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 [3H]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 [3H]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 of 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 Macaca 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 μg/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.

**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). Cultures were grown in 5% WBS. The percentage of cells are in the contractile state, 3-5% of nuclei are labeled with [3H]thymidine (Fig. 1). However, on day 8, the day after phenotypic modulation to the synthetic state has occurred. This is the subject of a separate report.1

**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 resuspended 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 A) myosin-containing filaments and thin (50-80 A) 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 × 10⁶ 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 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.
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Secondly, proliferating endothelial cells maybe a contrib-

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the vessel wall, the injury must fulfill at least two criteria:

Firstly, the smooth muscle cells must be stimulated to pheno-

tically modulate to the synthetic state; and secondly, mito-

gens, that is, at least 7 d after the time of

animals platelets are more fragile or, at the very least, that

be the PDGF and not the LDL that was inducing the effect.

It would then

stimulate to divide by the PDGF, because its

state smooth muscle independent of the PDGF, because its

argin 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 pheno-

tically modulate to the synthetic state; and secondly, mito-

gens 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]thy midine into DNA. This

may be caused by one or more factors. Firstly, a small per-

centage 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 contrib-

uting 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

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]thy midine, 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 subcul-

tured 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, hyperlipemic 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

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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REFERENCES

1. Barnes, M. J., L. F. Morton, and C. I. Levene. 1976. Synthesis of collagens types I and III

by pig medial smooth muscle cells in culture. Biochem. Biophys. Res. Commun. 70:339-

74.

2. Burke, J. M., G. Balian, R. Rosa, and P. Borstein. 1977. Synthesis of types I and III procollagen and collagen by monkey aortic smooth muscle cells in vitro. Biochemistry. 16:3245-

3249.

3. Campbell, G. R., J. H. Chamley, and G. Burnstock. 1974. Development of smooth muscle cells in tissue culture. J. Anat. 117:295-312.

4. Carvalho, A. C. A., R. W. Colman, and R. S. Lees. 1974. Platelet function in hyperlipo-

proteinemia. N. Eng. J. Med. 290:434-438.

5. Chamley-Campbell, J. H., and G. R. Campbell. 1974. Mitosis of contractile smooth muscle cells in tissue culture. Exp. Cell Res. 84:105-110.

6. Chamley-Campbell, J., G. R. Campbell, and R. Ross. 1979. The smooth muscle cell in

culture. Physiol. Rev. 59:1-61.

7. Chamley, J. H., and D. McConnell, and U. Groschel-Stewart, 1977. Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in primary culture and in subculture. Cell Tissue Res. 177:503-522.

8. Doga, K., R. W. Wisser, and D. Veneslinovich. 1971. The effect of normal and hyperlipemic low density lipoprotein fractions on aortic tissue culture cells. Circulation. 44. Suppl. 116.

9. Fischer-Drga, K., and R. W. Wisser. 1976. Stimulation of proliferation in stationary

primary cultures of monkey aortic smooth muscle cells. Part 2. Effect of varying contrac-
tions of hyperlipemic serum and low density lipoproteins of varying dietary fat origins. Arteriosclerosis. 24:515-525.

10. Fischer-Drga, K., R. Chen, and R. W. Wisser. 1974. Effects of serum lipoproteins on the morphology, growth and metabolism of arterial smooth muscle cells. In Arterial Menenchyme and Arteriosclerosis. W. D. Wagner and T. B. Clarke, editors. Plenum Press, New York. 299-311.

11. Fischer-Drga, K., R. Fraser, and R. W. Wisser. 1975. Stimulation of proliferation in stationary primary cultures of monkey and rabbit aortic smooth muscle cells. Effect of lipoprotein fractions of hyperlipemic serum and lymph. Exp. Mol. Pathol. 24:346-359.

12. Groschel-Stewart, U., J. H. Chamley, G. R. Campbell, and G. Burnstock. 1975. Changes in migration distribution in differentiating and redifferentiating smooth muscle cells in tissue culture. Cell Tissue Res. 165:13-22.

13. Groschel-Stewart, U., J. Schreiber, C. H. Mahrleiner, and K. Weber. 1976. Production of specific antibodies to contractile proteins, and their use in immunofluorescence mi-

croscopy. I. Antibodies to smooth and striated chicken muscle myosins. Histochemistry. 46:229-236.

14. Holley, R. W., and J. A. Kierman. 1974. Control of the initiation of DNA synthesis in 3T3 cells: serum factors. Proc. Natl Acad. Sci. U. S. A. 71:2908-2911.

15. Jones, J. H., G. Dooner, R. Kirkpatrick-Kirkoune, and J. F. Mustard. 1976. Effect of dist-

induced hyperlipidemia on in vitro-function of rabbit platelets. Thromb. Res. 9:435-445.

16. Layman, D. L., E. H. Epstein, R. F. Donlod, and J. L. Titus. 1977. Biosynthesis of type I and IV collagens by cultured smooth muscle cells from human aorta. Proc. Natl Acad. Sci. U. S. A. 74:671-675.

17. Martin, G. M., and C. A. Sprague. 1973. Symposium on in vitro studies related to atherogenesis. Life histories of hyperplastic cells line from aorta and skin. Exp. Mol. Pathol. 18:125-141.

18. Mauger, J. F., M. Worell, J. Tanasi, and Y. Courbon. 1975. Contractility of smooth muscle cells of rabbit aorta in tissue culture. Nature (London). 255:337-338.

19. Pease, D. C., and W. J. Paulus. 1960. Electron microscopy of elastic arteries. The thoracic aorta of the rat. J. Ultrasound. Res. 3:469-483.

20. Poole, J. C. F., S. B. Cromwell, and E. P. Benditt. 1971. Behaviour of smooth muscle cells.
and formation of extracellular structures in the reaction of arterial walls to injury. Am. J. Pathol. 62:391-414.
21. Reidy, M. A., and S. M. Schwartz. 1980. Endothelial regeneration. III. Time course of intimal changes after small defined injury to rat aortic endothelium. Lab. Invest. In press.
22. Ronnemaa, T., and N. S. Doherty. 1977. Effect of serum and liver extracts from hypercholesterolemic rats on the synthesis of collagen by isolated aortas and cultured aortic smooth muscle cells. Atherosclerosis. 26:261-272.
23. Ross, R. 1971. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. J. Cell Biol. 59:172-186.
24. Ross, R., and J. A. Glomset. 1976. The pathogenesis of atherosclerosis. I. and II. N. Engl. J. Med. 295:369-377; 420-425.
25. Ross, R., and A. Vogel. 1978. The platelet-derived growth factor. Cell. 14:203-210.
26. Ross, R., J. Glomset, B. Kariya, and L. Harker. 1974. A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc. Natl. Acad. Sci. U. S. A. 71:1207-1210.
27. Ross, R., C. Nist, B. Kariya, M.-J. Rivest, E. Raines, and J. Callis. 1978. Physiological quiescence in plasma-derived serum: influence of platelet-derived growth factor on growth in culture. J. Cell Physiol. 97:497-508.
28. Rutherford, R. B., and R. Ross. 1976. Platelet factors stimulate fibroblasts and smooth muscle cells quiescent in plasma to proliferate. J. Cell Biol. 69:196-203.
29. Shattil, S. J., R. A. Anaya-Galindo, J. Bennett, R. W. Colman, and R. A. Cooper. 1975. Platelet hyperreactivity induced by cholesterol incorporation. J. Clin. Invest. 55:636-643.
30. Spranger, S. C., V. P. Bond, and L. K. Dahl. 1962. Role of hyperplasia in vascular lesions of cholesterol-fed rabbits studied with thymidine-H1 autoradiography. Circ. Res. 11:329-336.
31. Vogel, A., E. Raines, B. Kariya, M.-J. Rivest, and R. Ross. 1978. Coordinate control of 3T3 cell proliferation by platelet-derived growth factor and plasma components. Proc. Natl. Acad. Sci. U. S. A. 75:2810-2814.
32. Wight, T., and R. Ross. 1975. Proteoglycans in primate arteries. II. Synthesis and secretion of glycosaminoglycans by arterial smooth muscle cells in culture. J. Cell Biol. 67:675-686.
33. Wissler, R. W. 1968. The arterial medial cell. smooth muscle or multifunctional mesenchyme? J. Atheroscler. Res.