0.5% glycerol. The gels were dried onto filter paper under vacuum and heat. Fluorography was performed with preexposed Kodak X-omat AR-5 film (Eastman Kodak Co., Rochester, NY) and Kyokko BX-III intensifying screens. Exposures were made at −70°C for 9 d. The fluorograms were scanned with a densitometer (model No. CS-930; Shimadzu Scientific Instruments, Inc., Columbia, MD) to determine the relative intensities of the bands. For estimation of molecular weights, the gels were calibrated with protein standard kits from Bio-Rad Laboratories (Richmond, CA) or Pharmacia Fine Chemicals.

ELISA

Freshly isolated SMCs were seeded in 35-mm plastic petri dishes covered with appropriate substrates and grown in medium F-12/0.1% BSA (changed each day). Media were collected (days 1-4), centrifuged at 10,000 g for 5 min, and the supernatants were frozen at −20°C. The cells were detached by trypsinization and counted in a hemocytometer. The fibronectin content of the samples was determined in duplicate using ELISA as follows. Microtiter wells were coated overnight at 4°C with 3 μg/ml of affinity-purified chicken IgG against a 65-kD cell-binding fragment of fibronectin. Unoccupied sites were blocked with PBS/1% BSA for 90 min at 37°C. After exposure to culture media (diluted 1:10), bound fibronectin was detected by sequential incubations with rabbit antiserum against rat fibronectin (diluted 1:200) and goat anti-rabbit IgG conjugated with alkaline phosphatase (diluted 1:1,000; Sigma Chemical Co.); all dilutions were made in PBS/1% BSA and the incubations were performed at 37°C for 90 min. p-Nitrophenylphosphate in diethanolamine (pH 9.8) was added and after 5-10 min the reaction was stopped with 3 M NaOH. Absorbance was measured at 405 nm and the fibronectin content of the samples was calculated from a standard curve set up in parallel with known concentrations of human plasma fibronectin.

Immunofluorescence Microscopy

SMCs were seeded in 24-well multidishes on substrate-coated glass coverslips and grown in medium F-12/0.1% BSA. After 1, 2, 4, and 6 d, the cultures were washed twice with PBS, fixed in 3% formaldehyde in PBS for 10 min at 20°C, and washed again in PBS. Fibronectin and laminin were visualized by indirect immunofluorescence microscopy, using a double-

staining protocol. The specimens were exposed to chicken antibodies against a 31-kD heparin-binding fragment of fibronectin (100 μg/ml) and rabbit antiserum against laminin (diluted 1:100) in PBS for 60 min at 37°C; for control purposes, the primary antibodies were preadsorbed with fibronectin and laminin, respectively. The coverslips were then rinsed three times in PBS for 20 min and exposed to FITC-conjugated goat anti-chicken IgG and rhodamine-labeled goat anti-rabbit IgG (both diluted 1:50 in PBS) for 60 min at 37°C. After rinsing in PBS, the coverslips were mounted in 90% glycerol with 0.1% paraphenylenediamine. The specimens were examined in a Zeiss Standard Junior fluorescence microscope and photographs were taken on Kodak Tri-X-Pan film.

Electron Microscopy

The cells were fixed in situ with 3% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) with 0.05 M sucrose for 1–2 h, scraped off the petri dishes with a rubber policeman, and transferred to small plastic tubes in fresh fixative. After rinsing in buffer, the specimens were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) with 0.5% potassium ferrocyanate for 1 h at 4°C, dehydrated in ethanol, stained with 2% uranyl acetate in ethanol, and embedded in low viscosity epoxy resin. Thin sections were cut on an LKB Ultrotome IV, stained with alkaline lead citrate, and examined in an electron microscope (model EM300; Philips, Eindhoven, Netherlands) operated at 80 kV. To follow the structural transformation of the SMCs, one large section from each culture was scanned without overlapping and all cells (200–400) were registered as being either in a contractile (cytoplasm dominated by myofilaments) or a synthetic (cytoplasm dominated by RER cisternae and a large Golgi complex) phenotype. Cells in intermediate stages accounted for a low percentage of the total and it was seldom difficult to assign the cells to one phenotype or the other.

Results

Substrate Influences on Arterial SMC Morphology

SMCs enzymatically isolated from the aorta of adult rats are distinguished by a cytoplasm filled with microfilaments and
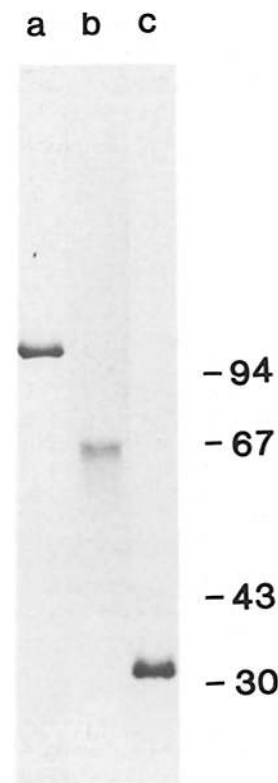
Figure 1. SDS-PAGE (7–15% gel) of isolated fibronectin fragments. Lane *a*, 105-kD cell-binding fragment; lane *b*, 70-kD collagen-binding fragment; lane *c*, 31-kD heparin-binding fragment.
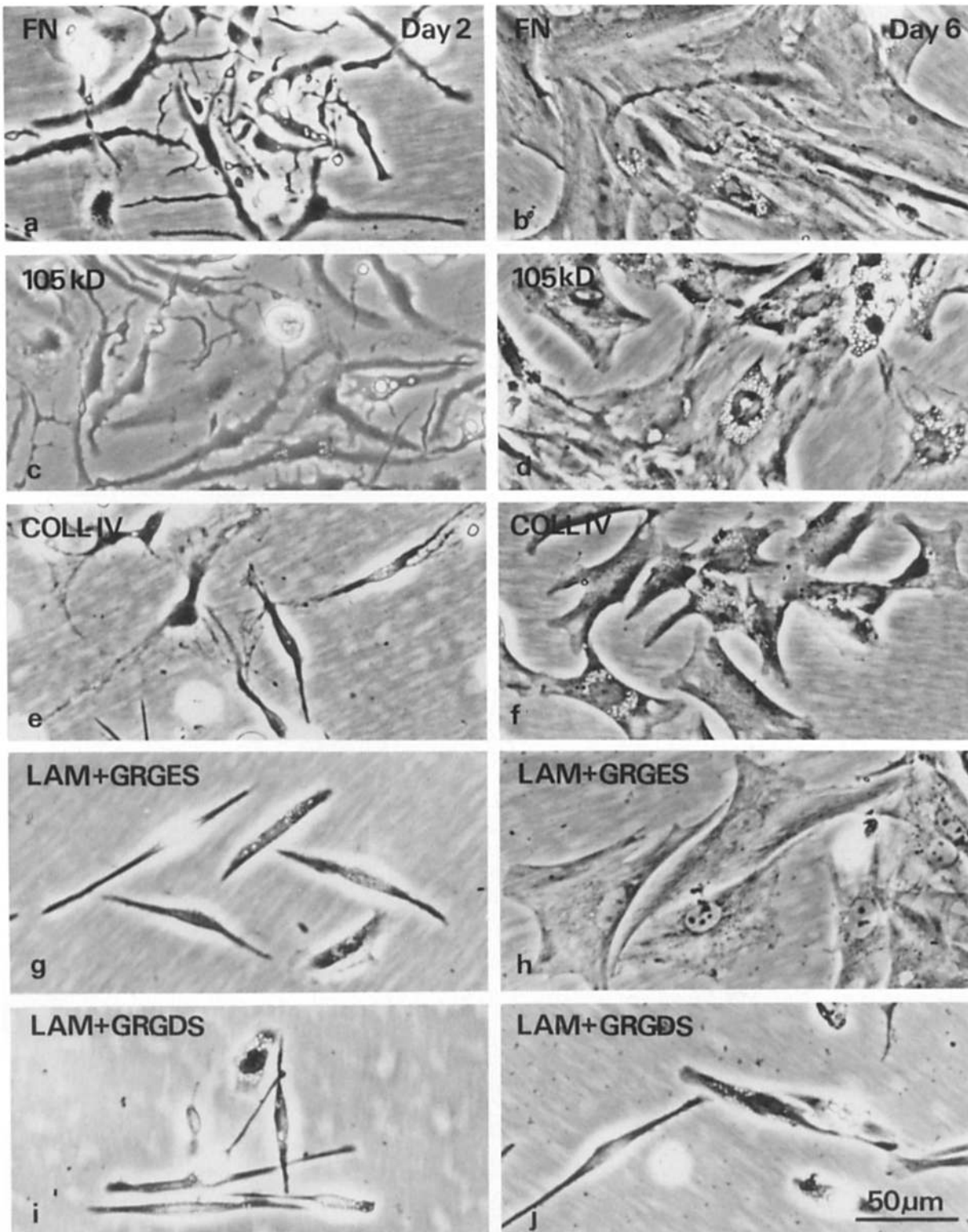
Figure 2. Attachment and spreading of arterial SMCs. The cells were cultivated on substrates of plasma fibronectin (*FN*), a 105-kD cell-binding fragment of plasma fibronectin (*105 kD*), type IV collagen (*COLL IV*), or laminin (*LAM*) with addition of the synthetic peptide GRGES (*LAM + GRGES*) or GRGDS (*LAM + GRGDS*) to the medium (250 µg/ml). Phase–contrast micrographs were taken on days 2 and 6 of culture.

numerous mitochondria. When seeded on a substrate of plasma fibronectin in serum-free medium, the cells attach, rapidly start to spread out, and within 3–4 d go through a marked structural transformation. This process includes a distinct increase in cell size, a relative loss of microfilaments, and formation of a widespread RER and a large juxtanuclear Golgi complex (Hedin and Thyberg, 1987). To define further the cell–extracellular matrix interactions involved in this

modulation of the SMCs from a contractile to a synthetic phenotype, we have used three different fibronectin fragments (Fig. 1), as well as type IV collagen and laminin, two basement membrane components.

The results show that the 105-kD cell-binding fragment of fibronectin was as active as the intact molecule in promoting attachment and spreading of the freshly isolated SMCs (Fig. 2, a and c; Table I). On the other hand, it did not support the long-term performance of the cells to the same extent (Fig. 2, b and d). The 70-kD collagen-binding fragment and the 31-kD heparin-binding fragment produced less efficient attachment and little or no spreading compared to intact fibronectin (Table I). In agreement with these findings, fine structural analysis indicated that fibronectin and the 105-kD cell-binding fragment were equally potent in bringing about modulation from a contractile to a synthetic phenotype (Fig. 3 a; Table I). In contrast, the cells remained in the former state and started to degenerate after a few days on the 70-kD collagen-binding fragment or the 31-kD heparin-binding fragment (Table I). Fibronectin isolated from confluent SMC cultures was equivalent to human plasma fibronectin in its ability to promote attachment, spreading, and phenotypic modulation early in primary culture (Table I).

The cells attached partly but spread poorly on substrates of type IV collagen and laminin, at least for the first few days (Fig. 2, e and g; Table I). After 4–6 d of culture, the spreading had advanced considerably and the cells showed a tendency to arrange in clusters, especially on laminin (Fig. 2, f and h). In a similar manner, the transition from a contractile to a synthetic phenotype was initially slow, but later showed a partial "catch-up" (Fig. 3 b; Table I). To test if this behavior could be due to secretion of fibronectin by the cells themselves, the peptide GRGDS, which contains the cell-attachment sequence of fibronectin (RGDS), was used. Addition of GRGDS to the culture medium did not interfere with initial cell attachment to laminin, but opposed the delayed spreading on days 4–6 (Fig. 2, i and j; Table I) and suppressed the phenotypic modulation (Fig. 3 c; Table I). The control peptide GRGES showed no similar effects (Figs. 2, g and h, and 3 b; Table I).

Activation of RNA, Protein, and DNA Synthesis

Previous reports have indicated that the morphological transformation of the SMCs early in primary culture is associated with onset of synthetic activities and replication in response to exogenous mitogens (Thyberg et al., 1983; Palmberg et al., 1985; Sjölund et al., 1986; Hedin and Thyberg, 1987). Here, RNA, protein, and DNA synthesis were assayed in SMCs cultivated under serum-free conditions on substrates of fibronectin, the 105-kD cell-binding fragment of fibronectin, or laminin. The results demonstrate that the incorporation of tritiated uridine (Fig. 4 a) and leucine (data not shown) into TCA-insoluble material, as well as the net accumulation of protein in the cultures (Fig. 4 b), increased in parallel with the modulation into a synthetic phenotype and thus were distinctly higher in cells cultured on fibronectin or the 105-kD fragment than on laminin. The DNA synthetic response to stimulation with PDGF differed in a similar way among the groups (Fig. 4 c); without exogenous mitogen the level of DNA synthesis was low in all groups (data not shown).

Extracellular Deposition of Fibronectin and Laminin

To investigate to what extent the synthetic activation of the SMCs on the different substrates involved production of extracellular matrix components, the deposition of fibronectin and laminin in the extracellular matrix was studied by double indirect immunofluorescence microscopy (days 1, 2, 4, and 6). With cells grown on fibronectin or the 105-kD fragment, no fibronectin fibrils were detected until day 2 (Figs. 5, a and b, and 6, a and b). Later on, a typical intercellular meshwork was formed (Figs. 5 c and 6 c). Laminin appeared in a patchlike fashion over the cell surface already on day 1 (Figs. 5 d and 6 d), and then remained bound to the cell surface and regions of close cell–cell contact (Figs. 5, e and f, and 6, e and f). When cultured on laminin, the SMCs laid down much more fibronectin than on the two other substrates. As early as day 1, fibrils were visible on the surface of spread cells (Fig. 7 a), and on the following days a tight fibrillar network was organized (Fig. 7, b and c). In contrast, the cell

Table I. Effects of Substrates of Fibronectin, Type IV Collagen, and Laminin on Attachment, Spreading, and Phenotypic Modulation of Arterial SMCs

Substrate	Cell attachment		Degree of cell spreading				Synthetic phenotype		
	6 h*	24 h	24 h	48 h	96 h	144 h	48 h	96 h	144 h
	%							%	
Fibronectin	77	+ + +	+ +	+ + +	+ + +	+ + +	45	80	95
105 kD	74	+ + +	+ +	+ + +	+ + +	+ + +	39	77	90
70 kD	ND	+ +	±	+	+	+	7	30	ND
31 kD	ND	+ +	±	±	+	+	2	17	ND
SMC fibronectin	ND	+ + +	+ +	+ + +	+ + +	+ + +	35	76	93
Type IV collagen	ND	+ +	+	+	+ +	+ +	23	35	65
Laminin	31	+ +	+	+	+ +	+ + +	12	36	70
Laminin + GRGES	ND	+ +	+	+	+ +	+ + +	6	44	77
Laminin + GRGDS	ND	+ +	+	+	+	+	8	28	43

Freshly isolated SMCs were seeded in plastic petri dishes on different substrates in medium F-12/0.1% BSA. At the indicated times the cultures were inspected in an inverted microscope and evaluated for the degree of cell attachment and cell spreading. Alternatively, they were fixed and processed for electron microscopy and the phenotypic state of the cells determined as described in Materials and Methods. (Cell attachment and spreading) ±, mainly rounded cells; +, rounded or elongated cells; + +, 20–50% attached, elongated, or partly flattened cells; + + +, >50% attached, elongated, and flattened cells. For determination of plating efficiency, duplicate cultures were trypsinized and the cells counted in a hemocytometer. (Phenotypic state) Each value indicated for fibronectin, the 105-kD fragment, and laminin represents the mean of two experiments.
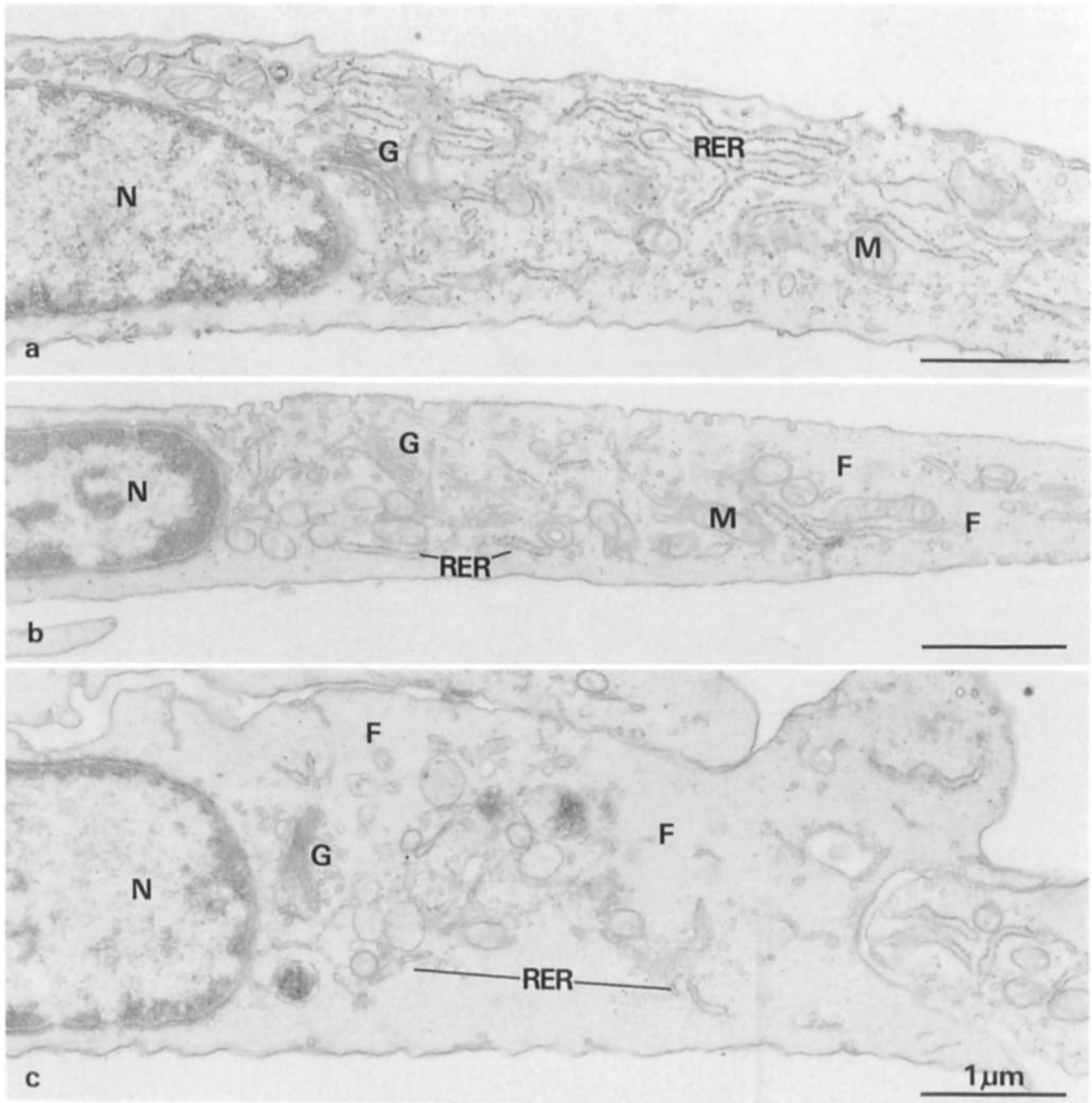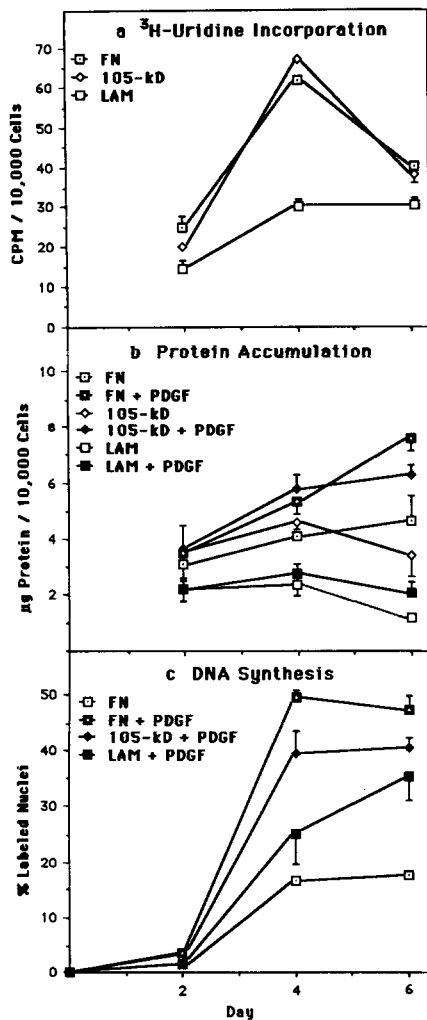* Hours in culture.

Figure 3. Substrate influences on the fine structure of arterial SMCs. (*a*) SMC in a synthetic phenotype from a culture fixed after 2 d on a substrate of the 105-kD cell-binding fragment of plasma fibronectin. Overview showing a flattened cell with numerous free ribosomes, an extensive RER, and stacked Golgi cisternae (*G*). *M*, mitochondria; *N*, nucleus. (*b*) SMC from a culture fixed after 4 d on a substrate of laminin with the peptide GRGES added to the medium (250 µg/ml). The cell has started but not yet completed the transition from a contractile to a synthetic phenotype. RER cisternae and stacked Golgi cisternae (*G*) are seen, but large parts of the cytoplasm are still occupied by microfilament bundles (*F*). (*c*) SMC in a contractile phenotype from a culture fixed after 6 d on a substrate of laminin with the peptide GRGDS added to the medium (250 µg/ml). The cytoplasm is dominated by microfilament bundles (*F*), but a few RER cisternae and a small stack of Golgi cisternae (*G*) are also observed.

surface staining for laminin was weak throughout the observation period, possibly due to intense background staining (Fig. 7, *d–f*).

With regard to fibronectin fibril formation, no clear differences were observed between cultures kept on fibronectin (Fig. 5, *a–c*) and the 105-kD fragment (Fig. 6, *a–c*). In view

of the findings of Woods et al. (1988), it cannot be excluded that cells cultured on fibronectin produced some fibrils by reorganization of the coated substrate. However, the fibrils made by cells grown on the 105-kD fragment were most likely derived from fibronectin, synthesized de novo, since the former molecule does not appear able to form fibrils on

Figure 4. (a) RNA synthesis and (b) protein accumulation in primary cultures of arterial SMCs grown on different substrates. On the indicated days (24 h), the cells were labeled with [³H]uridine for the last 6 h and acid-precipitable material was analyzed for radioactivity and protein content. In b, half of the cultures were stimulated with PDGF (20 ng/ml) on the indicated days (24 h). (c) Activation of DNA synthesis. On the indicated days (24 h), the cells were exposed to PDGF (20 ng/ml) in the presence of [³H]thymidine and then processed for autoradiographic determination of the percentage of labeled nuclei. For further experimental details, see Materials and Methods. Each point represents the mean of triplicate (a and b) or quadruplicate cultures (c) with the standard deviation indicated as a vertical line.
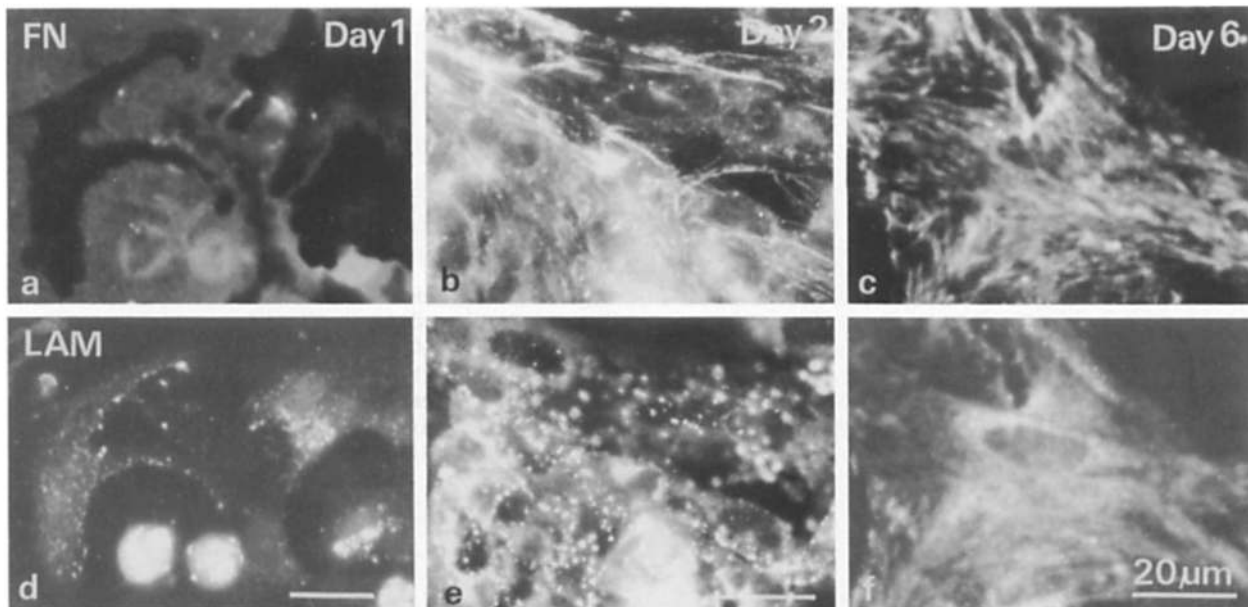
its own (Woods et al., 1988). Moreover, the antibody used in the immunocytochemical procedure was directed against the 31-kD heparin-binding fragment of fibronectin and unable to react with the 105-kD fragment (Woods et al., 1986).

Effects of the Peptides GRGDS and GRGES on Fibronectin Fibril Formation

As reported above the synthetic peptide GRGDS, which contains the cell-attachment sequence (RGDS) of fibronectin (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984), slowed down the phenotypic modulation of SMCs cultured on a substrate of laminin. In view of this finding it was of interest to determine if the peptide interfered with fibronectin fibril formation. For this purpose, SMCs were seeded on laminin and cultured in medium F-12/0.1% BSA with addition of 500 μg/ml of either GRGDS or the control peptide GRGES. After 1, 2, or 4 d the cells were fixed and processed for indirect immunofluorescence microscopy,

Figure 5. Extracellular deposition of fibronectin (a–c) and laminin (d–f) in primary cultures of arterial SMCs grown on a substrate of plasma fibronectin. Double immunofluorescence staining for fibronectin (FN) and laminin (LAM) was performed on days 1, 2, and 6 of culture, using a chicken antibody against the 31-kD heparin-binding fragment of fibronectin and a rabbit antiserum against laminin. For further experimental details, see Materials and Methods. Note the decrease in the background staining for fibronectin over the time period studied.
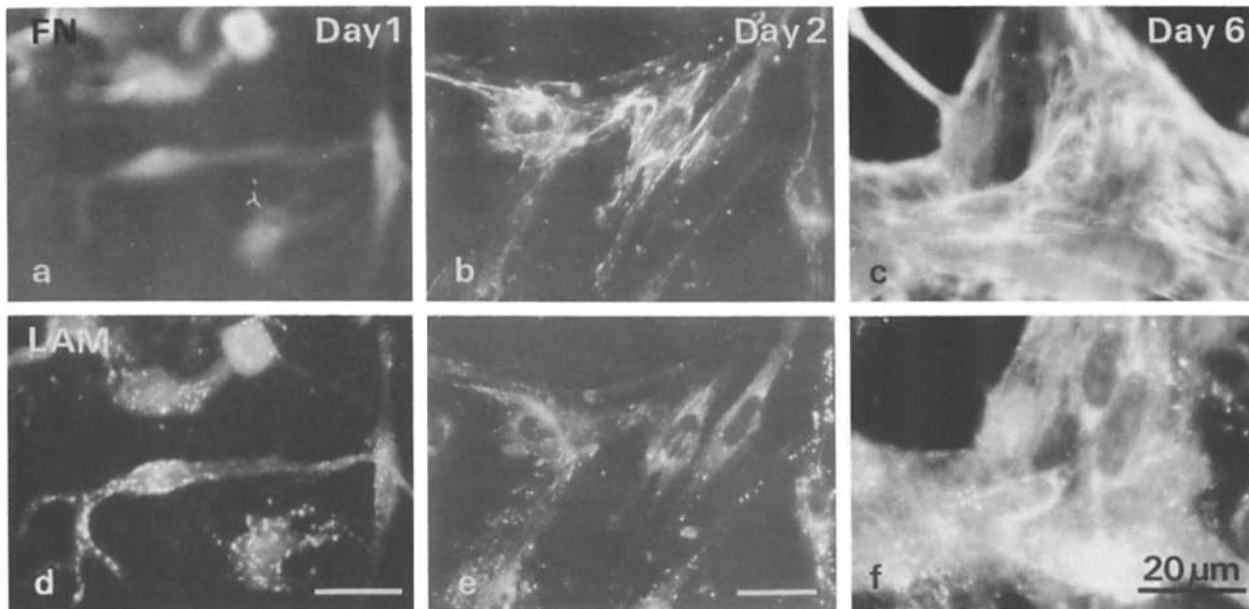
Figure 6. Extracellular deposition of fibronectin (*a–c*) and laminin (*d–f*) in primary cultures or arterial SMCs grown on a substrate of the 105-kD cell-binding fragment of plasma fibronectin. Double immunofluorescence staining for fibronectin (*FN*) and laminin (*LAM*) was performed on days 1, 2, and 6 of culture, using a chicken antibody against the 31-kD heparin-binding fragment of fibronectin and a rabbit antiserum against laminin. For further experimental details, see Materials and Methods. Note the absence of background staining.

using a primary antibody directed against the 31-kD heparin-binding fragment of fibronectin. In the presence of the control peptide, fibronectin fibrils were formed to the same extent as in the absence of peptide (Fig. 8 *a*; cf. Fig. 7, *a–c*). In contrast, no fibronectin fibrils were detected in the presence of GRGDS (Fig. 8 *b*).

Synthesis of Fibronectin and Laminin

To supplement the immunocytochemical observations, the fibronectin and laminin production was investigated biochemically. SMC fibronectin was isolated from pooled culture media and cell layer extracts (2-wk-old cultures grown in the presence of fibronectin-depleted serum) by gelatin
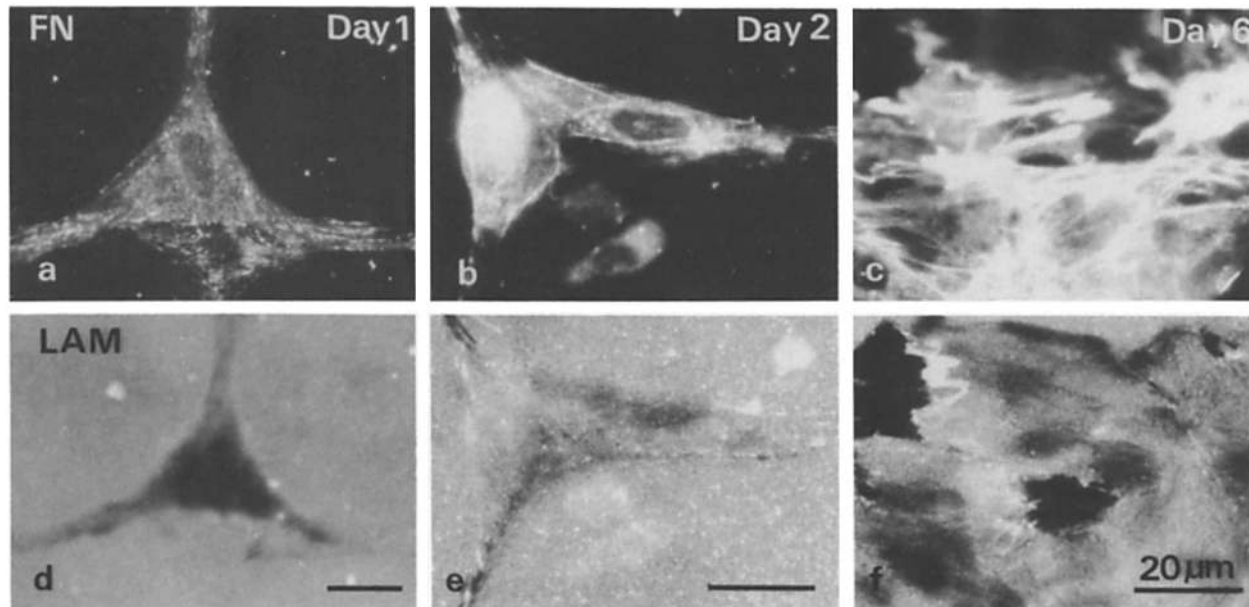

Figure 7. Extracellular deposition of fibronectin (*a–c*) and laminin (*d–f*) in primary cultures of arterial SMCs grown on a substrate of laminin. Double immunofluorescence staining for fibronectin (*FN*) and laminin (*LAM*) was performed on days 1, 2, and 6 of culture, using a chicken antibody against the 31-kD heparin-binding fragment of fibronectin and a rabbit antiserum against laminin. For further experimental details, see Materials and Methods. Note the intense background staining for laminin.
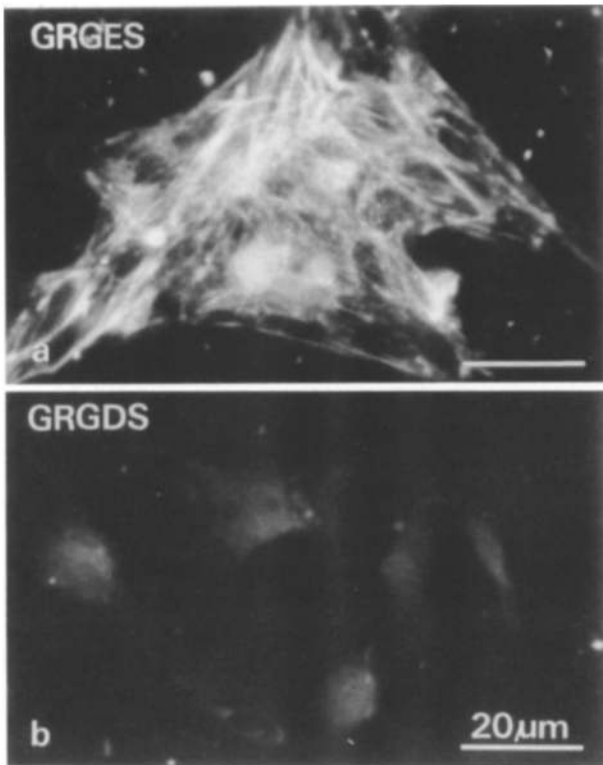
Figure 9. SDS-PAGE (5–12% gel) of fibronectin isolated from rat arterial SMC cultures (*SMC FN*) and human plasma fibronectin (*FN*). (*a*) Gel stained with Coomassie Brilliant Blue R-250. (*b*) The corresponding fluorogram. For further experimental details, see Materials and Methods.

Figure 8. Extracellular deposition of fibronectin in primary cultures of arterial SMCs grown on a substrate of laminin in medium containing 500 μg/ml of GRGES (*a*) or GRGDS (*b*). Immunofluorescence staining for fibronectin was performed on day 4, using a chicken antibody against the 31-kD heparin-binding fragment of fibronectin. For further experimental details, see Materials and Methods.

affinity chromatography and eluted as a single peak. SDS-PAGE of this material under reducing conditions demonstrated a doublet with an apparent molecular mass of 240 kD; the weaker band in the doublet comigrated with human plasma fibronectin (Fig. 9 *a*). A comparison with the corresponding fluorogram revealed that all of the detectable radiolabel colocalized with the upper band in the doublet (Fig. 9 *b*); this was thus the actual product of the smooth muscle cells, and the lower band probably represented residual plasma fibronectin detached from the culture dish.

Cellular production of fibronectin and laminin on the three different substrates (fibronectin, 105-kD cell-binding fragment, or laminin) was followed on days 1–4 by biosynthetic labeling (24-h intervals) with [³⁵S]L-methionine, immunoprecipitation with specific antisera, SDS-PAGE, and fluorography. Controls with rabbit preimmune serum indicated that the rabbit anti–rat fibronectin antiserum specifically recognized proteins of ~240 and 295 kD (Fig. 10). This finding raises the possibility that the SMCs early in primary culture and in close association with the phenotypic modulation produce fibronectin subunits of different sizes, perhaps as a result of variable RNA splicing (Hynes, 1985). Later on, after a number of divisions and when they are firmly established in a synthetic phenotype, the cells may shift to producing fibronectin subunits of only one size, namely 240 kD (Fig. 9). Alternatively, the 240-kD component represented a partially degraded product and the 295-kD component did not
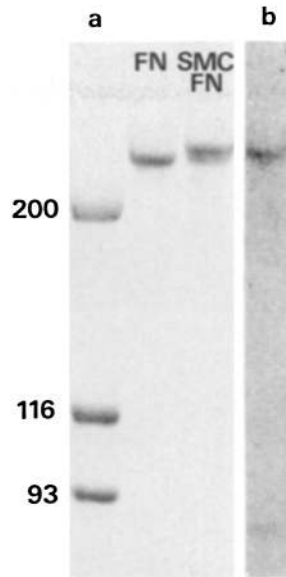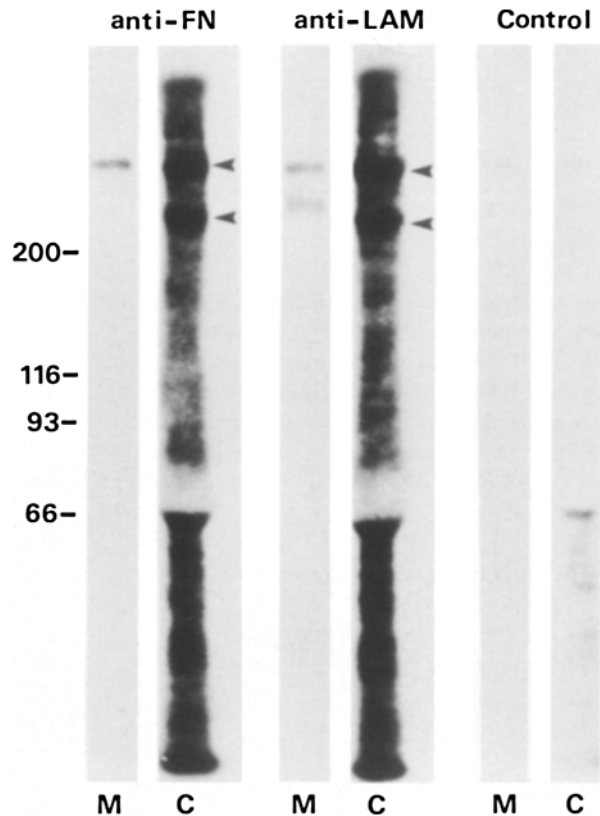

Figure 10. SDS-PAGE (5–12% gel) and fluorography of cell layer (*C*) and medium samples (*M*) from arterial SMCs labeled with [³⁵S]methionine on day 4 of culture. Immunoprecipitation was performed with antiserum against fibronectin (*anti-FN*), antiserum against laminin (*anti-LAM*), or preimmune serum (*Control*). Fibronectin and laminin B-chain bands are indicated by arrowheads.

appear in sufficient amounts to be detectable under the experimental conditions used for the isolation of fibronectin from the cultures.

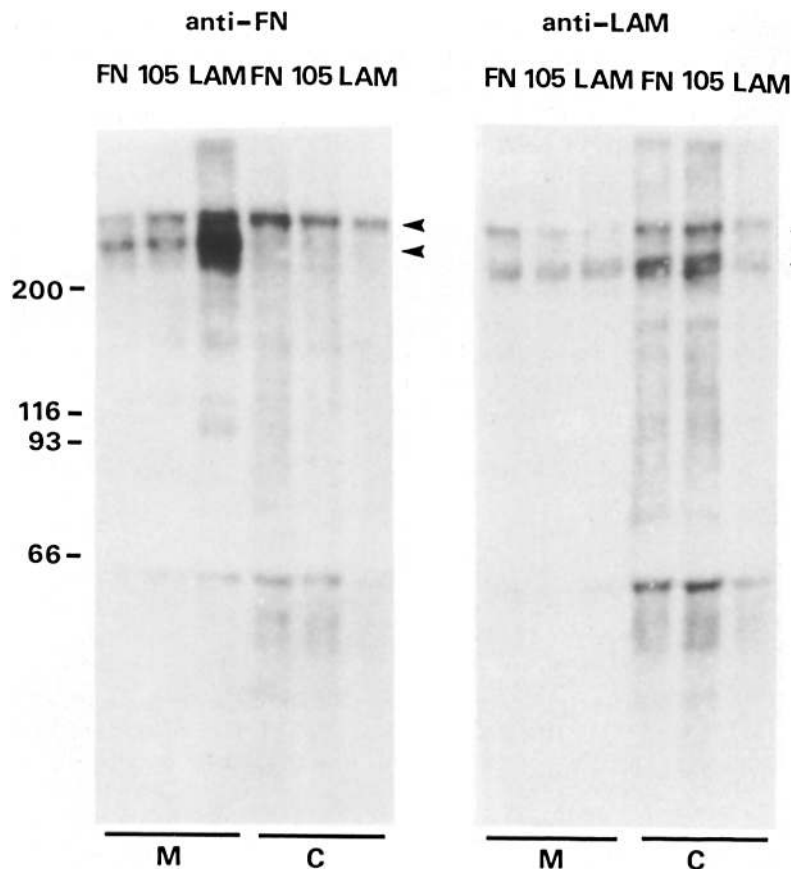The rabbit anti–mouse laminin antiserum specifically rec-

Figure 11. SDS-PAGE (5–12% gel) and fluorography of cell layer (*C*) and medium samples (*M*) from arterial SMCs labeled with [³⁵S]methionine on day 3 of culture. The cells were grown on substrates of plasma fibronectin (*FN*), the 105-kD cell-binding fragment of plasma fibronectin (*105*), or laminin (*LAM*). Immunoprecipitation was performed with antiserum against fibronectin (*anti-FN*) or laminin (*anti-LAM*). The bands used in the scanning densitometry are marked by arrowheads (cf. Fig. 10).

ognized proteins of ∿230 and 280 kD. The relative amounts of these two components were the same in the cell layer and the medium. Possibly, they represented the B1 and B2 chains of laminin, which frequently show different mobilities after metabolic labeling (Timpl and Dziadek, 1986). Alternatively, the smaller component could indicate a partially degraded product. No synthesis of the A chain of laminin

(440 kD) was detected, possibly due to lack of penetration into the gel or proteolysis; similar observations have previously been made in studies on other cell types (Warburton et al., 1982; Kühl et al., 1982).

As a representative example of the results, the fluorograms of the day 3 samples are shown (Fig. 11). Quantitative evaluation by densitometry indicated that the overall production as

Table II. Production of Fibronectin and Laminin by Arterial SMCs Cultured on Different Substrates

Protein synthesized	Substrate											
	Fibronectin				105 kD				Laminin			
	*1 d**	*2 d*	*3 d*	*4 d*	*1 d*	*2 d*	*3 d*	*4 d*	*1 d*	*2 d*	*3 d*	*4 d*
	Immunoprecipitation (relative amount per cell)											
Cell layer												
Fibronectin	4.5	7.7	16	ND	7.7	5.7	11	16	1.1	14	11	36
Laminin	1.9	3.0	15	21	2.0	3.2	18	13	2.2	3.3	8.9	ND
Medium												
Fibronectin	3.0	ND	11	48	6.6	4.5	15	ND	12	17	40	100
Laminin	1.5	10	10	16	4.0	2.8	7.0	14	3.2	3.3	11	28
	ELISA (µg/10⁶ cells)											
Medium												
Fibronectin	ND	0.2	0.3	1.3	ND	0.1	0.3	1.2	ND	0.1	0.5	1.9

Freshly isolated SMCs were seeded in plastic petri dishes on different substrates in medium F-12/0.1% BSA. The production of fibronectin and laminin was measured by biosynthetic labeling and immunoprecipitation in combination with SDS-PAGE and fluorography (see Materials and Methods). The intensity of the specific bands on the fluorograms were digitized by scanning densitometry and related to the cell number in parallel petri dishes (maximum value set to 100). The amount of fibronectin released into the media on the indicated days was also determined by an ELISA and related to the cell number in the respective dishes (see Materials and Methods).
* Days in culture.

well as the release into the medium of fibronectin and laminin increased day by day on all three substrates (Table II). It was further evident that cells cultured on fibronectin or the 105-kD fragment produced less fibronectin than cells cultured on laminin (Table II). On the other hand, it was not possible to decide for certain if cells kept on laminin produced less laminin than cells kept on fibronectin or the 105-kD fragment (Table II). The results of the ELISA were in good agreement with these findings, indicating that cells grown on laminin were those which most actively released fibronectin into the medium (Table II).

Discussion

The Phenotypic Properties of Arterial SMCs Are Age Dependent and Change in a Similar Way during Atherogenesis and In Vitro Cultivation

During fetal and early postnatal life, arterial SMCs have a fibroblast-like appearance, and their main functions are to multiply and to produce extracellular matrix components (synthetic phenotype). In the adolescent, they turn into highly specialized muscle cells and become engaged in the regulation of blood pressure and flow (contractile phenotype). Nevertheless, they are able to return to a proliferative and secretory state, and this appears to be one of the initial events in atherogenesis (Ross, 1986; Schwartz et al., 1986). A similar change in the differentiated properties of the cells occurs during in vitro cultivation (Chamley-Campbell et al., 1979; Thyberg et al., 1983). It includes marked structural changes and leads to the cells losing the ability to contract and gaining the ability to proliferate in response to appropriate mitogens and to secrete extracellular matrix components like collagen and elastin (Chamley-Campbell et al., 1979; Thyberg et al., 1983; Sjölund et al., 1986). Moreover, after a few divisions the cells start to produce a PDGF-like protein and to stimulate themselves in an autocrine manner (Nilsson et al., 1985; Sejersen et al., 1986; Sjölund et al., 1988).

The Ability of Fibronectin to Induce Modulation of the SMCs from a Contractile to a Synthetic Phenotype Resides in a 105-kD Cell-binding Fragment

In a recent report, we demonstrated that plasma fibronectin promotes modulation of isolated rat arterial SMCs from a contractile to a synthetic phenotype during the first few days in primary culture (Hedin and Thyberg, 1987). Here, this interaction was studied further by using chymotryptic fragments of plasma fibronectin as substrates for serum-free culture (Woods et al., 1986; Perris and Johansson, 1987). The results indicate that the modulating potential of fibronectin resides in a 105-kD cell-binding fragment. A 70-kD collagen-binding fragment and a 31-kD heparin-binding fragment allowed attachment of the cells, possibly via newly produced basement membrane collagen and cell surface glycoconjugates, respectively. However, the cells remained in a contractile phenotype and, after a few days, started to degenerate. These findings suggest that a specific receptor–ligand interaction is required to induce transition of the cells from a contractile to a synthetic phenotype.

The morphological transformation of the SMCs, which included a relative loss of myofilaments and formation of a widespread RER and a prominent Golgi complex, was as complete on the 105-kD cell-binding fragment as on intact fibronectin. The early functional activation of the cells (days 2–4), with increased RNA and protein synthesis and replication of DNA in response to PDGF, was likewise of equal magnitude on the two above-mentioned substrates. However, with increasing time (day 6), these functions showed a tendency to lag behind in cells grown on the 105-kD cell-binding fragment. Hence, it is possible that a cooperative action of more than one domain in the fibronectin molecule is required to fully support the long-term performance of the SMCs. In a similar way, it has been found that more than one domain is needed for complete adhesion of fibroblasts to fibronectin (Izzard et al., 1986; Woods et al., 1986) and for reorganization of substrate fibronectin into pericellular fibrils by fibroblasts (McDonald et al., 1987; Woods et al., 1988).

Laminin Maintains the SMCs in a Contractile Phenotype and the Delayed Modulation on Laminin Can Be Explained by Endogenous Production of Fibronectin

The specificity of the effect of fibronectin was confirmed by experiments with laminin, a 900-kD glycoprotein and a major component of the basement membrane that normally surrounds arterial smooth muscle (Chamley-Campbell et al., 1979; Timpl and Dziadek, 1986). When seeded on laminin, the cells attached and spread out, although less efficiently than on fibronectin. Likewise, the phenotypic modulation was slow and incomplete, but towards the end of the observation period (6 d) a partial catch-up was noted. Similar findings were made with type IV collagen, another basement membrane component (Timpl and Dziadek, 1986).

The delayed modulation on laminin was counteracted by a synthetic peptide (GRGDS) containing the cell-attachment sequence (RGDS) of fibronectin (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984), but not by a control peptide (GRGES). These observations suggest that laminin did not itself bring about any change in the differentiated characteristics of the isolated SMCs. However, it may have allowed them to synthesize and secrete fibronectin and to initiate, in an autocrine manner, a transition from a contractile to a synthetic phenotype. In accordance with this notion, fibronectin isolated from primary SMC cultures was found to be as effective as plasma fibronectin in supporting the phenotypic modulation. Moreover, as further discussed below, SMCs cultured on laminin actively secreted fibronectin, and fibronectin fibril formation by these cells was strongly inhibited by the peptide GRGDS. The latter finding corroborates earlier results with fibronectin fragments and domain-specific antibodies indicating that fibronectin fibril formation involves an interaction between the molecule and its cell surface receptor (McDonald et al., 1987; Woods et al., 1988).

The experiments with the synthetic peptides further indicated that the cell-attachment sequence of fibronectin (RGDS) was not itself sufficient to set forth a shift in the phenotype of the SMCs, at least not when used in soluble form; further work is in progress to see if it may do so when anchored to a substrate. Likewise, the cell-attachment sequence of fibronectin (RGDS) did not interfere with the at-

tachment of the cells to laminin. In agreement with studies on other cell types (Carlsson et al., 1981; Johansson et al., 1981; Couchman et al., 1983), this suggests that the adhesion of the SMCs to fibronectin and laminin is mediated via distinct cell surface receptors. Accordingly, it was recently demonstrated that the cell-attachment sequence of laminin is markedly different from that of fibronectin (Graf et al., 1987).

Production of Fibronectin and Laminin by the SMCs Is Influenced by the Substrate on Which the Cells Grow

In spite of distinct differences in the time sequences of the fine structural modification and the rates of overall RNA and protein synthesis, the SMCs started to produce fibronectin and laminin early in primary culture when they were grown on substrates of plasma fibronectin, the 105-kD cell-binding fragment, or laminin. This indicates that cells with a poorly developed RER and Golgi complex, classified morphologically as being in a contractile phenotype, were nevertheless able to synthesize and secrete fibronectin and laminin. Correspondingly, cells with an extensive RER and a large Golgi complex, classified morphologically as being in a synthetic phenotype, must have used only a part of their secretory capacity for this purpose.

Independently of the substrate on which the SMCs were grown, part of the newly produced fibronectin and laminin was released into the culture medium and part was incorporated into the cell layer. Within the latter compartment, fibronectin was arranged in fibrils which first associated closely with the cells, but later also spanned the gap between neighboring cells. In this way, a fine pericellular and intercellular network was created. Laminin initially formed patches dispersed over the cell surface, which subsequently fused into a diffuse layer, generating strong staining around the circumference of the cells and in areas of close cell–cell contact. These observations suggest that laminin was deposited in a basement membrane, in direct apposition to the plasma membrane, and fibronectin was deposited in the extracellular matrix located immediately outside the basement membrane. A similar situation has been reported to exist in the intact arterial wall (Voss and Rauterberg, 1986).

As a whole, the synthetic patterns of SMCs cultured on plasma fibronectin and the 105-kD fragment followed each other closely. Although these cells modulated into a synthetic phenotype at a considerably higher rate than cells cultured on laminin and showed a distinctly higher metabolic activity, they produced less fibronectin. Similarly, it was previously shown that subcultivated SMCs in preconfluent stages synthesized less fibronectin and collagen when grown on a fibronectin-coated surface than when grown on an albumin-coated surface (Holderbaum and Ehrhart, 1986). In view of these findings, it seems likely that the cells are able to sense the macromolecular composition of their environment and to change their secretion of extracellular matrix components accordingly. However, this regulatory mechanism may be discriminative, and not of general applicability. Thus, we are not able to conclude that cells cultured on laminin produced less laminin than cells cultured on fibronectin or the 105-kD fragment.

Concluding Remarks

The results of this study indicate that the adhesive glycopro-

teins fibronectin and laminin play an important role in determining the phenotypic properties of cultured rat arterial SMCs. Fibronectin stimulated modulation of the cells from a contractile to a synthetic phenotype and this activity was found to reside in a 105-kD cell-binding fragment of the molecule. In contrast, laminin held the cells back in a contractile phenotype. However, cells cultured on laminin secreted fibronectin and thereby promoted their own delayed modulation. Since SMC proliferation and secretion of extracellular matrix components are major early events in the development of atherosclerotic lesions (Ross, 1986; Schwartz et al., 1986), these findings suggest opposite roles of fibronectin and laminin in atherogenesis.

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