Phenotype-dependent Response of Cultured Aortic Smooth Muscle to Serum Mitogens

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ABSTRACT   Smooth muscle cells from the aortic media of adult pigs and monkeys have been grown in primary culture by plating cells enzymatically dissociated from the intact aorta. During the first 6 d these cells are in the "contractile" phenotype. That is, they contract slowly in response to angiotensin II and their cytoplasm is filled with both thick and thin myofilaments. In this state they do not incorporate [^{3}H]thymidine into DNA or proliferate in response to normolipemic or hyperlipemic whole blood serum (WBS). After 7 d in culture the cells undergo a spontaneous modulation of phenotype to a "synthetic" state where they cannot be stimulated to contract and their cytoplasm is filled with organelles usually associated with synthesis of secretory protein. Thick myosin-containing filaments can no longer be demonstrated. When challenged with normolipemic or hyperlipemic WBS the cells incorporate [^{3}H]thymidine into DNA and undergo logarithmic growth. It is suggested that when smooth muscle is in the contractile phenotype (as normally exists for most cells in the aortic media of adult animals) it does not divide when challenged with serum mitogens but can undergo a change of phenotype to a synthetic state in which division can be stimulated.

Differentiation of a cell involves transition from an initially multipotential state to the specialized form typical of the adult. A fully differentiated cell is one therefore that has lost all potentiality to develop into another cell type. The concept of cell modulation encompasses the fact that a fully differentiated cell can assume a different function (with associated morphological changes) in response to an altered environment without any change in its type-specific character. Modulation is therefore reversible.

The smooth muscle cell is the only cell type present in the media of mammalian arteries (19). It must therefore be responsible not only for maintaining artery wall tension via contraction-relaxation but also for vascular remodeling, repair, and growth. These latter functions require the retention in the adult of certain basic mesenchymal properties, namely the abilities to synthesize extracellular matrix and to divide (33). This multiplicity of functions requires a whole spectrum of variation in morphology or phenotype. At one end of the spectrum is the smooth muscle cell whose major function is contraction. The cytoplasm of this cell is largely filled with thick and thin myofilaments and it has been described as being in the "contractile" state (6). The vast majority of cells in the media of the adult aorta are in this phenotype. At the other end of the spectrum is the smooth muscle cell engaged in the synthesis of extracellular matrix and/or division. The cytoplasm of this cell contains few myofilaments but large amounts of rough endoplasmic reticulum (RER), free ribosomes, and Golgi complex. These cells have been described as being in the "synthetic" state or phenotype. This phenotype is present during wound repair (6, 20).

Smooth muscle cells that have migrated from aortic explants or have been subcultured contain large numbers of organelles usually associated with synthesis and lack thick myosin-containing filaments (6). They do not contract in response to angiotensin II or norepinephrine or to electrical or mechanical stimulation (7, 17, 18) but readily synthesize collagen, elastin, and glycosaminoglycans (1, 2, 16, 22, 23, 32) and are thus in a synthetic state. Subcultured aortic smooth muscle cells exposed to the platelet-derived growth factor (PDGF) in whole blood serum (WBS) proliferate in a dose-dependent manner with cell division commencing within 24 to 36 h of challenge (25–28), and smooth muscle which has migrated from aortic explants, proliferated, and reached a stationary growth phase can be stimulated to another proliferative phase by increased amounts of PDGF, hyperlipemic serum, or its low-density lipoprotein (LDL) within 1–2 d of challenge (8–11).

The present study was undertaken to determine the effect of the PDGF in WBS and hyperlipemic LDL on smooth muscle cells which have been enzymatically dissociated from the aortic media of mature animals and placed in primary culture. In the

first 6 d these cells contract in response to angiotensin II and norepinephrine, contain both thick and thin myofilaments, and are therefore in the contractile phenotype (see reference 7). The effect of these substances on smooth muscle cells in the phenotype they normally express in the adult artery wall is important in view of suggestions that the PDGF and hyperlipemic LDL play a role in stimulation of proliferation and migration of smooth muscle cells from the media into the intima in developing atherosclerotic plaques (see reference 24).

MATERIALS AND METHODS

Cell Culture

Thoracic aortas from 1- to 5-yr-old slaughterhouse swine (J. H. Ralph and Sons, Melbourne) and 1- to 9-yr-old Macacca Nemestrina monkeys (Regional Primate Center, University of Washington, Seattle) were opened longitudinally and the intima and inner two-thirds media gently peeled off in narrow strips. This tissue was placed into 3 mg/ml collagenase (148 μ/mg, 4196 CLS 40J292, Millipore Corp., Bedford, Mass.) in serum-free medium 199 at 37°C for 0.5–1.5 h, depending on donor species and age, followed by 0.5 mg/ml elastase (type III, E-0127, Sigma Chemical Co., St. Louis, Mo.) for 1 h, then the entire tissue was dispersed into single cells with fresh collagenase. No attempt was made to strip away endothelium because the smooth muscle closest to the endothelium would also be removed, resulting in a biased population. Most of the endothelium was removed in the first collagenase and elastase digestions, leaving a >99% pure population of smooth muscle cells as observed with smooth muscle-specific antibodies (see reference 7). The cells were centrifuged at 900 rpm for 4 min, resuspended in medium 199 + 5% monkey, rabbit, or fetal calf serum, and seeded into 30-mm plastic culture dishes (Sterilin). Approximately 95% of cells in the suspension excluded trypan blue and 60–70% attached to the culture substratum within 24 h. It was difficult to determine the form of the plating selection, for although more cells upon initial plating did not stain with fluorescein isothiocyanate (FITC)-labeled antibodies to smooth muscle myosin than those in the cell suspension, the difference was not statistically significant. Dishes were taken for pulse labeling with [³H]thymidine or for cell counts each morning. The remaining cells were refed in the late afternoon of days 2, 5, 8, 11, and 14 so that the pattern of [³H]thymidine uptake could not be attributed to the feeding regimen.

Preparation of Serum

WBS and platelet-deficient serum (PDS) were prepared from monkeys and rabbits according to the method previously described (31).

Hyperlipemic serum (900–2,000 mg cholesterol/100 ml) from clotted whole blood was obtained from rabbits fed 2% cholesterol and 2% peanut oil in rabbit chow.

Antibody Staining

The γ-globulin-enriched fraction of a rabbit antibody against highly purified chicken gizzard myosin (13) and FITC-labeled sheep anti-rabbit immunoglobulin (Wellcome) were used in the double-staining technique to determine the phenotypic state of cultured smooth muscle (see references 6 and 12).

Electron Microscopy

Cultures were fixed and prepared for electron microscopy as described previously (3).

[³H]thymidine Incorporation into DNA

Cells on glass coverslips were pulse-labeled for 4 h with [³H]thymidine (0.3 μCi/ml) in the appropriate medium, washed with phosphate-buffered saline (PBS), and placed in Holley's fixative (14) for 10 min. The coverslips were washed twice with PBS, air-dried, mounted onto glass slides with polyvinylpyrrolidone, sealed, then dipped in Kodak NTB2 emulsion and exposed for 14 d at 4°C. After development of silver grains, the cells were stained with haematoxylin-eosin and the percentage of labeled nuclei was determined by counting 1,000 cells in radial sections of each coverslip.

Growth Curves

Dishes were drained of medium and 1 ml trypsin-versene added. After 10 min at 37°C the cells were counted in a haemocytometer, with four counts for each dish.

RESULTS

Spontaneous Change in Phenotype

In the first 6 d of primary culture the isolated aortic smooth muscle cells from adult pig and monkey respond to 10^{-7} g/ml angiotensin II by a slow contraction (7). This is observed in ~85% of the cells and consists of a shortening to 60–80% of the original length over a period of 5–8 s. Reintroduction of agonist-free medium allows the cells to return to approximately their original length. However, to shorten, the cells partially detach from the substratum, so respreading often takes 1–2 h, at which time the same cells can be restimulated. The cells stain intensely with FITC-labeled antibodies to smooth muscle myosin, and ultrastructurally their cytoplasm is filled with thick (120–180 Å) myosin-containing filaments and thin (50–80 Å) actin-containing filaments, with other organelles located primarily in the perinuclear region. No deposition of extracellular matrix on the culture dish is observed ultrastructurally during this period, and the cells do not migrate when examined over a 24-h period with time-lapse microcinematography. These cells are in the contractile phenotype (see references 6 and 7).

On day 7, a morphological and functional change becomes apparent in the majority of the isolated cells, in that their contractility and their staining reaction with FITC-labeled smooth muscle myosin antibody are lost. This does not occur uniformly throughout the culture. In ~10% of cells the changes are apparent on day 6, increasing to ~90% on day 7, then to 95% on day 8. The remaining 5% of cells appear to remain indefinitely in the contractile state (see references 5 and 7). Ultrastructurally thick myofilaments can no longer be demonstrated in the altered cells, and bundles of thin myofilaments are found scattered throughout the cytoplasm which is filled with organelles usually associated with synthesis such as rough endoplasmic reticulum, free ribosomes, and Golgi complex. They actively migrate as viewed with time-lapse microcinematography and deposit a visible extracellular matrix on the culture dish in the presence of WBS. These cells are in the synthetic phenotype.

With isolated smooth muscle cells seeded at <5 × 10⁵ cells/ml medium, this spontaneous change in phenotype is first evident morphologically on day 7 irrespective of whether WBS or PDS is present in the culture medium and whether the serum concentration is 0.5, 5, 10, or 20%. It is also first apparent on day 7 with cells in serum-free medium, indicating that serum factors are not involved in inducing the change in phenotype.

Under certain conditions, smooth muscle cells will not undergo spontaneous change of phenotype in culture nor can they be stimulated to revert back to the contractile state after modulation to the synthetic state has occurred. This is the subject of a separate report.[1]

Response to the PDGF

In the first 6 d of primary culture, while the cells are in the contractile phenotype, 3–5% of nuclei are labeled with [³H]thymidine (Fig. 1). However, on day 8, the day after phenotypic modulation to the synthetic state is first evident morphologically, the number of labeled nuclei increases to 10–20% when the cells are grown in 5% WBS. The percentage of cells

incorporating [³H]thymidine into DNA then increases in a linear fashion with time in culture, until on day 14 40–60% of cell nuclei are labeled during the 4-h pulse-labeling period. The pattern of labeling is roughly homogeneous throughout each culture. That is, new cells are being recruited into the cell cycle rather than a rapid cycling of the progeny of the original 3–5% whose nuclei incorporated [³H]thymidine. In the presence of 5% PDS, the number of labeled nuclei does not exceed 15% of the total cell number throughout a 14-d culture period, even though modulation of phenotype has occurred on day 7.

Cell number remains constant while the cells are in the contractile state, then 1–2 d after modulation to the synthetic state has occurred, they begin to proliferate logarithmically in the presence of 5% WBS (Fig. 2). In the presence of 5% PDS, synthetic state cells do not proliferate, with the cell number remaining relatively constant over a 15-d culture period (Fig. 2). Synthetic state cells maintained quiescent in 5% PDS for 24 d and then challenged with 5% WBS respond within 36–48 h by logarithmic growth (27).

Response to Hyperlipemic Serum

Contractile state smooth muscle cells exposed to 5% hyperlipemic WBS do not take up [³H]thymidine or undergo proliferation (Fig. 3). However, after modulation to the synthetic phenotypic state has occurred on day 7, the cells undergo significantly more intense proliferation than in the presence of 5% normal WBS.

When normolipemic and hyperlipemic LDL (gifts of Dr. R. Wissler) are added to 5% PDS on a volume basis with no adjustment for differences in protein or cholesterol levels, the number of cells per plate after 14 d in culture does not increase
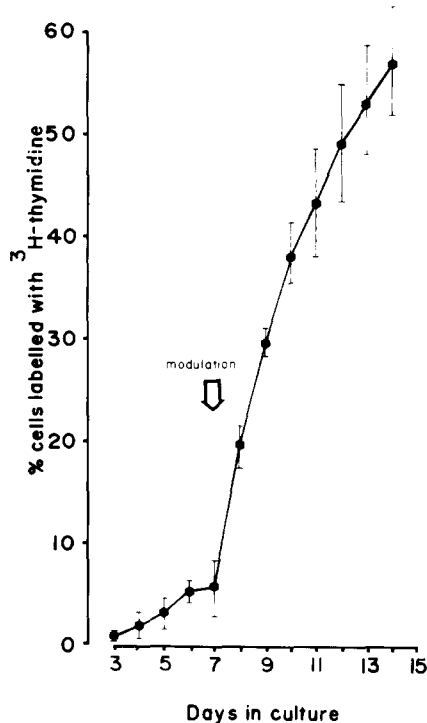

FIGURE 1 Monkey aortic smooth muscle cells in medium 199 + 5% monkey WBS labeled after a 4-h pulse with [³H]thymidine each day in culture. Modulation of phenotype from the contractile to synthetic state occurs on day 7. Each point represents the mean ± SD of four dishes.
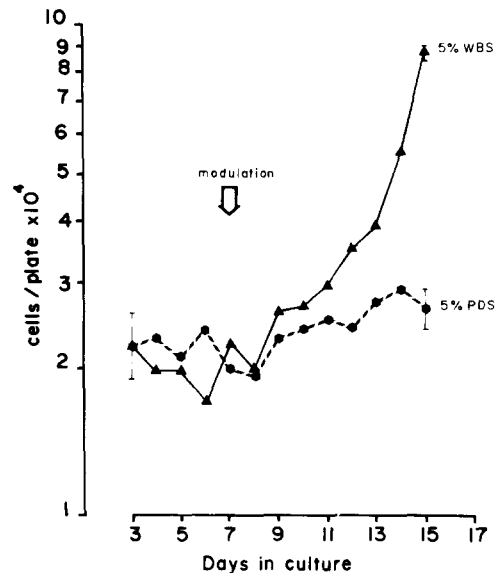

FIGURE 2 Growth curves of monkey aortic smooth muscle cells in medium 199 + 5% monkey WBS and 5% monkey PDS. Modulation of phenotype occurs on day 7. Each point represents the mean of three dishes, ± SD on the 3rd and 15th day.
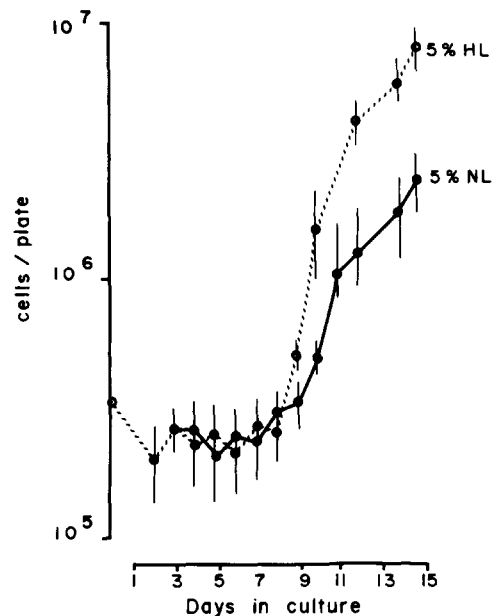

FIGURE 3 Growth curves of pig aortic smooth muscle in 5% hyperlipemic (HL) rabbit serum (1,260 mg cholesterol/100 ml) and 5% normolipemic (NL) rabbit serum (150 mg cholesterol/100 ml). Each point represents the mean of three dishes ± SD.

with normolipemic LDL, but there is a significant increase with hyperlipemic LDL (Fig. 4). The extent of this proliferation is below that with 5% WBS or with 5% PDS plus the PDGF in an equivalent level to 5% WBS.

The lack of response of the smooth muscle cells to normolipemic or hyperlipemic serum in the first 7 d of culture is not caused by a loss or alteration of mitogenic cell surface receptors by contaminant proteolytic or lipolytic enzymes in collagenase or elastase, because subcultured smooth muscle (which appears to be permanently in a synthetic state) proliferates in the presence of 5% WBS within 36–48 h after the same enzyme treatment.
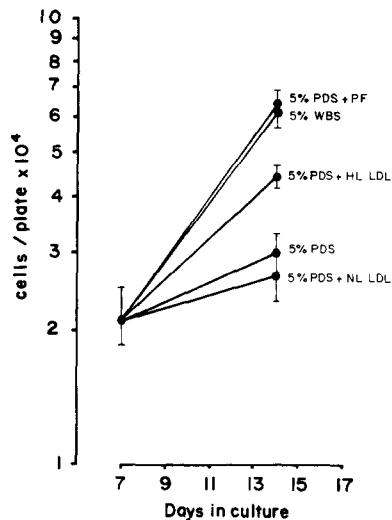
FIGURE 4 Growth of synthetic state monkey aortic smooth muscle cells in the presence of 5% monkey PDS or WBS with added normolipemic (NL) or hyperlipemic (HL) LDL or the platelet-derived growth factor (PF). Each point represents the mean of three dishes ± SD.

DISCUSSION

The present report demonstrates three important findings that may be relevant to the study of atherogenesis. (*a*) Smooth muscle in the contractile phenotypic state (as it usually exists in the adult blood vessel wall) is not stimulated to divide by the PDGF, hyperlipemic LDL, or other serum-derived factors. (*b*) Phenotypic modulation from the contractile state to the synthetic state appears to be a necessary prerequisite for smooth muscle cells in culture to become responsive to mitogens from the serum. (*c*) Neither platelet- nor plasma-derived factors are involved in the modulation process, as it occurs at the same time in the presence of different concentrations of WBS or PDS and in the total absence of serum.

If such a requirement is necessary for smooth muscle cell migration and proliferation to occur as a result of damage to the vessel wall, the injury must fulfill at least two criteria: Firstly, the smooth muscle cells must be stimulated to phenotypically modulate to the synthetic state; and secondly, mitogens from the blood must be able to contact these cells after they have modulated, that is, at least 7 d after the time of injury. This may explain recent findings that no intimal smooth muscle cell proliferation occurs when a narrow band (two cells wide) of endothelium is stripped from the rat aorta, enabling rapid restitution of the endothelial barrier (21).

In the first 6 d of culture (before phenotypic modulation to the synthetic state is evident), between 3 and 5% of aortic smooth muscle cells incorporate [³H]thymidine into DNA. This may be caused by one or more factors. Firstly, a small percentage of aortic smooth muscle cells may be capable of division in the contractile state, because it has been shown that 0.2 to 0.5% of spontaneously contracting smooth muscle cells from the newborn guinea pig vas deferens divide in 10% WBS while the majority of cells must first phenotypically modulate (5). Secondly, proliferating endothelial cells may be a contributing factor. However, rigorous scanning of the cultures both with phase-contrast microscopy and after staining with FITC-labeled antibodies to smooth muscle actin (which stain smooth muscle but not endothelial cells or fibroblasts) has shown that

the cultures are considerably >99% pure smooth muscle (see reference 7). The third and most likely explanation is that there is a small number of smooth muscle cells in the adult aorta normally in the synthetic phenotype. Spraragen et al. (30) demonstrated, using [³H]thymidine, that there is a constant turnover of smooth muscle cells in the rabbit aorta; in fact, approximately one cell is labeled per 200 high-power light microscope fields. The proportion of synthetic to contractile state cells is probably exaggerated in culture because subcultured and therefore synthetic state smooth muscle cells have a higher plating efficiency (80%) than freshly dissociated aorta (65%) (J. H. Chamley-Campbell, unpublished observation).

Finally, hyperlipimec LDL may be mitogenic for synthetic state smooth muscle independent of the PDGF, because its addition to 5% PDS is growth stimulatory while the same amount of normolipemic LDL has no effect. However, data are accumulating (4, 15, 29) to show that in hyperlipemic animals platelets are more fragile or, at the very least, that platelet survival is decreased. If this is the case, there could conceivably be more PDGF associated with the hyperlipemic serum and, in fact, with the hyperlipemic LDL. It would then be the PDGF and not the LDL that was inducing the effect.

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