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Arterial smooth muscle cells in primary culture produce a platelet-derived growth factor-like protein

(phenotype modulation/autocrine stimulation/DNA synthesis)

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ABSTRACT **Adult rat arterial smooth muscle cells (SMC) in primary culture modulate from contractile to synthetic phenotype. This process includes partial loss of myofilaments and formation of an extensive rough endoplasmic reticulum and a large Golgi complex. It gives the cells the ability to initiate DNA synthesis and actively proliferate when stimulated with serum or isolated growth factors. After a few divisions, growth becomes partly independent of exogenous mitogens and does not cease until multiple cell layers have been formed. Here, it is demonstrated that serum-free conditioned medium from primary cultures of adult rat arterial SMC contains a factor that initiates DNA synthesis in growth-arrested secondary cultures of SMC. The mitogenic activity was neutralized by antibodies to platelet-derived growth factor (PDGF), and no mitogenic activity occurred in conditioned medium from cultures pretreated with actinomycin D, excluding release into the medium of PDGF adsorbed to the plastic vessels during the initial culture in serum-containing medium. Exposure of human fibroblasts to samples of the conditioned medium at 4°C inhibited subsequent binding of ^{125}I-labeled PDGF. It was further shown that the SMC of the primary cultures were able to initiate DNA synthesis in a chemically defined medium lacking PDGF and other growth factors. During the early, most active, and partly autonomous growth phase, the SMC had a low binding capacity for ^{125}I-labeled PDGF and responded but little to stimulation with exogenous PDGF. Later on, with increasing cell density and decreasing growth rate, the ability to bind and respond to exogenous PDGF increased. Taken together, the observations suggest that modulation of SMC from contractile to synthetic phenotype is accompanied by production of a PDGF-like protein and autocrine or possibly by mitogen-independent initiation of DNA synthesis. Functionally, this may be important during wound healing and in the development of atherosclerotic lesions.**

Smooth muscle cells (SMC) build up the media of mammalian arteries and are the principal cells of atherosclerotic lesions (1). *In vitro* techniques have been widely applied in the study of these cells (2). In primary culture, SMC pass through a modulation from contractile to synthetic phenotype, a process that includes loss of myofilament bundles and formation of an extensive rough endoplasmic reticulum and a large Golgi complex (3, 4). This modulation gives the cells the ability to synthesize DNA and divide when stimulated with serum or purified growth factors.

Platelet-derived growth factor (PDGF) has been identified as the major mitogen in serum for SMC (5, 6) and other connective tissue cells (7). PDGF is structurally related to the putative transforming protein of simian sarcoma virus (SSV; refs. 8–10), and cells transformed by SSV and other tumor viruses produce PDGF-like molecules (11–14). It has also been shown that some sarcoma and glioma cell lines produce PDGF-like proteins (15–18) and contain transcripts that hybridize with the transforming gene of SSV, v-*sis* (19). These findings suggest that autocrine stimulation of growth, involving production of PDGF-like mitogens, may in certain cases be part of the transformed phenotype. Additionally, production of PDGF-like proteins has been observed in normal endothelial cells (14, 20) and arterial SMC from young but not adult rats (21). Endothelial cells have also been found to express a 3.7-kilobase RNA homologous to the c-*sis* gene, the cellular equivalent of v-*sis* (22).

In a previous study on adult rat arterial SMC in primary culture (4), we found that transition into synthetic phenotype was followed by rapid PDGF-initiated cell growth. After one or a few divisions, the need for exogenous growth factor was diminished, and growth continued at an unchanged rate in serum-containing medium to which PDGF antibodies had been added and in plasma-containing medium without addition of PDGF. The object of the present investigation was to study whether this reflects autocrine secretion of growth factors by the SMC.

MATERIALS AND METHODS

Cell Culture. SMC were isolated from the aortic media of 7- to 9-month-old male Sprague–Dawley rats by collagenase digestion (4). The freed cells were suspended in medium F-12 supplemented with 10 mM Hepes, 10 mM 2-{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (Tes) (pH 7.3), L-ascorbic acid (50 μg/ml), gentamycin sulfate (50 μg/ml), and 10% human whole blood serum; seeded into Falcon plastic flasks or Petri dishes (5×10^4 cells per cm^2); and grown at 37°C in 5% CO_2/95% air (medium changed every second day).

Collection of Conditioned Media. Cultures were rinsed four times and incubated with medium MCDB 104 (GIBCO) supplemented with L-ascorbic acid, gentamycin sulfate (see above), and 1% bovine serum albumin (Sigma; 7638) for 3 or 5 days. Media were collected, centrifuged at 200 × *g* for 10 min, frozen, and stored at −20°C. Cells were detached from the culture vessels by trypsinization and counted in an electronic cell counter. Control cultures were treated with actinomycin D at 5 μg/ml (Sigma) for 60 min in medium MCDB 104 containing 1% bovine serum albumin, rinsed four times, transferred to fresh medium without actinomycin D for 3 days, and the medium was collected as above.

Assay of DNA Synthesis. Subconfluent secondary cultures of SMC (grown on glass coverslips) were used to assay the DNA synthesis-stimulating activity of the conditioned media. The cells were growth-arrested by incubation in medium MCDB 104 containing 1% bovine serum albumin for 24 hr.

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Abbreviations: PDGF, platelet-derived growth factor; SMC, smooth muscle cells; SSV, simian sarcoma virus.

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They were then given fresh or conditioned medium MCDB 104 with or without 50 μg of PDGF antibodies per ml (ref. 23; the gamma globulin fraction was purified as described in ref. 6), exposed to 1.0 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine (Amersham; 5.0 Ci/mmol) per ml for 24 hr, and processed for autoradiographic determination of the percentage of labeled nuclei (24).

Partial Purification of PDGF-Like Material Released by SMC. Serum-free conditioned medium (160 ml) from primary SMC cultures (day 4–8) was pumped through a 10-ml column of Sulphadex (sulfated Sephadex G-50 medium; see ref. 25) at a flow rate of 30 ml/hr. After the column was washed with 30 ml of 0.15 M NaCl/0.01 M phosphate buffer, pH 7.4, the active fraction was eluted with 20 ml of 1.5 M NaCl/0.01 M phosphate buffer, pH 7.4, and then dialyzed against 0.15 M NaCl/0.01 M phosphate buffer, pH 7.4.

Assay of Competition for the PDGF Receptor. PDGF was purified to homogeneity (26) and labeled with ^{125}I (27) (^{125}I-PDGF) to a specific activity of about 30,000 cpm/ng. Confluent cultures of human foreskin fibroblasts (AG 1523; Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ) were rinsed with phosphate-buffered saline containing 0.9 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 0.1% bovine serum albumin (binding medium) and exposed to test samples (diluted in binding medium) for 2 hr at 4°C. After the cells were rinsed with binding medium, they were exposed for 60 min at 4°C to ^{125}I-PDGF (2 ng/ml) in binding medium. They were then washed five times with binding medium and lysed in 1.0% Triton X-100/10% glycerol/20 mM Hepes, pH 7.4. ^{125}I radioactivity was determined in a γ counter.

Binding of PDGF to SMC. SMC (grown in 4-cm^2 Costar 12-well plates) were washed twice with binding medium and exposed for 2 hr at 4°C to ^{125}I-PDGF (2 ng/ml) in binding medium with or without an excess of unlabeled PDGF (500 ng/ml). The cells were then washed and lysed as described above for the determination of cell-bound radioactivity.

Electron Microscopy. The cells were fixed in cacodylate-buffered glutaraldehyde, postfixed in cacodylate-buffered osmium tetroxide, stained with uranyl acetate, dehydrated in ethanol, and embedded in low-viscosity epoxy resin (4). Thin sections were cut on an LKB Ultrotome IV, stained with lead citrate, and examined in a Philips electron microscope 300.

RESULTS

Fine Structure of SMC in Primary Culture. Freshly isolated cells were rounded and partly contracted. The nuclei had a wide peripheral zone of heterochromatin and small nucleoli, while the cytoplasm was dominated by myofilament bundles with associated dense bodies. Within 1–2 days of culture, the cells attached to the substrate and spread out. At the same time, a structural reorganization took place, including acquisition of euchromatic nuclei with large nucleoli, regression of the myofilament system, and formation of an extensive rough endoplasmic reticulum and a large Golgi complex. This transition from contractile to synthetic phenotype was completed in 2–3 days and gave the cells the ability to actively proliferate and to produce extracellular matrix components (for further details, see ref. 4).

Initiation of DNA Synthesis in Growth-Arrested Secondary SMC Cultures by Conditioned Medium from Primary SMC Cultures. After 2–3 days in primary culture in the presence of 10% whole blood serum, the SMC started to proliferate rapidly and reached confluence after 7–9 days of culture (cf. ref. 4). Serum-free conditioned medium from the active growth period of the primary cultures initiated DNA synthesis in subconfluent, growth-arrested secondary cultures of SMC. The effect was concentration-dependent and was completely blocked by PDGF antibodies (Fig. 1). Moreover, no or only little stimulatory activity appeared in conditioned medium from primary cultures pretreated with actinomycin D or in conditioned medium from secondary cultures of SMC in active growth phase (Table 1).

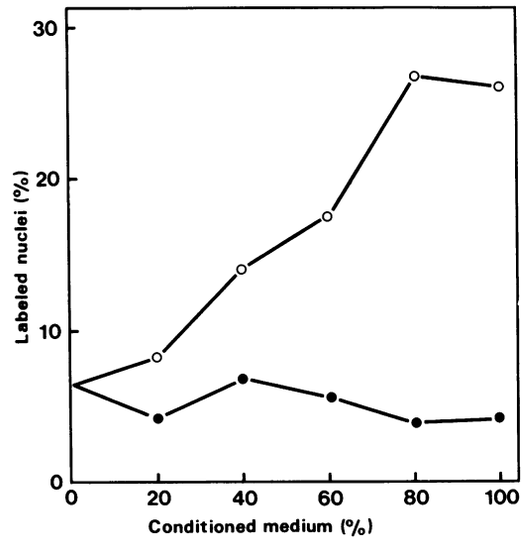

FIG. 1. Autoradiographic analysis of the initiation of DNA synthesis in growth-arrested secondary cultures of SMC exposed to serum-free conditioned medium (diluted with medium MCDB 104 containing 1% bovine serum albumin) from primary cultures of SMC (days 4–6). ○, Conditioned medium; ●, conditioned medium with PDGF antibodies at 50 μg/ml. Each value is the mean of triplicate cultures.

Competition Activity of Conditioned Media for the PDGF Receptor. Human foreskin fibroblasts preincubated with partially purified material from conditioned medium of primary SMC cultures showed a reduced ability to bind ^{125}I-PDGF (Fig. 2). Competition for the PDGF receptor in the preparation was equal to 8 ng of PDGF per ml, which would correspond to about 1 ng/ml in the conditioned medium, if full recovery in the Sulphadex step is assumed. Competition for the PDGF receptor was fully neutralized by PDGF antibodies and was not found in conditioned medium from SMC cultures pretreated with actinomycin D (not shown).

DNA Synthesis of SMC in Primary Culture. To test the

Table 1. Initiation of DNA synthesis in growth-arrested secondary cultures of SMC exposed to serum-free conditioned media from primary or secondary cultures of SMC

Conditioned medium	Addition	Labeled nuclei, %
Control medium (not conditioned)	None	6.5 (3.4)
Primary culture day 4–6	None	41.9 (1.2)
	Actinomycin D pretreatment	8.3 (0.8)
	Anti-PDGF (50 μg/ml)	11.6 (0.6)
Primary culture day 7–9	None	42.5 (2.5)
Nonconfluent secondary culture (3 days)	None	16.3 (5.8)

Conditioned media were collected and tested for stimulatory effect on the initiation of DNA synthesis as described. Each value represents the mean of triplicate cultures with the SD in parenthesis. The cell number at the end of the conditioning period was 3×10^6 per flask (75 cm^2) after 6 days of primary culture, 8×10^6 per flask after 9 days of primary culture, and 3×10^6 per flask in the secondary culture. In the culture pretreated with actinomycin D, no signs of general toxicity or cell detachment were observed.

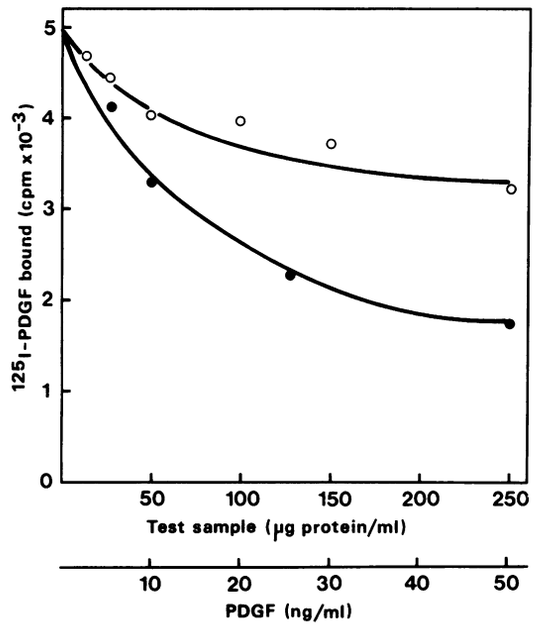

FIG. 2. Effect of conditioned medium of primary SMC cultures (days 4–8) and unlabeled PDGF on the binding of ^{125}I-PDGF to human foreskin fibroblasts. Active material in the medium was partially purified on a column of Sulphadex and assayed for PDGF-receptor competition as described. ○, Material from conditioned medium; ●, unlabeled PDGF. Each value is the mean of triplicate cultures.

ability of the cells to stimulate their own growth and respond to exogenous growth factors, two sets of experiments were performed. SMC were set up in primary culture in medium F-12 containing 10% whole blood serum at high initial density (1.0×10^5 cells per cm^2). After 2, 4, 6, 8, and 10 days, the cells were rinsed twice with serum-free medium MCDB 104 containing 0.1% bovine serum albumin (rinsing medium), incubated in this medium for 24 hr, and rinsed twice again. They then were exposed for 24 hr to rinsing medium containing 1.0 μCi of [^{3}H]thymidine per ml and processed for autoradiography. The cultures were divided into three groups: one control group, one group treated with 1.0 μg of actinomycin D per ml for 1 hr before the 24-hr incubation in serum-free medium, and one group in which the exposure to [^{3}H]thymidine was done in the presence of 50 μg of PDGF antibodies per ml. Early in culture (day 4), the rate of initiation of DNA synthesis was high and but little affected by actinomycin D or PDGF antibodies. Subsequently, the cells rapidly became confluent and ceased to grow. By scraping away the cells in a 2- to 3-mm-wide corridor in the confluent cultures (just before the transfer to serum-free medium), cellular growth could be reinitiated. This process was inhibited by pretreatment with actinomycin D but was not affected by PDGF antibodies (Table 2).

In the second set of experiments, SMC in primary culture (lower initial density, 5×10^4 cells per cm^2) were rinsed twice with rinsing medium (see above) after 2, 4, 6, 8, and 10 days, incubated in this medium for 24 hr, and rinsed twice again. They were then exposed for 24 hr to rinsing medium containing 1.0 μCi of [^{3}H]thymidine per ml either without further additions or with 25 ng of PDGF per ml or 10% whole blood serum and were processed for autoradiography. The results show that the cells had a high rate of initiation of DNA synthesis on day 4, which then decreased with increasing density of the cultures. The relative stimulatory effect of PDGF and whole blood serum was low in the early, most active growth phase but then increased successively with time, although the total percentage of labeled nuclei decreased at the same time (Table 3).

Table 2. Initiation of DNA synthesis in primary cultures of SMC (high initial density) under serum-free conditions and after pretreatment with actinomycin D or in the presence of PDGF antibodies

Day of exposure to [^{3}H]thymidine	Labeled nuclei, %		
	Control	Actinomycin D	PDGF antibodies
4	52.0 (5.3)	43.3 (3.1)	41.3 (2.3)
6	<4	12.3 (2.5)	<3
8	<3	<3	<3
10	<1	<1	<1
10S	16.0 (4.2)	<1	19.0 (1.8)
12	<1	<1	<1
12S	12.7 (2.3)	<1	16.2 (2.0)

For experimental details, see the text. In the cultures where cells were scraped from the vessels, here designated 10S and 12S, the percentage of labeled nuclei was determined in the central corridor into which cells had migrated from the neighboring, confluent parts. Each value represents the mean of triplicate cultures with the SD in parenthesis.

Binding of PDGF to SMC. Early in primary culture, the SMC showed a low but significant binding of ^{125}I-PDGF. During the phase of active growth, the specific binding of ^{125}I-PDGF to the SMC remained low but then increased >100% when growth ceased at confluence (Fig. 3). Thus, the increase in responsiveness to exogenously added PDGF with time (Table 3) was accompanied by an increase in PDGF receptor number per cell.

DISCUSSION

Conditioned medium from primary cultures of adult rat arterial SMC was shown to contain a factor that stimulates DNA synthesis in growth-arrested secondary cultures of the same cells. This factor competed with PDGF for binding sites on the surface of human fibroblasts, and its biological activity was neutralized by PDGF antibodies. The appearance of the factor in the conditioned medium was inhibited by pretreatment of the cells with actinomycin D, an inhibitor of RNA synthesis and, as a consequence thereof, protein synthesis. Hence, the results cannot be explained by release into the conditioned medium of PDGF adsorbed to the plastic vessels during the initial culture in serum-containing medium. The rate of production of DNA synthesis-stimulating activity decreased with time and increasing density of the primary cultures but did not cease entirely. Moreover, cells in nonconfluent secondary cultures released smaller amounts of stimulatory activity and, in agreement with earlier observations (6), cells in subconfluent secondary cultures did not

Table 3. Initiation of DNA synthesis in primary cultures of SMC (lower initial density) under serum-free conditions and response to stimulation with PDGF and whole blood serum

Day of exposure to [^{3}H]thymidine	Labeled nuclei, %		
	Control	PDGF	WBS
4	46.2 (2.6)	53.0 (1.0)	55.3 (1.7)
6	25.7 (2.1)	65.9 (2.0)	59.0 (2.6)
8	8.5 (1.6)	50.6 (1.7)	41.9 (1.1)
10	7.7 (1.5)	39.4 (2.0)	37.5 (1.1)
12	<1	19.7 (1.2)	29.2 (1.6)

For experimental details, see the text. Each value represents the mean of triplicate cultures with the SD in parenthesis. WBS, whole blood serum.

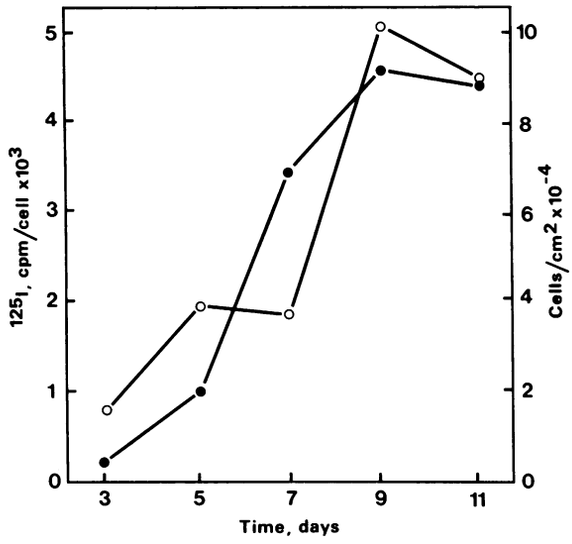

FIG. 3. Binding of ^{125}I-PDGF to SMC in primary culture. On the indicated days, cultures were exposed to ^{125}I-PDGF, and cell-associated radioactivity was determined as described. Nonspecific binding, measured in the presence of a 250-fold excess of unlabeled PDGF, was subtracted. In parallel cultures (triplicates), the cells were detached by trypsinization and counted in an electronic cell counter. ○, ^{125}I-PDGF bound; ●, cells per cm^2. Each value is the mean of triplicate cultures.

become completely quiescent by serum starvation. Taken together, these findings suggest that the SMC produce a PDGF-like protein, the function of which could be to initiate DNA synthesis in an autocrine or paracrine manner. Preliminary observations indicate that a similar phenomenon occurs in cultures of adult human arterial SMC (unpublished data).

PDGF was shown recently to show sequence homology with the transforming protein encoded by v-*sis* (8–10), the oncogene of SSV (28). The cellular homologue to v-*sis*, c-*sis*, corresponds to gene sequences coding for one of the two polypeptide chains of PDGF, the B chain (29, 30). The viral product p28^{sis} is dimerized after synthesis and proteolytically modified to a p24^{sis} species (10). This product probably resembles a PDGF B–B chain homodimer, whereas PDGF more likely is composed of one A and one B chain. Contrary, the PDGF-like growth factor produced by certain human tumor cells of mesenchymal origin resembles PDGF proper (17). The structural relationship between the PDGF-like factor produced by SMC and PDGF remains to be elucidated.

Early in primary culture, the SMC showed a high rate of DNA synthesis in serum-free medium, which was only little affected by actinomycin D. This suggests that the cells had entered an autonomous state in which entrance into the S phase of the cell cycle did not require exogenous growth factors or continuous synthesis of RNA. Later on, when a confluent stage with multiple cell layers had been reached, the rate of DNA synthesis ceased. However, after generation of a cell-free corridor by scraping, the cells adjacent to the corridor reinitiated DNA synthesis, although kept in serum-free medium. In this case, actinomycin D had a distinct inhibitory effect, indicating that synthesis of RNA and probably also protein was now required.

Exogenously added PDGF antibodies had no effect on DNA synthesis in the primary SMC cultures, neither early in the most active growth phase nor later after scraping of the confluent cultures. Similarly, PDGF antibodies added to the medium of a human osteosarcoma cell line that produces a PDGF-like mitogen have been found not to affect the growth rate of these cells (31). This argues against a significant role of the secreted PDGF-like mitogen in the stimulation of the proliferation of the producer cell. Instead, it is possible that the factor interacts with and activates the PDGF receptor in a compartment where it is inaccessible to exogenously added antibodies—e.g., inside the cell—after synthesis and during transport to the cell membrane. Other possible explanations are that the cells produce growth factors distinct from PDGF or that their growth is independent of mitogens in the abovementioned cases.

In parallel with the increasing density of the primary SMC cultures and the decreasing growth rate, the ability of the cells to bind and respond functionally to exogenous PDGF increased. The low binding capacity in the early phases of culture could be due to blocking and/or down-regulation of the PDGF receptor caused by the endogenously produced factor (32–34).

The secretion of the PDGF-like mitogen was most prominent early in primary culture, suggesting that this function is concentrated to SMC that have just passed through the modulation from contractile to synthetic phenotype. This process is defined by a structural transformation into a fibroblast-like state and gives the cells potential to proliferate and to produce extracellular matrix components, while losing their contractility (2–4). In essence, it represents a return of the cells to a structural and functional state resembling that of arterial SMC in the embryo and in the young, growing organism (35–39); interestingly, cultured arterial SMC from young rats were recently found to secrete a PDGF-like mitogen (21). Such a change in the phenotypic properties of the cells necessitates conspicuous alterations in gene expression. Our results suggest that activation or reactivation of the gene for PDGF or a closely related locus is part of these events.

Two interesting features of this gene activation are its occurrence in primary cultures of normal diploid SMC and its apparent connection to the transition of the cells from contractile to synthetic phenotype, a process believed to represent an early key event in the healing of wounds in the arterial wall and the development of atherosclerotic lesions (1). The observations are in good agreement with the idea that the primary role of the platelet in wound healing and atherosclerosis is to adhere to the site of injury, stop the bleeding, and provide factors that help to recruit and to activate growth of cells that repair the defect in the tissue. Once this activation has occurred, the SMC may start to produce their own growth factors and, as a result, become less dependent on exogenous stimulation. In accordance with the concept established by Benditt and Gown (40), such a partial autonomy of the SMC could be one of several factors promoting the tumor-like development of a lesion within the arterial wall.

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