

Rat Aortic Smooth Muscle Cells in Serum-free Primary Cultures Enter the Cell Cycle after a Short Exposure to PDGF-BB and Continue to Replicate after Withdrawal of the Mitogen

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Abstract

The ability of a few major peptide mitogens to induce DNA replication in primary and secondary cultures of rat aortic smooth muscle cells was studied. Freshly isolated cells were seeded on a substrate of fibronectin in a serum-free medium and allowed to go through the transition from a contractile to a synthetic phenotype. A large fraction of the cells entered the cell cycle and synthesized DNA following a short treatment with PDGF-BB (30-60 min). Moreover, a high replicative activity persisted for at least 2-3 days after removal of the mitogen. A distinct response was also obtained with bFGF, EGF and PDGF-AA, but a longer treatment was required for maximal effect (4-8 h), and the replicative activity rapidly returned to control levels after washing away the exogenous stimulus. On the other hand, only a weak effect was obtained with IGF-I, IL-1 α and TGF- β 1 (24-48 h). In secondary cultures, the mitogens had to be present throughout the prereplicative period for maximal effect and only PDGF-BB gave rise to a moderately raised level of continuing DNA synthesis. Taken together, these findings indicate that PDGF-BB is a most potent mitogen for vascular smooth muscle cells and that the cells are particularly responsive shortly after the conversion into a synthetic phenotype. In addition, PDGF-BB appears to be unique in its capacity to make the cells able to replicate in the absence of an exogenous stimulus. This may be important in the proliferative response of smooth muscle cells to vascular injury.

Key words: smooth muscle cells, peptide mitogens, autocrine growth stimulation.

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Early in primary culture, vascular smooth muscle cells (SMCs) are converted from a contractile to a synthetic phenotype [9, 60]. This process shows striking similarities to the alterations in SMC morphology and function that occur during atherogenesis [44]. It includes a structural reorganization of the cells with loss of actin filament bundles and formation of an extensive endoplasmic reticulum and a prominent Golgi complex. At the same time, the expression of SMC α -actin decreases, while the expression of nonmuscle β - and γ -actin increases. As a consequence, the cells lose their contractility and instead become able to proliferate in response to exogenous mitogens and to secrete extracellular matrix components. Moreover, as soon as they have entered the division cycle, the cells start to produce mitogens on their own and to replicate in the absence of exogenous stimuli [5, 60].

Previous investigations in our laboratory have indicated a role for extracellular matrix components in the control of

the differentiated properties of rat aortic SMCs. Under serum-free conditions, a substrate of fibronectin was found to promote the transition of freshly isolated cells into a synthetic phenotype, whereas a substrate of the basement membrane components laminin and collagen type IV held them back in a contractile phenotype [20]. Subsequently, fibronectin was demonstrated to exert its effect via the RGD cell attachment sequence and by interaction with a cell surface receptor belonging to the integrin family of proteins [7, 21]. The shift in phenotype on fibronectin made the cells competent to respond to a mitogenic stimulus but did not induce DNA replication in itself. These observations confirm the notion that the passage from a contractile to a synthetic phenotype is a necessary but not sufficient requirement for initiation of SMC proliferation and suggest that these processes are regulated separately [60].

In parallel, it was demonstrated that the shift in phenotype of the SMCs early in primary culture is associated with an increased expression of platelet-derived growth factor (PDGF) α - and β -receptors and mRNA for the A- but not the B-chain of PDGF. Accordingly, exposure of the cells to PDGF isolated from human platelets (principally PDGF-AB) resulted in their entering the cell cycle and starting to divide. Moreover, after withdrawal of the stimulus they started to secrete a PDGF-like protein into the medium and continued to replicate in the absence of exogenous mitogens. This process of autocrine or paracrine growth stimulation was most prominent directly after the transition of the cells into a synthetic phenotype and ceased as they were grown to confluence and passaged by trypsinization [52, 53].

The object of the present investigation was to examine the ability of a few major peptide mitogens to induce replication of SMCs early in primary culture and to make the cells able to continue to replicate after withdrawal of the stimulus. For this purpose, freshly isolated rat aortic SMCs were seeded on a substrate of fibronectin in a serum-free medium, allowed to pass through the transition into a synthetic phenotype, and then exposed to the mitogens. The DNA synthetic activity of the cells was determined by thymidine autoradiography both in direct connection with the exposure to the mitogens and after an intervening incubation in medium to which no mitogens had been added. Comparative studies were made on cells in secondary culture. The following mitogens were used: basic fibroblast growth factor, bFGF [1]; epidermal growth factor, EGF [30]; insulin-like growth factor-I, IGF-I [10]; interleukin-1 α , IL-1 α [49]; PDGF-AA [39]; PDGF-BB [39]; and transforming growth factor- β 1, TGF- β 1 [55]. The results indicate that PDGF-BB is a most potent mitogen for SMCs both in primary and secondary cultures and that cells in primary culture require a much shorter exposure than cells in secondary culture for a maximal response. In addition, PDGF-BB is clearly more potent than PDGF-AA and other mitogens in making cells in primary culture able to enter a state of autocrine or paracrine growth stimulation.

Materials and Methods

Materials

Ham's medium F-12, newborn calf serum (NCS) and collagenase were from Gibco Brl (Paisley, Scotland), bovine serum albumin (BSA) and soybean trypsin inhibitor from Sigma (St. Louis, Mo., USA), trypsin from Difco (Detroit, Mi., USA), [^{3} H]-thymidine (5 Ci/mmol) from Amersham International (Amersham, England), and cell culture plasticware from Nunc (Roskilde, Denmark). The medium was supplemented with 10 mM each of the organic buffers Hepes and Tes (pH 7.3), 50 μ g/ml of L-ascorbic acid, and 50 μ g/ml of gentamycin sulfate (below referred to as medium F-12). Fibronectin was isolated from human plasma by affinity chromatography on gelatin-Sepharose and diluted in Dulbecco's phosphate-buffered

saline (PBS, pH 7.3) to a final concentration of 20 μ g/ml [19]. The protein (0.5 ml = 10 μ g) was added to 12-mm glass coverslips and allowed to adsorb for 16 h at 20°C. The coverslips were then rinsed twice with PBS and incubated in medium F-12/0.1% BSA for 30 min to block unspecific binding sites. Recombinant human PDGF-AA (short form) and PDGF-BB were produced in yeast [35]. Recombinant bovine bFGF, human EGF, human IGF-I, and human IL-1 α were from Boehringer Mannheim (Mannheim, Germany). TGF- β 1 was isolated from human platelets [31]. Stock solutions of the mitogens were prepared in dilute acetic acid, PBS, or medium F-12 and stored frozen at -20°C before use.

Cell culture

SMCs were isolated from the aortic media of 300-350 g male Sprague-Dawley rats by collagenase digestion [59], seeded on fibronectin-coated glass coverslips (3x10⁴ cells/cm²), and incubated in medium F-12/0.1% BSA at 37°C in an atmosphere of 5% CO₂ in air for 96 h before the DNA synthesis experiments. For comparative purposes, growth-arrested secondary cultures were also used. In this case, freshly isolated SMCs were first grown to confluence in medium F-12/10% NCS. After detachment of the cells with 0.1% trypsin and 0.02% EDTA in calcium- and magnesium-free PBS, they were reseeded on fibronectin-coated glass coverslips in medium F-12/0.1% BSA with 20 μ g/ml trypsin inhibitor (3x10⁴ cells/cm²) and incubated for 48 h at 37°C before the experiments.

Electron microscopy

For a fine structural analysis of their phenotypic state, the cells were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate-HCl buffer (pH 7.3) with 0.05 M sucrose, postfixed in 2% osmium tetroxide in cacodylate buffer with 0.5% potassium ferrocyanate, dehydrated in ethanol, stained with 2% uranyl acetate in ethanol, and embedded in Spurr low-viscosity epoxy resin. Thin sections were cut with a diamond knife using an LKB Ultratome IV, picked up on carbon-coated formvar films, stained with alkaline lead citrate, and examined in a JEOL EM 100CX at 60 kV.

Thymidine autoradiography

To study the initiation of DNA synthesis, the cultures were exposed to 1 μ Ci/ml of tritiated thymidine in medium F-12/0.1% BSA with or without mitogens and then fixed in 3% cacodylate-buffered glutaraldehyde (pH 7.3). After dehydration in ethanol, the coverslips were mounted on glass slides, dipped in Kodak NTB2 emulsion, left in the dark for 48 h at 4°C, and developed in Kodak D-19. The cells were stained with 1% methylene blue and the fraction of labelled nuclei was determined by counting 500 cells on each coverslip. All experiments were repeated at least twice with similar results.

Results

Phenotypic modulation in primary cultures

The freshly isolated SMCs were all in a contractile phenotype with a cytoplasm largely filled up with myofilament bundles coalescing in dense bodies and abundant mitochondria. After seeding on a substrate of fibronectin in medium F-12/0.1% BSA, the cells attached and spread out at a somewhat variable rate. At the same time, they were converted into a synthetic phenotype, a process morphologically characterized by a successive loss of myofilaments and formation of an extensive endoplasmic reticulum and a large Golgi complex (cf. [19, 58]). After 48 h of culture, about two thirds of the cells still remained in a contractile phenotype (Fig. 1A), while about one third of them had adopted a synthetic phenotype (Fig. 1B). After 96 h of culture, the structural reorganization of the cells had progressed further and more than two thirds of them were now in a synthetic phenotype. After 144 h of culture, no or only very occasional cells in a contractile phenotype were observed.

Induction of DNA synthesis by peptide mitogens

Freshly isolated aortic SMCs were seeded on a substrate of fibronectin, incubated in medium F-12/0.1% BSA for 96 h, and then studied by thymidine autoradiography. In the first place, the fraction of cells that synthesized DNA after stimulation with peptide mitogens for 24 or 48 h was determined. The mitogens were used at concentrations previously found optimal both in primary and subcultured cells and the assay times were selected in order to detect the direct response to the exogenous stimuli as well as possible indirect responses due to endogenous production of mitogens [60]. Control cells incubated in medium F-12/0.1% BSA showed but a low labelling index (< 5% labelled nuclei), confirming that they were in a quiescent state. Of the mitogens tested, PDGF-BB (10-20 ng/ml) showed the most prominent effect, closely followed by bFGF (2-10 ng/ml). A less marked effect was obtained with EGF (20-50 ng/ml), whereas IGF-I (20 ng/ml), IL-1 α (1-2 ng/ml), and TGF- β 1 (1-2 ng/ml) raised the labelling index only little over the controls (data not shown). Similar results were previously obtained with cells in serum-starved secondary cultures [57, 60]. In the second place, it was examined how long the cells had to be exposed to PDGF-BB, bFGF, and EGF to enter the cell cycle and replicate their DNA. A 30 min exposure to PDGF-BB was found sufficient for maximal effect in primary cultures, whereas 8 h were required with bFGF and EGF (Fig. 2). In secondary cultures, the cells had to be exposed to the mitogens for 8 h to reach a maximal response (Fig. 3). Finally, the effects of the two homodimeric forms of PDGF were compared in primary cultures. The results showed that PDGF-AA was clearly less stimulatory than PDGF-BB and that a longer exposure was required with PDGF-AA than with PDGF-BB for maximal effect (Fig. 4).

Decay of DNA synthetic activity after mitogen stimulation

In this part of the work, freshly isolated SMCs were first incubated on a substrate of fibronectin in medium F-12/0.1% BSA for 96 h. They were then exposed to mitogens for 24 h, rinsed twice, and incubated without exogenous mitogens for another 24 h. After renewed rinsing, they were exposed to tritiated thymidine for 24 h and finally processed for autoradiography. The results indicated that nonstimulated control cells remained quiescent under these conditions (< 5% labelled nuclei). After treatment with and withdrawal of PDGF-BB, a large fraction of the cells continued to replicate their DNA (a similar result was obtained with serum). With bFGF only a weak stimulatory effect on this persisting DNA synthetic activity was seen and with EGF practically no effect at all (Table 1). In secondary cultures, the replicative activity had in most cases returned to control levels on the second day after removal of the exogenous mitogens. Thus, only a low remaining activity was found in cultures treated with PDGF-BB and no activity at all in cultures treated with bFGF or EGF (Table 1). A direct comparison of the effects of the two homodimeric forms of PDGF in primary cultures showed that PDGF-BB supported a persisting DNA synthetic activity at a distinctly higher level than PDGF-AA (Table 2). Furthermore, the effect of PDGF-BB was found to be largely independent of time, with similar results after 1, 4 and 20 h of exposure (Table 3).

Table 1. DNA synthetic activity on the second day following exposure to peptide mitogens in primary and secondary cultures of rat aortic smooth muscle cells.

Mitogen	Primary cultures ¹		Secondary cultures ²	
	% labelled nuclei ³			
Control	1.3	(0.3)	4.9	(0.4)
PDGF-BB (10 ng/ml)	42.9	(1.9)	8.1	(1.5)
PDGF-BB (20 ng/ml)	45.9	(2.1)	10.5	(1.2)
bFGF (2 ng/ml)	5.3	(1.8)	5.1	(1.4)
bFGF (10 ng/ml)	11.2	(2.5)	7.5	(1.3)
EGF (20 ng/ml)	2.9	(0.4)	7.8	(0.3)

¹ Freshly isolated cells were seeded on a substrate of fibronectin, kept in serum-free medium for 4 days, stimulated with the mitogens for 24 h, incubated in mitogen-free medium for 24 h, and then exposed to tritiated thymidine in mitogen-free medium for 24 h. The cells were finally fixed and processed for autoradiography.

² Freshly isolated cells were first grown to confluence in the presence of serum, trypsinized, reseeded on a substrate of fibronectin, kept in serum-free medium for 2 days, and then further treated in the same way as the primary cultures.

³ The results are given as means of triplicate cultures with the standard deviation in parenthesis.

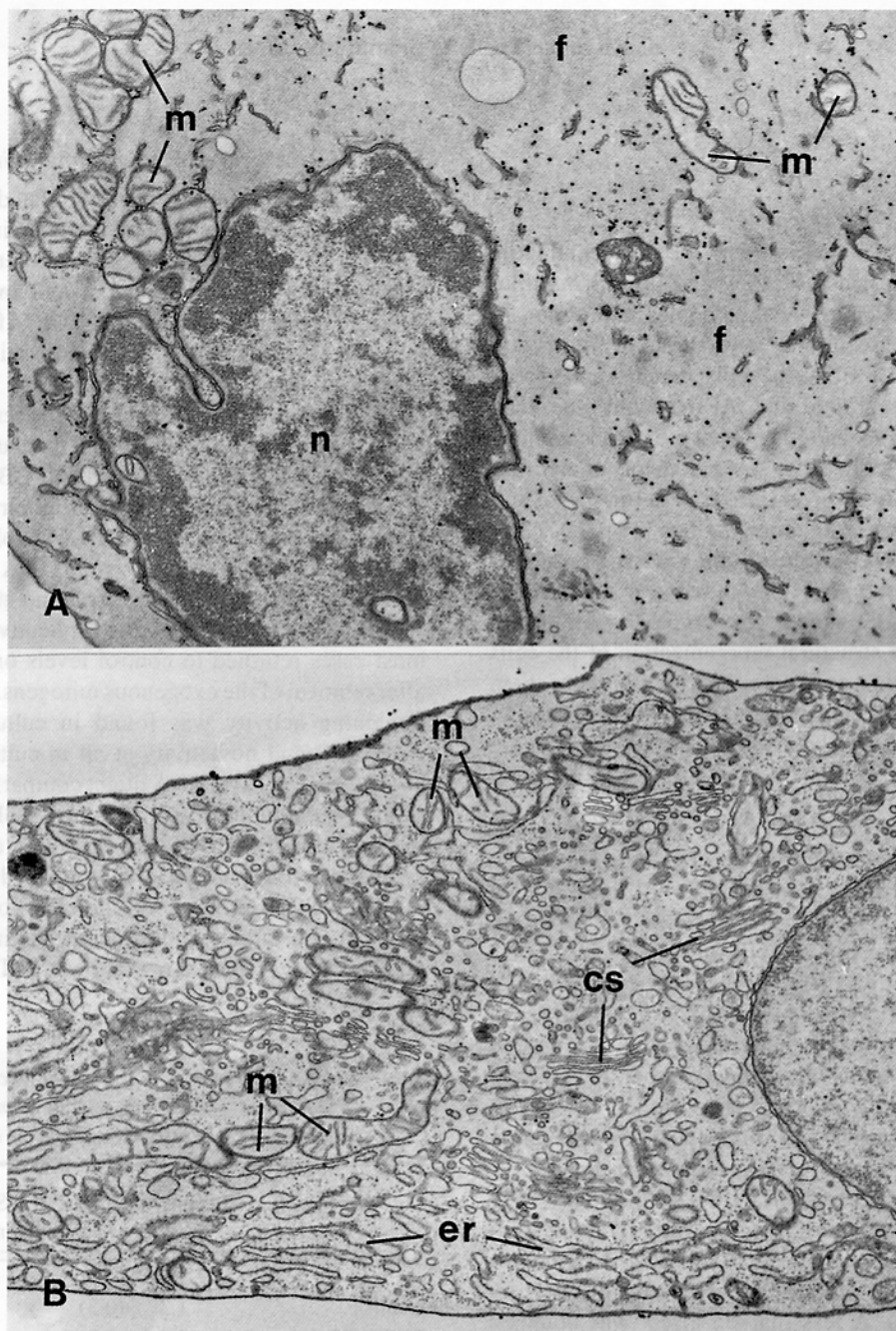


Figure 1. Fine structure of rat aortic SMCs seeded on a substrate of fibronectin in a serum-free medium and fixed for electron microscopic analysis after 48 h of culture. (A) This cell still remains in a contractile phenotype with a heterochromatic nucleus (n) and a cytoplasm largely occupied by myofilament bundles (f). (B) This cell has passed through the transition into a synthetic phenotype and is distinguished by an euchromatic nucleus (n) and a cytoplasm with a widespread endoplasmic reticulum (er) and a large Golgi complex consisting of cisternal stacks (cs) grouped together in the juxtanuclear region. Mitochondria (m) are abundant in both cells. Bars 1 μ m.

Discussion

General conclusions

The findings of this study indicate that PDGF-BB is a most potent mitogen for rat aortic SMCs both in primary and secondary cultures. An almost equipotent effect was obtained with bFGF and a less prominent effect with EGF and PDGF-AA. On the other hand, IGF-I, IL-1 α , and TGF- β 1 raised the thymidine labelling index only little over the controls when used alone in a serum-free medium.

Similar findings were recently reported by Grainger et al. [15]. Noticeably, cells in primary cultures required a much shorter exposure to PDGF-BB than cells in secondary cultures to initiate DNA synthesis. Therefore, it seems as if the SMCs are in a most highly responsive state immediately after the transition from a contractile to a synthetic phenotype and then become less responsive with an increasing number of divisions. Moreover, cells in primary cultures continued to synthesize DNA for a few days after a short exposure to and removal of PDGF-BB, whereas no

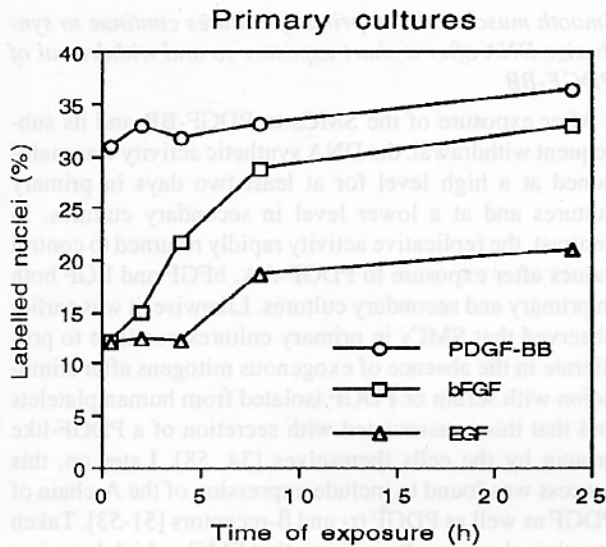


Figure 2. Time dependence of induction of DNA synthesis by PDGF-BB (10 ng/ml), bFGF (5 ng/ml), and EGF (40 ng/ml) in serum-free primary cultures of rat aortic SMCs. Following the exposure to the mitogens, the cultures were rinsed twice and the incubations were continued in medium F-12/0.1% BSA. Tritiated thymidine was added 10 h after the start of the experiment and the cells were fixed for autoradiography 14 h later. The results are given as means of triplicate cultures (standard deviation < 15% of indicated values).

such effect was obtained with bFGF, EGF, and PDGF-AA. These findings suggest that PDGF-BB in addition to stimulating the cells to enter the cell cycle makes them able to go on to replicate in the absence of exogenous stimulation for some time. In the following, these results are discussed in further detail and with special reference to the possible role of PDGF-BB in evoking a state of autocrine or paracrine growth stimulation.

Smooth muscle cells in primary cultures synthesize DNA after a short exposure to PDGF-BB

Freshly isolated rat aortic SMCs seeded on a substrate of fibronectin in a serum-free medium and allowed to pass through the transition from a contractile to a synthetic phenotype showed a stronger DNA synthetic response to PDGF-BB than to PDGF-AA. This is in good agreement with previous observations of a higher binding of PDGF-BB than of PDGF-AA to these cells [53]. Similar findings have been made in serum-starved subcultures of SMCs from several species [16, 22, 43, 46, 53]. Recent studies on rat aortic SMCs further indicate that the different forms of PDGF use separate signalling pathways [27, 47]. Hence, differences both in receptor numbers and mechanisms of action could explain the lower mitogenic potency of PDGF-AA as compared with PDGF-BB.

Another noticeable finding was that PDGF-BB gave a maximum DNA synthetic response already after 30 min of

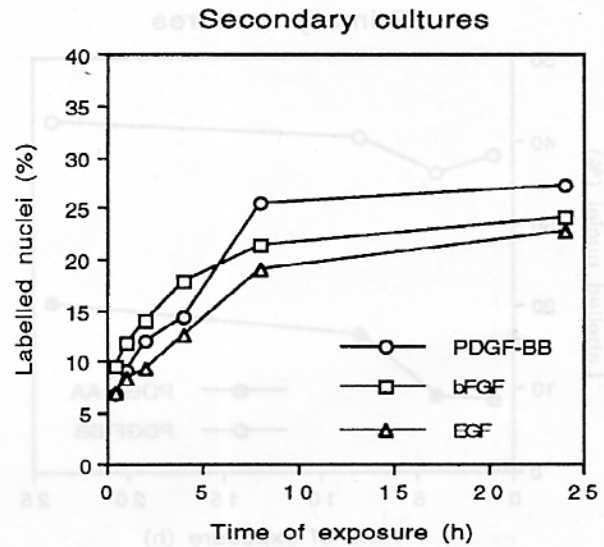


Figure 3. Time dependence of induction of DNA synthesis by PDGF-BB (10 ng/ml), bFGF (5 ng/ml), and EGF (40 ng/ml) in secondary cultures of rat aortic SMCs. Following the exposure to the mitogens, the cultures were rinsed twice and the incubations were continued in medium F-12/0.1% BSA. Tritiated thymidine was added 10 h after the start of the experiment and the cells were fixed for autoradiography 14 h later. The results are given as means of triplicate cultures (standard deviation < 20% of indicated values).

exposure in primary cultures, whereas at least 4-8 h were required with PDGF-AA. In contrast, the cells had to be exposed continually to PDGF-BB for maximal effect in secondary cultures, i.e. throughout the prereplicative phase of 10-15 h [57]. Similar observations have been made in studies on human fibroblasts [63] and rat aortic SMCs [6] stimulated with PDGF isolated from human platelets (principally PDGF-AB). In part, these variations in responsiveness could be due to variations in the number of receptors for PDGF-AA and PDGF-BB between primary and passaged cells [53]. The aforementioned differences in signal transduction between PDGF-AA and PDGF-BB could also be important in this context. Moreover, the responsiveness of the cells may be affected by the number of doublings they have undergone prior to the experiments. Thus, even if the total fraction of cells which were able to replicate did not differ between primary and secondary cultures, the need for a longer exposure to the mitogens in the latter could reflect an early stage in the aging process which restricts the number of divisions the cells pass through before they become senescent and stop to proliferate [8].

Among the other mitogens tested, bFGF had a strong and EGF a weak effect on the replication of the SMCs. On the other hand, IGF-I, IL-1 α , and TGF- β 1 lacked distinct effect both in primary and secondary cultures under the

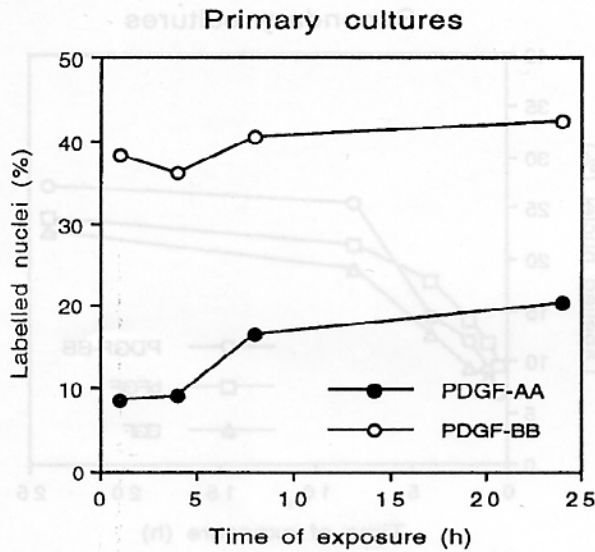


Figure 4. Time dependence of induction of DNA synthesis by PDGF-AA (10 ng/ml) and PDGF-BB (10 ng/ml) in serum-free primary cultures of rat aortic SMCs. Following the exposure to the mitogens, the cultures were rinsed twice and the incubations were continued in medium F-12/0.1% BSA. Tritiated thymidine was added 10 h after the start of the experiment and the cells were fixed for autoradiography 14 h later. The results are given as means of triplicate cultures (standard deviation < 15% of indicated values).

serum-free conditions used here. Even though somewhat varying results can be found in the literature depending on species, cellular origin and experimental protocol, similar potencies of these mitogens have been reported previously (reviewed in [5]). The net effect of bFGF approached that of PDGF-BB, but a considerably longer exposure was required with the former factor in primary cultures (in secondary cultures both had to be present continually). As in the case with PDGF-AA and PDGF-BB, this could be due to differences in receptor numbers as well as signalling mechanisms. The weakness of EGF and IGF-I to induce DNA synthesis under serum-free conditions is in accord with the observation that these agents are poor smooth muscle mitogens on their own, but may interact with PDGF to stimulate cell replication [11]. As further discussed below, IL-1 α [38] and TGF- β 1 [3, 28] have been demonstrated to stimulate production of PDGF-AA by subcultured rat and human SMCs. Nevertheless, both IL-1 α and TGF- β 1 showed only a weak mitogenic effect, perhaps due to low intrinsic activities as well as a weak response of the cells to PDGF-AA (see above). In a similar manner, TGF- β 1 has in several recent studies been found unable to stimulate SMC replication on its own and to either inhibit or enhance the response to other mitogens [4, 23, 24, 48].

Smooth muscle cells in primary cultures continue to synthesize DNA after a short exposure to and withdrawal of PDGF-BB

After exposure of the SMCs to PDGF-BB and its subsequent withdrawal, the DNA synthetic activity was maintained at a high level for at least two days in primary cultures and at a lower level in secondary cultures. In contrast, the replicative activity rapidly returned to control values after exposure to PDGF-AA, bFGF and EGF both in primary and secondary cultures. Likewise, it was earlier observed that SMCs in primary cultures continue to proliferate in the absence of exogenous mitogens after stimulation with serum or PDGF isolated from human platelets and that this is associated with secretion of a PDGF-like protein by the cells themselves [34, 58]. Later on, this process was found to include expression of the A-chain of PDGF as well as PDGF α - and β -receptors [51-53]. Taken together, these results indicate that SMCs which have just passed through the modulation from a contractile to a synthetic phenotype may respond to PDGF by entering a state of autocrine or paracrine growth regulation during which they produce PDGF on their own. After a limited number of divisions this state comes to an end and the cells then need to be exposed to PDGF or other mitogens continually in order to replicate. It remains to be clarified if this is the result of a normal aging process or the loss of a specific function turned on for a short time in connection with the shift in phenotype. In partial support of the former alternative, it has been observed that SMCs from the aorta of young but not adult rats go on to secrete a PDGF-like mitogen during repeated passages in culture [50]. However, the situation is probably more complex than so. Thus, SMCs isolated from the intima of rat arteries two weeks after balloon injury were found to secrete much more of a PDGF-like mitogen than cells isolated from the media of uninjured arteries [62]. This suggests that the ability of the SMCs to secrete PDGF in culture may also depend on whether the preceding change into a synthetic phenotype has taken place in vivo (injured artery) or in vitro (normal media).

Our present and earlier results further point out that the B-chain of PDGF may be of particular importance in bringing about an autocrine or paracrine growth stimulation of the SMCs, implicating the β -receptor in this process. Nevertheless, it should be kept in mind that a multitude of agents have been reported to stimulate PDGF A-chain expression in cultured SMCs, including angiotensin II [33], IL-1 [38], TGF- β [3, 28], FGF [66], TNF- α [66], endothelin [18], thrombin [25], uric acid [41], and mechanical strain [65]. However, it is unclear to what extent endogenous PDGF-AA is responsible for the delayed growth response. As shown here and in previous studies, PDGF-AA is a relatively weak smooth muscle mitogen (depending on receptor number) and antibodies against PDGF have not been found to consistently inhibit the delayed growth response. Moreover, after exposure to various stimuli, the SMCs may produce other mitogens as

Smooth muscle cells in primary cultures

Table 2. DNA synthetic activity on the second day following exposure to PDGF-AA or PDGF-BB in primary cultures of rat aortic smooth muscle cells.

Mitogens ¹	% labelled nuclei ²	
Control	1.7	(0.4)
PDGF-AA (10 ng/ml)	3.9	(0.9)
PDGF-AA (20 ng/ml)	4.7	(2.8)
PDGF-BB (10 ng/ml)	26.0	(3.5)
PDGF-BB (20 ng/ml)	30.9	(3.3)

¹ Freshly isolated cells were seeded on a substrate of fibronectin, kept in serum-free medium for 4 days, stimulated with the mitogens for 24 h, incubated in mitogen-free medium for 24 h, and then exposed to tritiated thymidine in mitogen-free medium for 24 h. The cells were finally fixed and processed for autoradiography.

² The results are given as means of triplicate cultures with the standard deviation in parenthesis.

well, including FGF [14, 67], SDGF [32], endothelin [17, 42], and HB-EGF [12, 56].

The cellular response to growth factors may also be influenced by binding of the latter to the culture substrate. For example, FGF and PDGF have been found to adhere to the surface of plastic dishes and still remain active [54]. In addition, these factors both interact with glycoconjugates in the extracellular matrix. This could give rise to a local depot from which active mitogen is released enzymatically and made available for high affinity receptors on the surface of adjacent cells [61]. In the case of PDGF it has further been noted that the tendency to interact with extracellular matrix components is isoform-dependent, with PDGF-BB showing the strongest binding [26, 36, 37, 40].

Such circumstances may have influenced the reactivity of the cells to the mitogens employed in the present investigation. However, they could not explain why cells in primary cultures required a shorter exposure than cells in secondary cultures to replicate their DNA and why a continued replicative response was only obtained in primary cultures and only after exposure to PDGF-BB.

Possible *in vivo* functions

Summing up, the results of this study indicate that PDGF-BB is a most potent mitogen for cultured rat aortic SMCs and that the cells are as most responsive directly after the conversion from a contractile to a synthetic phenotype. At that time, they enter the cell cycle following a short exposure to the mitogen (30-60 min) and then go on to replicate in the absence of exogenous stimulation for a few days, apparently due to activation of an autocrine or paracrine loop. Later on, longer exposures are required to induce a new replicative cycle and the cells rapidly return to a quiescent state in the washout period. Several observations suggest that this is not merely an *in vitro* phenome-

Table 3. DNA synthetic activity on the second day following exposure to PDGF-BB in primary cultures of rat aortic smooth muscle cells.

Mitogens ¹	Time of exposure	% labelled nuclei ²
Control	-	4.8 (0.8)
PDGF-BB (20 ng/ml)	1 h	18.5 (2.7)
	4 h	24.6 (4.5)
	20 h	21.5 (3.9)

¹ Freshly isolated cells were seeded on a substrate of fibronectin, kept in serum-free medium for 4 days, stimulated with PDGF-BB for the indicated times during day 5, incubated in mitogen-free medium for another 24 h, and exposed to tritiated thymidine in mitogen-free medium for 24 h. The cells were finally fixed and processed for autoradiography.

² The results are given as means of triplicate cultures with the standard deviation in parenthesis.

non and that a similar course of events may occur *in vivo* and be involved in atherogenesis as well as restenosis after angioplasty. In human atherosclerotic plaques, SMCs have been noted to express the A-chain of PDGF, whereas macrophages and endothelial cells express the B-chain of PDGF [2, 45, 64]. In a similar manner, SMCs in the rat carotid artery were observed to express both the A-chain of PDGF and the PDGF β -receptor at raised levels after balloon catheter injury, with particularly high levels in the growing neointima [29]. Further, the formation of such lesions was found to be inhibited by antibodies against PDGF, supposedly reflecting its action both as a chemoattractant and a mitogen for SMCs [13].

Together with the cell culture studies discussed above, these reports point to a dual role of PDGF in diseases with an abnormal proliferation of vascular SMCs, briefly summarized in the following four-stage model. (1) As a result of endothelial injury and penetration of blood components into the underlying arterial media, SMCs are induced to convert from a contractile to a synthetic phenotype. Basically, this represents a return of the cells to an earlier differentiated state, characteristic of the fetus and the young growing organism. (2) PDGF-AB or -BB (molecules containing at least one B-chain) released from platelets adhering to exposed subendothelial tissue, damaged endothelial cells, or macrophages stimulate synthetic state SMCs to migrate into the intima and start to proliferate. (3) Once cycling, the SMCs begin to produce PDGF-AA and other mitogens on their own. This leads to activation of an autocrine or paracrine loop and allows the cells to go on replicating even if the exogenous stimulation ceases. (4) After a restricted number of divisions the endogenous mitogen production comes to an end and the SMCs again become quiescent.

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