Arterial Smooth Muscle Cells Express Platelet-derived Growth Factor (PDGF) A Chain mRNA, Secrete a PDGF-like Mitogen, and Bind Exogenous PDGF in a Phenotype- and Growth State-dependent Manner

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Abstract. Adult rat arterial smooth muscle cells are shown to express platelet-derived growth factor (PDGF) A chain mRNA, to secrete a PDGF-like mitogen, and to bind exogenous PDGF in a phenotype- and growth state-dependent manner. In the intact aortic media, where the cells are in a contractile phenotype, only minute amounts of PDGF A chain and no B chain (*c-sis*) RNA were detected. After cultivation and modulation of the cells into a synthetic phenotype, the A chain gene was distinctly expressed, whereas the B chain gene remained unexpressed. Cells kept in serum-free medium on a substrate of plasma fibronectin showed high levels of A chain RNA and high PDGF receptor activity, but did not secrete detectable amounts of PDGF-like mitogen. After exposure to PDGF, which is itself sufficient to initiate DNA synthesis and mitosis in these cells, a PDGF-like mitogen was released into the extracellular medium. Concomitantly, the amount of A chain transcripts per cell and the ability of the cells to bind radioactive PDGF decreased. Similarly, smooth muscle cells initially grown in the presence of serum released more PDGF-like mitogen, contained fewer A chain transcripts, and bound more radioactive PDGF in proliferating than in stationary cultures. The findings confirm the notion that adult rat arterial smooth muscle cells are able to promote their own growth in an autocrine or paracrine manner. Furthermore, they reveal some basic principles in the control of this process.

PLATELET-derived growth factor (PDGF)[1] has been recognized as a powerful mitogen for cells of mesenchymal origin, such as arterial smooth muscle cells, fibroblasts, and glial cells. It is a cationic, 30-kD protein that binds to specific receptors on the surface of the responding cells and elicits a pleiotropic response that ultimately leads to DNA synthesis and mitosis (Heldin et al., 1985; Ross et al., 1986). Structurally, PDGF is a dimer of two polypeptide chains linked by disulfide bonds. Two types of subunit chains have been identified, A and B, which can be combined into heterodimers or homodimers. The B chain is encoded by the protooncogene *c-sis* (Doolittle et al., 1983; Waterfield et al., 1983) and the A chain by a gene locus that gives rise to a product with an overall amino acid sequence homology of 40% as compared with the B chain precursor (Betsholtz et al., 1986*b*). Several human tumor cell lines have been found to express either or both of the PDGF-genes, to produce PDGF-like proteins, and to promote their own growth in an autocrine or paracrine manner (Heldin and Westermark, 1984; Betsholtz et al., 1986*b*; Ross et al., 1986). Evidence is accumulating to indicate that a similar mechanism may also operate in normal cells during parts of their life cycles.

Arterial smooth muscle cells (SMC) make up one example. They form the media of the arterial wall and together with macrophages are the principal cells of atherosclerotic lesions (Ross, 1986; Schwartz et al., 1986). Dependent on age, the SMC appear in at least two different phenotypic states. In the fetus and in the young evolving organism, they have a fibroblast-like appearance, divide, and secrete extracellular matrix components; at this stage they are referred to as being in a synthetic phenotype. In the adult, the SMC become highly specialized muscle cells, which contract in response to chemical and mechanical stimuli and take part in the control of blood pressure and flow; at this stage they are referred to as being in a contractile phenotype (Chamley-Campbell et al., 1979; Schwartz et al., 1986). However, this change in differentiated characteristics with age is reversible. Thus, it is well established that SMC proliferation is an early key event in the formation of atherosclerotic lesions and that the SMC in the lesions are of a modified, fibroblast-like type

1. *Abbreviations used in this paper*: medium F-12, Ham's medium F-12 supplemented with 10 mM Hepes/10 mM Tes (pH 7.3), 50 μg/ml L-ascorbic acid, and 50 μg gentamycin; NCS, newborn calf serum; PDGF, platelet-derived growth factor; SMC, smooth muscle cells.

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(Ross, 1986; Schwartz et al., 1986). Likewise, studies of adult arterial SMC cultivated in vitro indicate that the cells normally go through a transition from a contractile to a synthetic phenotype before they become able to replicate (Fritz et al., 1970; Chamley-Campbell et al., 1979, 1981; Thyberg et al., 1983).

This process includes a prominent reorganization of the cytoskeleton (Palmberg et al., 1985) with changes in the expression of actin (Gabbiani et al., 1984; Owens et al., 1986), myosin (Chamley et al., 1977; Larson et al., 1984), and intermediate filament proteins (Travo et al., 1982; Skalli et al., 1986). Concomitantly, the volume fraction occupied by myofilaments is reduced and an extensive rough endoplasmic reticulum and a large Golgi complex are formed (Thyberg et al., 1983). As a result, the cells lose the ability to contract and start to secrete extracellular matrix components and divide (Burke and Ross, 1979; Chamley-Campbell et al., 1981; Thyberg et al., 1983; Sjölund et al., 1986). Furthermore, adult rat arterial SMC in primary culture express PDGF A chain mRNA (Sejersen et al., 1986) and produce a mitogen with structural and immunological properties similar to PDGF, which may stimulate cell growth in an autocrine or paracrine manner (Nilsson et al., 1985). However, this function appears transient and SMC from young rats were found to produce PDGF-like material in larger amounts and in a more stable way than cells from adult animals (Seifert et al., 1984). Similarly, SMC isolated from intimal thickenings of injured rat arteries released more PDGF-like material than medial cells isolated from uninjured vessels (Walker et al., 1986).

Recent observations suggest that plasma fibronectin may be a major determinant of the phenotypic properties of adult arterial SMC (Hedin and Thyberg, 1987). When seeded on a substrate of this glycoprotein and cultured in a serum-free medium, the cells attached with high efficiency, and within 3–4 d most of them modulated into a synthetic phenotype as resolved by electron microscopy. The structural transformation was not itself accompanied by a proliferative response, but when exposed to PDGF or serum the cells promptly started to synthesize DNA and divide. This supports the notion that the change in phenotype is an absolute but not sufficient requirement for initiation of SMC growth (Chamley-Campbell et al., 1981; Thyberg et al., 1983). Ongoing studies in our laboratory further indicate that the ability of fibronectin to promote the phenotypic modulation resides in the cell-binding domain of the molecule and that substrates of laminin and type IV collagen, two main constituents of the basal membrane that normally surrounds smooth muscle, retain the cells in a contractile phenotype (unpublished observations). This suggests that the phenotypic modulation is a result of a specific receptor-ligand interaction, and not just a general adaptation to the in vitro environment.

In the present investigation, we have studied the role of phenotypic properties and proliferative state in the control of PDGF gene expression and PDGF production by adult rat arterial SMC cultivated in vitro. The cells were used directly after isolation, kept in serum-free medium on a substrate of plasma fibronectin to allow modulation from a contractile to a synthetic phenotype, or kept on fibronectin and then exposed to PDGF to initiate DNA synthesis and mitosis. Alternatively, the cells were grown in serum-containing medium and used in primary or secondary culture, either in a proliferating or in a nonproliferating state (for an overview of the main experimental groups, see Table I). RNA was isolated and examined for the presence of transcripts hybridizing with cDNA probes for the PDGF A chain and B chain genes. Conditioned media were collected and assayed for mitogenic activity and content of material competing with authentic PDGF for binding to specific receptors on the surface of human fibroblasts. The ability of the SMC themselves to bind PDGF was also analyzed. Finally, we studied if the SMC are able to form colonies when grown in suspension in agar, a property normally associated with tumor cells (Shin et al., 1975) and found to correlate with autocrine secretion of growth factors (Sporn and Todaro, 1980). Taken together, the results indicate that the SMC express PDGF A chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype- and growth state–dependent manner.

Materials and Methods

Cell Culture

Ham's medium F-12 supplemented with 10 mM Hepes/10 mM Tes (pH 7.3), 50 μg/ml L-ascorbic acid, and 50 μg/ml gentamycin sulfate (medium F-12) was used. SMC were isolated from the aortic media of 4–5-mo-old male Sprague-Dawley rats by digestion with 0.1% collagenase (type I; Sigma Chemical Co., St. Louis, MO) in medium F-12 with 0.1% BSA at 25°C, first for 1 h to remove remaining endothelial cells and adventitial fibroblasts and then for another 15–20 h to obtain complete dissolution of the tissue. Routine examination by electron microscopy indicated that this procedure yielded a pure population of smooth muscle cells in a contractile phenotype (cf. Thyberg et al., 1983). The cells were seeded at 2×10^5 cells/cm^2 in Nunclon plastic petri dishes (Nunc, Roskilde, Denmark) in medium F-12 with 0.1% BSA or 10% newborn calf serum (NCS) and the cultures were kept at 37°C in an atmosphere of 5% CO_2 in air. Medium was changed after 1 d and thereafter every second day. To set up secondary cultures, the cells were detached by treatment with 0.25% trypsin (1:250; Difco Laboratories, Inc., Detroit, MI) and 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free PBS (pH 7.3).

Culture in soft agar was performed according to Benya and Shaffer (1982). 60-mm plastic petri dishes (Lux; Flow Laboratories, Irvine, Scotland) were coated with a bottom layer of 1% standard low relative molecular weight agarose (Bio-Rad Laboratories, Richmond, CA). Freshly isolated cells or cells obtained from confluent primary cultures by trypsinization were suspended in 0.5% low gel temperature agarose (Bio-Rad Laboratories) in medium F-12/10% NCS and 2 ml (3×10^5 cells) were poured into each dish. The dishes were kept at 37°C for 30 min to allow the cells to sediment and were then transferred to 4°C for 10 min to let the agar gel. The cultures were kept at 37°C in a humid chamber in an atmosphere of 5% CO_2 in air and medium was changed once a week.

Isolation of PDGF and Fibronectin

PDGF was isolated from fresh human platelets as described (Heldin et al., 1987) and labeled with ^{125}I to a specific activity of ~30,000 cpm/ng using the chloramine T method (Heldin et al., 1981*b*). Antibodies against PDGF were produced in rabbits (Heldin et al., 1981*a*) and immunoglobulins were purified from immune serum by chromatography on protein A-Sepharose (Pharmacia, Uppsala, Sweden). Fibronectin was purified from fresh-frozen human plasma by affinity chromatography on gelatin-Sepharose 4B (Pharmacia) as described (Ruoslahti et al., 1982; Hedin and Thyberg, 1987). To prepare cell culture substrates, fibronectin was dissolved in Dulbecco's PBS (PBS; pH 7.4) at 20 μg/ml, poured into the petri dishes to cover the bottom, and left at 25°C for 60 min. The dishes were then rinsed twice with PBS and once with medium F-12/0.1% BSA before the seeding of cells.

Collection of Conditioned Media

Cultures were rinsed four times with medium F-12 and then incubated with medium F-12/0.1% BSA for 48 h at 37°C. Media were collected, centrifuged at 200 *g* for 10 min, and used directly to assay PDGF receptor–competing

activity or frozen at −20°C for subsequent assay of mitogenic activity. The cells were detached by trypsinization and counted in a hemocytometer. Control media were prepared from cell-free dishes in the same manner.

RNA Isolation and Blot Analysis

The cells were scraped off the petri dishes with a rubber policeman and RNA was isolated by extraction with hot phenol (Edmonds and Caramela, 1969). For intact aortas, RNA was prepared using the LiCl/urea method (Auffrey and Rougeon, 1980). Samples of total cellular RNA (36 μg/lane) were fractionated by electrophoresis in agarose/formaldehyde (Lehrach et al., 1977) and transferred to nitrocellulose membranes (Thomas, 1980); the intactness of the RNA was verified in parallel gels stained with ethidium bromide (not shown). Hybridizations were done as described by Sejersen et al. (1985, 1986). cDNA probes were nick-translated in the presence of [^{32}P]dCTP (3,000 Ci/mmol) to specific activities of ~4 × 10^8 dpm/μg (Thomas, 1980). The following cloned DNA fragments were used as probes: human PDGF A chain, clone D1 (Betsholtz et al., 1986*b*), and human *c-sis*, clone pSM-1 (Clarke et al., 1984). The size of the transcripts was determined relative to rat 18S and 28S RNA.

Assay of PDGF Receptor-competing Activity

The ability of the conditioned media to compete with ^{125}I-PDGF for binding to human foreskin fibroblasts (AG 1523) was measured as described (Nistér et al., 1984). Confluent fibroblast cultures were rinsed with PBS containing 0.1% BSA (binding medium) and exposed to conditioned media or varying amounts of PDGF (diluted in binding medium) for 150 min at 4°C. After rinsing with binding medium, the cells were exposed to ^{125}I-PDGF (2 ng/ml) in binding medium for 45 min at 4°C. They were then rinsed five times with binding medium and lysed in 1% Triton X-100/10% glycerol/20 mM Hepes (pH 7.4). Finally, radioactivity was determined in a gamma spectrometer.

Assay of PDGF Receptor Binding

SMC were grown in 12-well multidishes (4 cm^2; Costar, Data Packaging Corp., Cambridge, MA). For the assay of PDGF receptor binding, the cultures were rinsed twice with binding medium (see above) and exposed to ^{125}I-PDGF (4 ng/ml), with or without various amounts of unlabeled PDGF, for 3 h at 4°C. The cells were then rinsed and lysed as described in the preceding paragraph and cell-bound radioactivity was determined. Cell numbers were estimated in parallel cultures by trypsinization and counting in a hemocytometer. In an attempt to detect binding sites occupied by endogenous or exogenous PDGF, some cultures were incubated with 200 μg/ml of suramin (Bayer AG, Wuppertal, Federal Republic of Germany) in binding medium for 30 min at 4°C before the exposure to ^{125}I-PDGF; suramin has been shown to inhibit receptor binding of PDGF and to displace prebound PDGF (Williams et al., 1984; Hosang, 1985).

Assay of DNA Synthesis

Confluent primary cultures were trypsinized and secondary cultures were set up on glass coverslips in Nunclon 24-well multidishes (2 cm^2; Nunc). The cells were grown to subconfluence in medium F-12/10% NCS and growth arrested by incubation in medium F-12/0.1% BSA for 40 h. They were exposed to experimental media in the presence of 2 μCi/ml of [^{3}H]thymidine (5 Ci/mmol; Amersham International, Amersham, United Kingdom) for 24 h, rinsed, and fixed in 3% cacodylate-buffered glutaraldehyde. After dehydration in ethanol, the coverslips were mounted on glass slides, dipped in NTB2 emulsion (Kodak Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY), and air dried. The specimens were exposed at 4°C for 2 d, developed in D-19 (Kodak Laboratory and Specialty Chemicals), and stained with methylene blue. To determine the labeling index, at least 500 cells on each coverslip were counted.

Electron Microscopy

Primary fixation was done in 3% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) with 50 mM sucrose. After postfixation in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) with 0.5% potassium ferrocyanate for 60 min at 4°C, the cells were dehydrated in ethanol, stained with 2% uranyl acetate in ethanol, and embedded in low viscosity epoxy resin. Thin sections were cut (Ultrotome IV; LKB, Bromma, Sweden), stained with alkaline lead citrate, and examined in a Philips EM 300.

Results

Expression of PDGF Gene Activity

Intact aortic tissue, freshly isolated SMC, and SMC grown in vitro under varying conditions were used to study the role of phenotypic properties and proliferative state in the control of PDGF gene expression (see Table I for further explanation of the experimental groups). Because of problems in accumulating large numbers of SMC in primary and secondary culture (especially under serum-free conditions), no detailed kinetic analysis could be performed. RNA prepared from the intact, adult aortic media (dissected and predigested with collagenase to remove remaining endothelial cells and adventitial fibroblasts) contained only small amounts of transcripts that hybridized with the cDNA probe for the PDGF A chain. Similarly, RNA prepared from freshly isolated cells (group I) reacted weakly with this probe. On the other hand, with one exception (group VII), all groups of cultivated cells (groups II–VI) showed an increased content of PDGF A chain transcripts (Fig. 1; Table II). There was a major band corresponding to a transcript of 1.7 kb. In a few groups there also appeared two minor bands, corresponding to transcripts of 1.3 and 2.3 kb, respectively (Fig. 1). A comparison of the relative strengths of the bands, as analyzed by scanning densitometry, and the RNA content of the cells, as analyzed by acridine orange staining and flow cytometry, indicated that the strongest expression of the PDGF A chain gene occurred in nonproliferating primary cultures (groups II and V; Table II). In contrast to these findings, no signs of expression of the PDGF B chain gene (*c-sis*) were detected, neither in the intact, adult aortic media nor in any of the experimental groups (Table I). In this context it should be stressed that the

Table I. Main Experimental Groups of Smooth Muscle Cells

Group	Culture conditions	Functional state of cells
I	Freshly isolated cells	Contractile phenotype
II	Cells seeded on fibronectin and cultured in serum-free medium for 4 d	Synthetic phenotype, nonproliferating (subconfluent)
III	Cells seeded on fibronectin, cultured in serum-free medium for 3 d, and then exposed to PDGF (20 ng/ml) for 24 h	Synthetic phenotype, proliferating (subconfluent)
IV	Primary cultures grown in serum-containing medium for 4–5 d	Synthetic phenotype, proliferating (subconfluent)
V	Primary cultures grown in serum-containing medium for 12–14 d	Synthetic phenotype, nonproliferating (confluent)
VI	Secondary cultures grown in serum-containing medium for 4–5 d	Synthetic phenotype, proliferating (subconfluent)
VII	Secondary cultures grown in serum-containing medium for 12–14 d	Synthetic phenotype, nonproliferating (confluent)

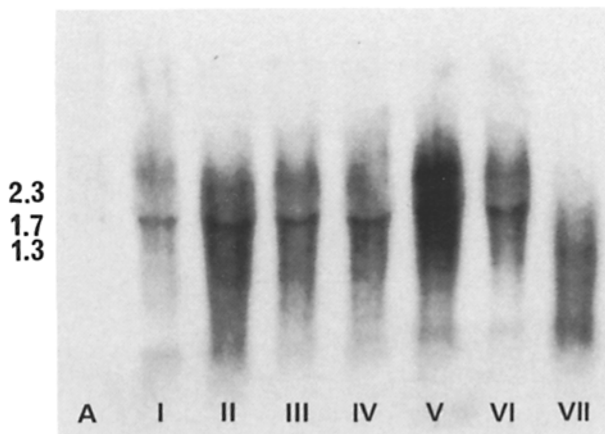

Figure 1. RNA blot analysis of samples from adult rat arterial SMC. RNA was isolated by phenol extraction, fractionated by electrophoresis in agarose/formaldehyde, and transferred to a nitrocellulose membrane. PDGF A chain transcripts were visualized by hybridization to a ^{32}P-labeled cDNA probe followed by autoradiography. A further description of the experimental groups is given in Table I. *A*, intact aorta. Sizes are shown in kilobases.

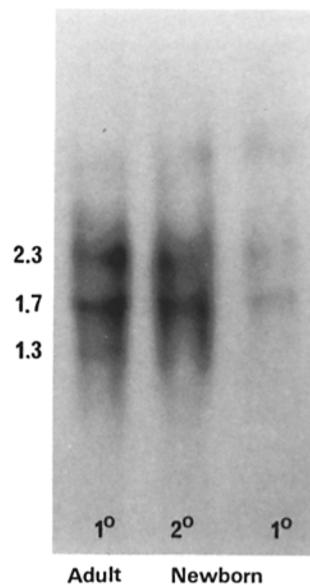

Figure 2. RNA blot analysis of samples from newborn rat arterial SMC. RNA was isolated by phenol extraction, fractionated by electrophoresis in agarose/formaldehyde, and transferred to a nitrocellulose membrane. PDGF A chain transcripts were visualized by hybridization to a ^{32}P-labeled cDNA probe followed by autoradiography. For comparison, a sample from a confluent primary culture of adult cells was run next to the samples from subconfluent primary and secondary cultures of newborn cells. Sizes are shown in kilobases.

rat genome has previously been shown to contain DNA sequences that hybridize with the human cDNA probe used here (Sejersen et al., 1986).

Analysis of RNA samples from proliferating primary and secondary cultures of arterial SMC from 10-d-old rats showed that these cells expressed the PDGF A chain gene (Fig. 2). The sizes of the transcripts were the same as in the adult cells, but the relative amount of transcripts per unit total RNA was markedly higher (5-10-fold) in secondary than in primary cultures (Fig. 2). Like the situation in the adult cells, no signs of expression of the PDGF B chain gene (*c-sis*) were detected.

Production of a PDGF-like Mitogen

To look for secretion of growth factors by the SMC, conditioned serum-free media were prepared (48 h) and tested for their ability to initiate DNA synthesis in serum-starved secondary SMC cultures. Stimulatory material occurred in the media from all proliferating cultures (groups III, IV, VI), and more so in primary than in secondary cultures (Fig. 3; see also Table IV). Media from confluent, nonproliferating primary cultures (group V) likewise produced a distinct effect, with a labeling index about half of that given by optimal concentrations of PDGF (10-20 ng/ml). Antibodies against PDGF (50 µg/ml) partially neutralized stimulatory effects in all groups (Fig. 3).

An important question related to these experiments is whether or not PDGF and other serum growth factors may attach to the bottom of the plastic dishes or the fibronectin substrate during the early phases of culture and then detach during the conditioning. To test this possibility, conditioned media were prepared from cell-free dishes and tested for DNA synthesis-stimulating activity as described above. The results of this control experiment disclosed that no functionally significant release of preadsorbed mitogens from the dishes occurred during the conditioning period (Fig. 4). Moreover, it has previously been shown that pretreatment with actinomycin D inhibits the secretion of PDGF-like material by the SMC, indicating that this is a metabolically active process (Nilsson et al., 1985).

*Table II. Expression of PDGF A Chain mRNA in Smooth Muscle Cells**

Experimental groups‡		Relative amount of A chain RNA per unit total RNA§	Relative amount of total RNA per cell‖	Relative amount of A chain RNA per cell
Group	Culture conditions			
I	Freshly isolated cells	1	1	1
II	Fibronectin 4 d	9	3	27
III	Fibronectin 4 d, PDGF final 24 h	4	3	12
IV	Primary cultures/serum subconfluent	3	5	15
V	Primary cultures/serum confluent	20	6	120
VI	Secondary cultures/serum subconfluent	4	5¶	20
VII	Secondary cultures/serum confluent	0	6¶	0

* Results of one experiment are shown (repeated once with similar results).
‡ For further details, see Table I.
§ Autoradiograms of RNA blots were scanned in a densitometer and the areas under the peaks were calculated. Group I was set equal to 1.
‖ Cells were stained with acridine orange and analyzed for relative content of RNA in a fluorescence-activated cell sorter (Traganos et al., 1977). Group I was set to equal to 1.
¶ Not determined, presumed to be approximately the same as in the primary cultures.

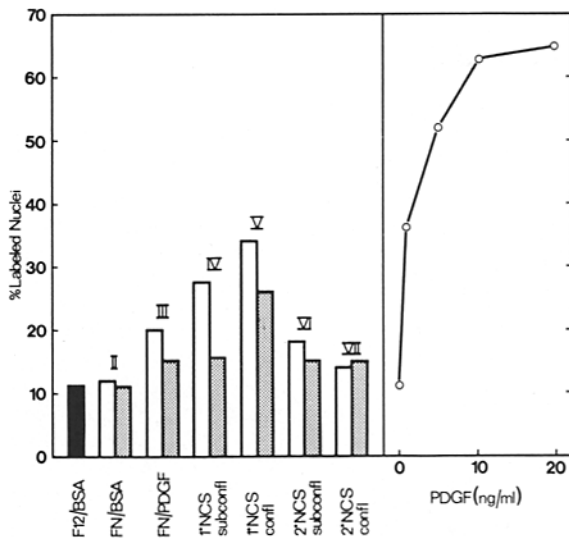

Figure 3. Initiation of DNA synthesis by conditioned media from adult rat arterial SMC. Serum-free conditioned media were prepared and tested for their ability to initiate DNA synthesis in serum-starved secondary SMC cultures (*left*). The cells were exposed to [^{3}H]thymidine for 24 h in conditioned media without (*open bars*) or with (*stippled bars*) 50 μg/ml of PDGF antibodies (immunoglobulin fraction) and processed for autoradiography. Medium without or with addition of increasing amounts of PDGF served as controls (*solid bar* in *left*, and *right*). The results are given as means of triplicate cultures. No adjustments for differences in cell numbers in the respective cultures were made (cf. Table IV). The experiment was repeated once with similar results.

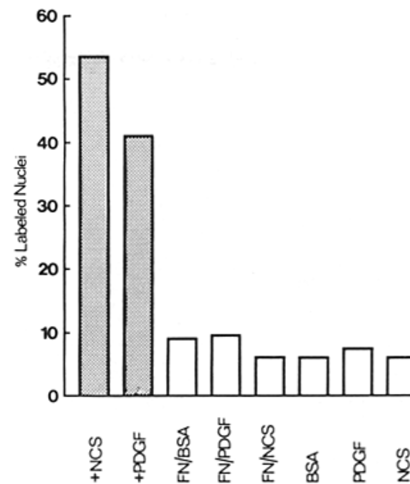

Figure 4. Lack of attachment and deattachment of PDGF to the cell culture substrates. Cell-free plastic petri dishes without and with (*FN*) a substrate of plasma fibronectin were prepared and exposed to medium F-12 with 0.1% BSA, 10% NCS, or 20 ng/ml of PDGF for 24 h at 37°C. The dishes were then rinsed four times and incubated with medium F-12/0.1% BSA for 48 h at 37°C. Serum-starved secondary SMC cultures were exposed to [^{3}H]thymidine in the conditioned media for 24 h and processed for autoradiography (*open bars*). Cultures exposed to [^{3}H]thymidine in medium F-12 with 10% NCS or 20 ng/ml of PDGF served as positive controls (*stippled bars*). The results are given as means of quadruplicate cultures.

PDGF Receptor-competing Activity

As a further test of the nature of the mitogen released by the SMC, conditioned media were examined in a radioreceptor assay. Human fibroblasts were exposed to the conditioned media or varying amounts of pure, unlabeled PDGF at 4°C and were then allowed to bind ^{125}I-PDGF at 4°C. In good agreement with the results of the DNA synthesis experiments, the cells in groups III–VI were found to have released material that competed with ^{125}I-PDGF for binding to receptors on the fibroblast surface (Table III). On a per cell basis, the largest amounts of such material occurred in the conditioned media of primary cultures that had been kept on a substrate of plasma fibronectin for 4 d and had been exposed to PDGF for the final 24 h (group III) and of primary and secondary cultures that had been grown in the presence of 10% serum for 4–5 d (groups IV and VI), i.e., in all cases cells that were in a subconfluent and proliferating state at the start of conditioning.

PDGF Receptor Expression

To analyze the ability of the SMC themselves to interact with PDGF, receptor-binding experiments with ^{125}I-PDGF were performed. These studies were restricted to the primary cultures (groups II–V), in which the strongest expression of the PDGF A chain gene and the most active release of a PDGF-like mitogen into the extracellular medium occurred. The highest specific binding was found in group II, i.e., cells kept on a substrate of plasma fibronectin for 4 d and at no time exposed to exogenous mitogen. In the other three groups, cells kept on fibronectin for 4 d and exposed to PDGF for the final 24 h (group III), subconfluent primary cultures (group IV), and confluent primary cultures (group V), the specific binding was lower (Fig. 5 *A*). Preincubation of the cells with suramin lacked effect on the binding of ^{125}I-PDGF in group II, whereas the binding increased in groups III–V (Fig. 5 *A*). If the cells were first transferred to serum- and PDGF-free medium for 48 h (corresponding to the conditioning period described above), the subsequent binding of

*Table III. Production of PDGF Receptor–competing Activity by Smooth Muscle Cells**

Experimental groups‡		
Group	Culture conditions	PDGF equivalents
		ng/ml/10⁶ cells
II	Fibronectin 4 d	0.0
III	Fibronectin 4 d, PDGF final 24 h	4.9
IV	Primary cultures/serum subconfluent	11.4
V	Primary cultures/serum confluent	0.3
VI	Secondary cultures/serum subconfluent	4.0
VII	Secondary cultures/serum confluent	0.1

Conditioned media (48 h) were prepared and analyzed for content of PDGF-like material using a radioreceptor assay. Pure unlabeled PDGF was used to construct a standard curve and convert the receptor-competing activity of the samples into PDGF equivalents (ng/ml).
* Results of one experiment are shown (repeated twice with similar results).
‡ For further details, see Table I.

*Table IV. Summary of Data on Expression of PDGF A Chain mRNA, Secretion of a PDGF-like Mitogen, and PDGF Receptor Activity in Rat Arterial Smooth Muscle Cells**

Experimental groups‡					
Group	Culture conditions	A Chain RNA per cell	PDGF receptor competing activity§	Mitogenic activity§	PDGF receptor activity‖
I	Freshly isolated cells	±	ND	ND	ND
II	Fibronectin 4 d	++	–	–	+++/+++
III	Fibronectin 4 d, PDGF final 24 h	+	++	++	+/++
IV	Primary cultures/serum subconfluent	+	+++	+++	+/++
V	Primary cultures/serum confluent	+++	+	++	+/++
VI	Secondary cultures/serum subconfluent	++	++	+	ND
VII	Secondary cultures/serum confluent	–	±	–	ND

* Based on the results in Table II, Table III, Fig. 3, and Fig. 5 (values transferred into a relative scale).
‡ For further details, see Table I.
§ On a per cell basis.
‖ Without and with preincubation with suramin.

^{125}I-PDGF increased in all groups. However, the relative differences between the groups and the effects of preincubation with suramin remained essentially the same (Fig. 5 *B*).

To ascertain that the differences in ^{125}I-PDGF binding described above were due to differences in receptor number and not differences in binding affinity, a Scatchard plot analysis was made on the cells in groups II and III; these cells were grown on a substrate of fibronectin in serum-free medium and showed a high and a low binding of ^{125}I-PDGF, respectively (Fig. 5). In both groups half-maximal binding was obtained at 1.5–2 nM and maximal binding at 4–6 nM. The binding data indicated a single class of receptors in both groups, with a K_d of 1.9 nM in group II and 1.8 nM in group III.

Growth of Cells in Soft Agar

The power of tumor cells to grow in an anchorage-independent manner has been found to correlate to autocrine secre-

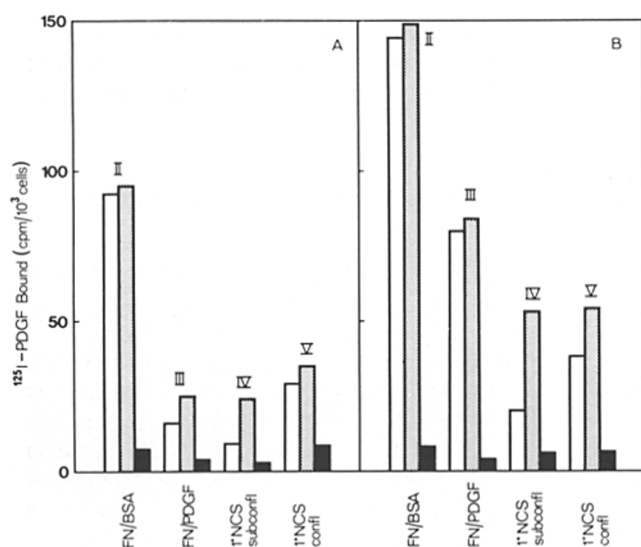

Figure 5. PDGF receptor expression of adult rat arterial SMC. Cells were set up in primary culture (groups II–V) and tested for their ability to bind ^{125}I-PDGF at 4°C, either directly (*left*) or after incubation in serum-free medium for an additional 48 h at 37°C (*right*). (*Open bars*) Cells exposed to ^{125}I-PDGF; (*stippled bars*) cells treated with suramin and then exposed to ^{125}I-PDGF; (*solid bars*) cells exposed to ^{125}I-PDGF in the presence of a 250-fold excess of unlabeled PDGF. The results are given as means of triplicate cultures. The experiment was repeated twice with similar results.

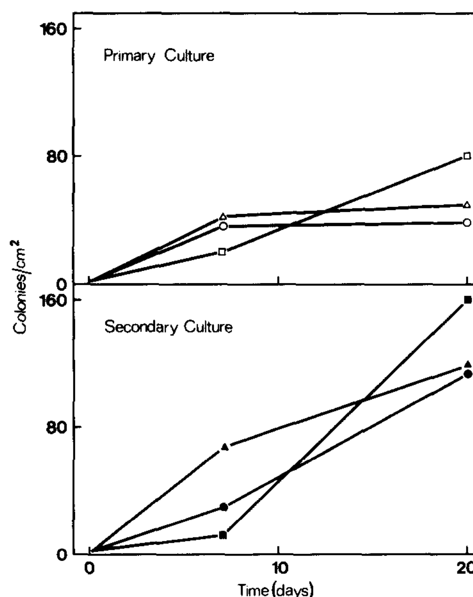

Figure 6. Colony formation during growth in agar. Freshly isolated adult rat arterial SMC (primary cultures) and SMC obtained from confluent monolayer cultures (secondary cultures) were suspended in agar and grown in normal medium (*circles*), medium with 50 μg/ml of PDGF antibodies (*triangles*), or medium with 200 μg/ml of suramin (*squares*). At the indicated times, the number of cell colonies (>10 cells) in 25 randomly selected grid squares (4 mm^2 each) was counted. Each value is the mean of duplicate or triplicate cultures.

tion of growth factors (Sporn and Todaro, 1980). Since the SMC secrete PDGF, it was considered of interest to examine if they share the ability to grow in suspension and if agents that are supposed to neutralize extracellular PDGF interfere with this process. Both freshly isolated cells (primary cultures) and cells obtained from confluent petri dishes by trypsinization (secondary cultures) were found to grow in soft agar in medium containing 10% serum. In primary cultures, most of the colonies (>10 cells) appeared to be formed already during the first week (Fig. 6). No further increase in the number of colonies was seen after that, but they continued to grow in size for at least 3 wk, reaching a maximal diameter of 100–150 μm (Fig. 7). Electron microscopic studies indicated that the cells were all in synthetic phenotype with an extensive rough endoplasmic reticulum, a large Golgi complex, and few myofilament bundles (Fig. 8). In secondary cultures, the colonies became more numerous but considerably smaller than in primary cultures, reaching a maximum number after 3 wk (Fig. 6). The colony-forming efficiency was 0.3–0.7% in primary cultures and 1.0–1.5% in secondary cultures. Antibodies against PDGF (50 μg/ml) lacked clear effect both on the number and size of the colonies. With suramin (200 μg/ml), the number of colonies formed during the first week was less than half of that in the control cultures. After 3 wk, the number of colonies was instead distinctly raised (Fig. 6). Compared with the controls, they were similar in overall size but more irregular in shape.

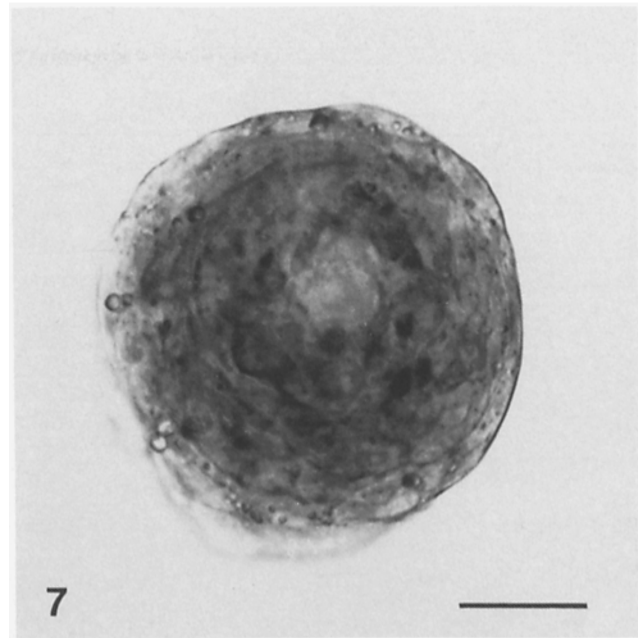

Figure 7. Light microscopy of agar colonies. Freshly isolated adult rat arterial SMC were suspended in agar and grown in normal medium for 3 wk. After fixation in 3% buffered glutaraldehyde, the cultures were stained with 0.05% methylene blue for 5 min. Bar, 25 μm.

Discussion

General Comments

The results of this study show that adult rat arterial SMC cultivated in vitro express PDGF A chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype- and growth state-dependent manner (summarized in Table IV). Together with the results of previous reports (Nilsson et al., 1985; Sejersen et al., 1986; Hedin and Thyberg, 1987), they suggest that plasma fibronectin and PDGF play an important role in controlling the phenotypic properties of the cells and act in concert to give them the ability to stimulate their own growth in an autocrine or paracrine manner. In the following discussion, we will briefly consider the role of age in this process. We will then discuss the observations on the adult cell system in some detail and end by presenting a tentative model of autocrine growth regulation in arterial smooth muscle.

On the Role of Age in PDGF Secretion by Arterial SMC

A comparison of SMC from adult and young animals revealed that both expressed PDGF A chain but not B chain (*c-sis*) mRNA. After transfer from primary to secondary culture, the content of A chain transcripts decreased in adult cells but increased in young cells. Accordingly, SMC from adult rats were found to release a PDGF-like mitogen into the medium only during a limited period in primary and secondary culture, whereas young SMC have been observed to do so continually during a large number of passages (Seifert et al., 1984). Taken together, these findings suggest that secretion of PDGF is a constitutive function of arterial SMC in the young and growing organism. In the adult, this function is normally lost and can be resumed only after return of the cells to a phenotypic state similar to that in the young (see below). Such a change in differentiated characteristics occurs as a consistent response to in vitro cultivation (Fritz et al., 1970; Chamley-Campbell et al., 1979; Thyberg et al., 1983), possibly as a result of interaction with plasma fibronectin (Hedin and Thyberg, 1987). In vivo a similar change may be induced by endothelial damage and penetration of blood components into the underlying media (Schwartz et al., 1986).

PDGF A Chain Gene Expression and PDGF Secretion by Adult Arterial SMC Is a Phenotype- and Growth State-dependent Process

Only minute amounts of PDGF A chain and no B chain (*c-sis*) transcripts were detected in RNA isolated from the intact aortic media. This is in good agreement with the notion that arterial SMC are normally in a highly differentiated, contractile, and nonproliferative state (Chamley-Campbell et al., 1979; Schwartz et al., 1986). In contrast, isolated and cultivated SMC distinctly expressed the PDGF A chain gene, whereas the B chain (*c-sis*) gene remained silent. A similar pattern of PDGF gene expression has been noted in several human tumor cell lines, but cell lines that express only the B chain gene or both the A and B chain genes have also been observed (Betsholtz et al., 1986*b*).

The discovery of raised levels of PDGF A chain transcripts in RNA from the newly isolated SMC (group I) was somewhat surprising. As judged by electron microscopy, these cells are in a contractile phenotype similar to that of the cells in the intact aortic media. They have an extensive myofilament system, few free ribosomes, and a poorly developed rough endoplasmic reticulum (Thyberg et al., 1983). Hence,

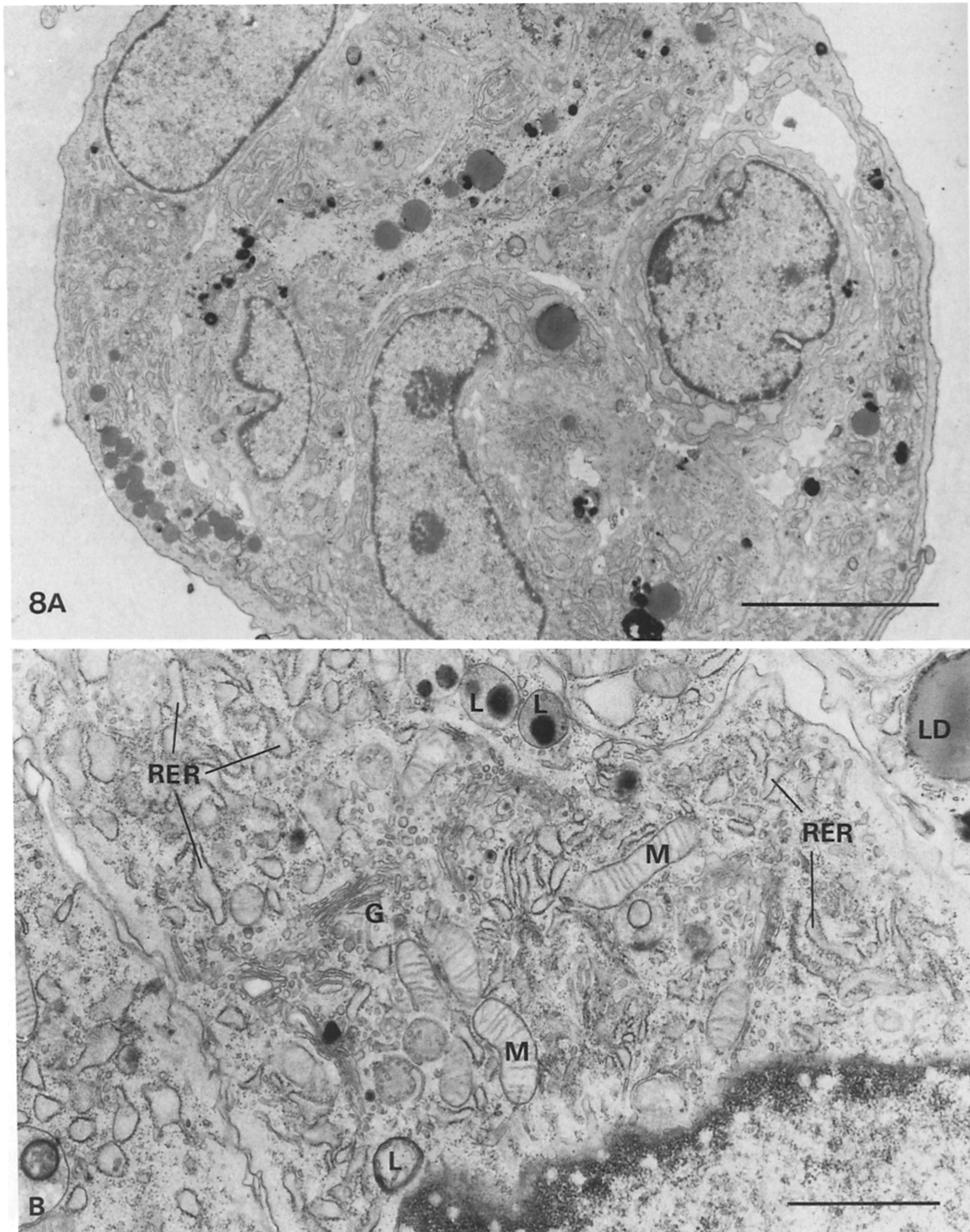

Figure 8. Electron microscopy of agar colonies. Freshly isolated adult rat arterial SMC were suspended in agar, grown in normal medium for 5 wk, and fixed in 3% buffered glutaraldehyde. (*A*) Overview; (*B*) detail of a rounded, central cell. All the cells in the colonies are in synthetic phenotype with an extensive rough endoplasmic reticulum (*RER*) and a large Golgi complex (*G*). *L*, lysosomes; *LD*, lipid droplet; *M*, mitochondria. Bars: (*A*) 5 μm; (*B*) 1 μm.

it seems as if the PDGF A chain gene is turned on in a sizable fraction of the cells already during the collagenase digestion, perhaps as one of the first events in the phenotypic modulation. More direct evidence for a link between these processes was obtained by the observation that cells kept in serum-free medium on a substrate of plasma fibronectin for 4 d (group II) were among those that most strongly expressed the PDGF A chain gene. Previous studies have shown that these cells rapidly convert into synthetic phenotype, but do not start to proliferate without exogenous mitogen stimulation (Hedin and Thyberg, 1987). Likewise, they were not found to release detectable amounts of PDGF-like material and showed a high PDGF receptor activity, without signs of receptor occupancy using the suramin displacement method (Williams et al., 1984; Hosang, 1985).

If, on the other hand, the SMC were kept on a substrate of plasma fibronectin for 4 d and were exposed to PDGF for the final 24 h (group III), they started to discharge a PDGF-like agonist into the culture medium (conceivably a PDGF A chain homodimer). Therefore, it is concluded that at least two requirements need to be fulfilled before the cells are able to synthesize and secrete their own PDGF. First, they must go through a transition from a contractile to a synthetic phenotype and, second, they have to be stimulated with PDGF, or possibly some other mitogen. Although detailed experimental data are still lacking, the findings further suggest that the transcription of the PDGF A chain gene and the subsequent synthesis and processing of the protein are controlled separately.

Concomitant with the secretion of a PDGF-like mitogen, the cellular content of PDGF A chain transcripts decreased. This suggests that the appearance of the gene product brings about a lowered rate of transcription of the gene or an increased rate of degradation of preexisting transcripts, or both. The actively secretory cells also showed a reduced ability to bind PDGF, evidently due to a reduced number of cell surface receptors as the binding constants did not differ (comparison between groups II and III). Previous studies have indicated that after uptake by endocytosis, both PDGF and its receptor are normally transferred to lysosomes and broken down by acid hydrolases (Heldin et al., 1982; Nilsson et al., 1983; Rosenfeld et al., 1984). Such a receptor down regulation could partly explain the decreased binding of the radioactive probe in cells exposed to PDGF (group III). Conceivably, PDGF produced by the cells themselves will likewise induce a receptor degradation and contribute to the reduced binding capacity. Moreover, part of the bound PDGF will remain on the cell surface for a considerable time and block some of the receptors (Heldin et al., 1982; Nilsson et al., 1983). Accordingly, preincubation of the cells with suramin, which dissociates PDGF from its receptor (Williams et al., 1984; Hosang, 1985), increased the subsequent binding of the radioactive probe. It should also be noted that incubation of the cells in PDGF-free medium for an additional 48 h before the binding experiment increased the PDGF receptor activity. This suggests that a net renewal of receptors occurred in the absence of exogenous PDGF, although production of a PDGF-like molecule by the cells themselves continued (group III).

A comparison of primary and secondary cultures kept in serum-containing medium and examined in different growth phases (group IV–VII) corroborated the above findings and provided additional information concerning the regulation of PDGF secretion in the SMC. In primary cultures, the release of PDGF-like material into the medium slowed down as the cells passed from a proliferating into a stationary phase, and in secondary cultures it seemed to stop completely (see Table IV). At the same time, the number of PDGF A chain transcripts per cell rose in primary cultures but dropped below the detection limit in secondary cultures. We interpret these findings to indicate that the ability of adult rat arterial SMC to produce PDGF during in vitro cultivation is transient and dependent on proliferative rate. It comes to a peak during the exponential growth phase early in primary culture and then declines as the cells become confluent. Nevertheless, PDGF mRNA is retained or synthesized de novo and as the cells are trypsinized, reseeded at lower density, and start again to divide, PDGF production is resumed, although now at a lower level.

Adult Arterial SMC Are Able to Grow in an Anchorage-independent Way

It is well established that tumor cells are able to grow in an anchorage-independent way (Shin et al., 1975). This has been found to correlate with autocrine secretion of growth factors (Sporn and Todaro, 1980). Accordingly, it was recently shown that human fibroblasts infected with simian sarcoma virus not only produce a protein similar to PDGF but also form colonies in suspension in agar (Johnsson et al., 1986). Here it is demonstrated that adult rat arterial SMC share this property. Similarly, it has previously been demonstrated that bovine arterial SMC are able to grow in soft agar when stimulated with a combination of PDGF, epidermal growth factor, and transforming growth factor β (Assoian and Sporn, 1986).

Our findings further indicate that the SMC do not need to be attached to a substrate to go through the modulation from a contractile to a synthetic phenotype. Thus, all cells in the colonies were in a synthetic state with an extensive rough endoplasmic reticulum, a large Golgi complex, and few myofilament bundles. The colony-forming efficiency was lower in primary than in secondary cultures, probably due to loss of cells during the initial phenotypic modulation in the former. PDGF antibodies lacked inhibitory effect on the colony formation and, likewise, have been found to lack effect on the autocrine stimulation of the cells in monolayer culture (Nilsson et al., 1985). A possible reason could be that the antibodies, due to their size, are unable to penetrate into the pericellular environment and react with PDGF released locally. The antiparasital drug suramin, a smaller molecule that is known to inhibit receptor binding of PDGF as well as to displace prebound PDGF (Williams et al., 1984; Hosang, 1985), slowed down colony formation during the first week of culture. After another 2 wk, and of unknown reason, the number of colonies was markedly higher in suramin-treated than in control cultures. In contrast to these findings, suramin was found to efficiently revert the transformed phenotype of human and rat fibroblasts infected with simian sarcoma virus, but in this case the growth properties of the cells were examined only in monolayer culture (Betsholtz et al., 1986*a*).

Tentative Model of Autocrine Growth Regulation in Adult Arterial SMC

Based on these and other recent findings (Nilsson et al., 1985; Sejersen et al., 1986; Hedin and Thyberg, 1987), and

as a framework for future studies, a tentative and simplified model of autocrine growth regulation in adult arterial SMC is presented. This model includes the following main steps: (*a*) Upon contact with plasma fibronectin, the smooth muscle cells modulate from a contractile to a synthetic phenotype. As parts of this process, PDGF receptors are expressed at the cell surface and transcription of the PDGF A chain gene is induced. (*b*) After stimulation with exogenous PDGF, the cells synthesize DNA and divide. Additionally, they start to produce PDGF (A chain homodimers). These molecules bind to and activate PDGF receptors on the cell itself (autocrine stimulation) or, after diffusion through the extracellular medium, PDGF receptors of neighboring cells (paracrine stimulation). (*c*) In response to the synthesis of PDGF, the transcription of the PDGF A chain gene decreases. The fate of preexisting transcripts may vary, for example depending on the number of previous cell divisions. However, ultimately the transcripts will be degraded. As a result, the synthesis of PDGF will terminate and the autocrine stimulation of the cells will cease.

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