Platelet-Derived Growth Factor (PDGF) Accelerates Induction of Competence, and Heparin Does Not Inhibit PDGF-Induced Competence in Primary Cultured Smooth Muscle Cells of Rat Aorta

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ABSTRACT—The time-dependent effect of platelet-derived growth factor (PDGF) on cell proliferation was investigated to clarify whether PDGF accelerates the rate of proliferation or its start in primary cultured smooth muscle cells (SMC) of rat aorta. In synchronized SMC at the Go phase, 1, 10 and 100 ng/ml PDGF started DNA synthesis at 24, 15–18 and 12 hr, respectively, after stimulation by 3% fetal bovine serum (FBS) or hypophysectomized rat plasma (deficient in insulin-like growth factor-I (IGF-I)). Heparin (1, 10 or 100 μg/ml) decreased only the rate of DNA synthesis stimulated by PDGF in synchronized SMC. DNA synthesis in non-synchronized cells stimulated by PDGF with FBS was determined up to 10 days in culture. The stimulation with 1% FBS plus 30 ng/ml PDGF potentiated the DNA synthesis which was saturated with stimulation by 10% FBS alone, suggesting that prolonged treatment of PDGF transforms SMC. These results demonstrated that PDGF concentration-dependently accelerated the induction of competence independently of IGF-I, and heparin did not inhibit PDGF-induced competence but inhibited progression in primary cultured SMC of rat aorta.

Keywords: Platelet-derived growth factor, Heparin, Vascular smooth muscle, Cell proliferation

Platelet-derived growth factor (PDGF) is produced in platelets (1), macrophages (2), endothelial cells (3) and vascular smooth muscle cells (SMC) (4, 5). PDGF stimulates proliferation of vascular SMC (6) and is closely related to the promotion of atherosclerosis (7). The role of PDGF is important for the proliferation of cells because PDGF with other progression factors synergistically stimulates proliferation of vascular SMC (8, 9). Although PDGF induces competence to replicate DNA synthesis in quiescent Balb/c 3T3 cells (10), the PDGF effect has not yet been established in vascular SMC. Competence is a state at which the cells can respond to progression factors, such as insulin-like growth factor-I (IGF-I) or platelet-poor plasma (10). Platelet-poor plasma accelerates only the rate of DNA synthesis in Balb/c 3T3 cells (11). Whether PDGF accelerates the rate of proliferation or its initiation remains unknown in vascular SMC.

Heparin inhibits the proliferation of SMC in bovine aorta (12). There are conflicting reports about the pattern of the inhibitory action of heparin because heparin may interfere with PDGF (a competence factor) binding (13) or it may inhibit serum-induced progression but not competence (14). Therefore, we tried to elucidate the inhibitory effect of heparin on PDGF-induced competence.

Primary cultured cells are useful for studying the pattern of SMC proliferation because these cells show contractile activity and change from the contractile state to the synthetic state in culture (15). Repeatedly subcultured cells can not return to the contractile state (16). In the present paper, PDGF-induced competence was determined by monitoring the time for the start of the DNA synthesis stimulated by PDGF and the effect of prolonged PDGF treatment on proliferation. Also the pattern of heparin inhibition of PDGF-induced competence was investigated in primary cultured SMC of rat aorta.

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MATERIALS AND METHODS

Cell culture

Rat aortic SMC in primary culture were prepared by the method of Chamley et al. (17), with the following modifications. The thoracic aortas of Wistar rats (9 weeks old, weighing 245–317 g) were placed in Hank's solution (pH 7.3, 136.8 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 0.4 mM KH2PO4, 1.3 mM CaCl2, 0.3 mM Na2HPO4, 5.6 mM glucose and 4.2 mM NaHCO3), and blood, fat and connective tissue were removed. They were then placed in Hank's solution containing 1 mg/ml collagenase type I (Sigma, St. Louis, MO, U.S.A.) and 0.1–0.8 U/ml elastase type I (Sigma) and incubated for 30 min at 37°C. The adventitia was cleanly stripped off from each aorta, and the remaining medial tissue was placed in Hank's solution containing 6 mg/ml collagenase type I (Sigma, St. Louis, MO, U.S.A.) and 0.25–2.5 U/ml elastase type I and incubated for 1.5 hr at 37°C with gentle shaking in order to obtain single cells and small cell clumps. The suspension of SMC was centrifuged at 150 X g for 10 min, resuspended at the density of 3 X 10^4 cells/0.5 ml/16 mm-well in Dulbecco's modified Eagle medium (DMEM, Nissui, Tokyo, Japan) supplemented with 160 U/ml penicillin G potassium (Banyu Seiyaku, Tokyo, Japan), 100 μg/ml streptomycin sulfate (Meiji Seika, Tokyo, Japan), 4.2 mM NaHCO3 (complete DMEM) and 5% fetal bovine serum (FBS, Whittaker Bioproducts, Walkersville, MD, U.S.A.), and injected into a 24-well plate (16-mm well, Corning, New York, NY, U.S.A.). The cells were cultured in a humidified atmosphere of 5% CO2 in air at 37°C. The cultured cells were identified as SMC because “hills and valleys” (15) were observed at confluence under an optical microscope.

Thymidine incorporation into synchronized SMC

The experimental procedures used for 3H-thymidine incorporation were based on the method of Stiles et al. (10), modified as follows: the SMC were cultured with 5% FBS-complete DMEM for 6 days to obtain nearly confluent growth and then synchronized by removing FBS (serum starvation) for 2 days. After this time, the cultures were arrested at the G0 phase (9). The synchronized cells were stimulated by 1, 10 or 100 ng/ml recombinant PDGF (rPDGF, c-sis, Amersham Japan, Tokyo) or 1, 10 or 100 μg/ml heparin (Sigma) in complete DMEM for 3 hr. After washing rPDGF with or without heparin, 3% FBS or hypophysectomized rat plasma (Imamichi Institute for Animal Reproduction, Ibaraki, Japan) was added into complete DMEM with 0.037 MBq [methyl-3H]-thymidine (925 GBq/mmol, Amersham Japan). The cells were incubated at 37°C for the indicated periods (continuous labeling). Tritium-thymidine incorporation in a well was stopped every 3 hr by removing the incubation medium and adding 0.5 ml of 5% trichloroacetic acid. After incubation for 30 min at 4°C, 3H-labeled cells were washed three times with 5% trichloroacetic acid at 4°C, and lysed in 0.5 ml of 1 N NaOH. The cell lysate was neutralized, and the radioactivity of the sample was counted with a scintillation spectrometer (LS 3801, Beckman, Clifton, U.S.A.).

Thymidine incorporation into non-synchronized SMC

In the individual wells of a 24-well plate, the SMC isolated from the aorta were resuspended for various periods at the density of 3 X 10^4 cells/0.5 ml/well in complete DMEM and 1%, 2%, 5% or 10% FBS. Then, 3, 10 or 30 ng/ml rPDGF was injected into the plates. After these procedures, the culture medium was removed every 24 hr from 3 wells, and the cells were washed with complete DMEM. Complete DMEM (0.5 ml) with 0.037 MBq 3H-thymidine was added to each well, incubation was carried out for 2 hr at 37°C (pulse labeling), and then 3H-thymidine incorporation was stopped. Subsequent procedures were the same as those described above for the synchronized SMC. Total incorporation of 3H-thymidine on a given day is determined as follows: 24 (hr) X 1/2 X [(dpm of the radioactivity per hr on the previous day) + (dpm of the radioactivity per hr on a given day)]. Total 3H-thymidine incorporation during 10 days was determined as the cumulative sum of incorporation on each day. Data are the means of triplicate assays.

RESULTS

rPDGF concentration-dependently accelerated the start of proliferation in primary cultured SMC of rat aorta

With pretreatment of 1, 10 or 100 ng/ml rPDGF for only 3 hr, 3H-thymidine incorporation into synchronized SMC started 24, 15–18 or 12 hr after 3% FBS stimulation, respectively (Fig. 1). The effect of rPDGF on the proliferation of SMC was observed after rPDGF was removed. Without rPDGF, the cells started to incorporate 3H-thymidine 24 and 18 hr after 5% and 10% FBS stimulation, respectively (data not shown). Differences in 3H-thymidine incorporation (delta values) were measured as the total incorporation obtained with rPDGF stimulation minus the incorporation obtained with 3% FBS stimulation, respectively (data not shown).
Hypophysectomized rat plasma is deficient in IGF-I (10). The effect of rPDGF combined with hypophysectomized rat plasma was investigated to exclude the effect of IGF-I, the major progression factor in serum. Pretreatment of 1, 10 and 100 ng/ml rPDGF also resulted in $^3$H-thymidine incorporation into synchronized SMC 24, 15 and 12 hr after stimulation by 3% hypophysectomized rat plasma, respectively (Fig. 2). The difference in the value of $^3$H-thymidine incorporation represented the total incorporation following rPDGF stimulation minus the incorporation stimulated with 3% hypophysectomized rat plasma. The net incorporation for 24 hr with 3% hypophysectomized rat plasma alone was $1.56 \times 10^5$ dpm/well. The results in Fig. 2 are consistent with those of Fig. 1, indicating that rPDGF-induced acceleration is independent of IGF-I.

Prolonged incubation with rPDGF and FBS abnormally stimulated the proliferation in primary cultured SMC of rat aorta.

The prolonged effect of rPDGF with FBS was investigated in non-synchronized cells. Tritium-thymidine incorporation stimulated by 1% (Fig. 3), 2%, 5% and 10% FBS (data not shown) was time-dependent (up to 10 days). The cell number stimulated by 1% and 10% FBS without rPDGF at day 7 was $3.82 \pm 0.12$ and $18.0 \pm 1.3 \times 10^4$ cells/well, respectively. Thymidine incorporation stimulated by 1% FBS was potentiated by rPDGF in a concentration-dependent manner (3, 10 and 30 ng/ml) (Fig. 3). Ten percent FBS-stimulated incorporation was saturated due to confluence. rPDGF stimulation also was saturated within 10 days of culture. rPDGF (30 ng/ml)-stimulated thymidine incorporation was not different on day 4 of culture, but was 2-fold higher on day 10 compared with 10% FBS-stimulated incorporation. rPDGF stimulated the proliferation of SMC over confluence, suggesting that the rPDGF may transform SMC in a longer culture time than 4 days.

Furthermore, FBS produced concentration-dependent potentiation of rPDGF-stimulated $^3$H-thymidine incorporation for 10 days of culture (Fig. 4). In the presence of 10% FBS, rPDGF (30 ng/ml)-stimulation potentiated incorporation by 2.9-fold compared with the incorporation which was saturated with 10% FBS stimulation. By an optical microscope, we observed abnormal proliferation in multilayers of SMC (Fig. 5). Prolonged rPDGF treatment caused the cell shapes to change from the spindle type to the elliptic type. In the presence of 10% FBS, even 3 ng/ml rPDGF also showed the same tendency (data not shown).

![Fig. 1](image1.png)  
**Fig. 1.** Recombinant platelet-derived growth factor (rPDGF) (1, 10 or 100 ng/ml) with 3% fetal bovine serum (FBS) accelerated the starting time of $^3$H-thymidine incorporation into the acid-insoluble fraction of primary cultured smooth muscle cells (SMC) of rat aorta. The cells were taken at the indicated times after serum starvation, as described under Materials and Methods. rPDGF was added to cultures at the Go phase, 3 hr before FBS-stimulation. The difference (delta) in $^3$H-thymidine incorporation represents the total incorporation following rPDGF-stimulation minus the incorporation following 3% FBS stimulation. The values are means ± S.E.M of triplicate assays.

![Fig. 2](image2.png)  
**Fig. 2.** rPDGF (1, 10 or 100 ng/ml) with hypophysectomized rat plasma accelerated the starting time of $^3$H-thymidine incorporation into the acid-insoluble fraction of primary cultured SMC of rat aorta. The experimental procedures were the same as those described in the legend to Fig. 1 except 3% hypophysectomized rat plasma was used instead of FBS. The difference (delta) is the same as in Fig. 1 except 3% hypophysectomized rat plasma was used instead of 3% FBS. The values are data of a single experiment.
Fig. 3. Time course of $^3$H-thymidine incorporation stimulated by rPDGF (3, 10 or 30 ng/ml) with 1% FBS (closed circle) or 10% FBS (open circle) into the acid-insoluble fraction of primary cultured SMC of rat aorta. In these experiments, the cells were not synchronized. rPDGF with FBS was freshly added to the culture medium every other day. The experimental procedures are described in detail in Materials and Methods. The values are the means of triplicate assays.

Fig. 4. Concentration-dependency of rPDGF-stimulated $^3$H-thymidine incorporation with FBS (1%, 2%, 5% or 10%) for 10 days into the acid-insoluble fraction of the primary cultured SMC of rat aorta. The experimental procedures were the same as those described in the legend to Fig. 3.

Fig. 5. Photographs of PDGF-induced abnormal proliferation (day 7) in primary cultured SMC of rat aorta. The bar represents 10 μm.

10% FBS

+PDGF (30 ng/ml)

Day 7
Heparin did not delay rPDGF-stimulated acceleration in the start of proliferation in primary cultured SMC of rat aorta

We tried to elucidate the effect of heparin on rPDGF-induced competence in synchronized SMC. Pretreatment with 30 ng/ml rPDGF started \(^{3}\)H-thymidine incorporation into synchronized SMC 15 hr after 1% FBS stimulation with or without heparin (Fig. 6). The difference in \(^{3}\)H-thymidine incorporation was the total incorporation with rPDGF and heparin stimulation minus the incorporation with 1% FBS-stimulation. The net incorporation for 24 hr with 1% FBS alone was 1.39 ± 0.15 \(\times 10^5\) dpm/well. Heparin (1, 10 or 100 \(\mu\)g/ml) tended to produce a concentration-dependent decrease only in the rate of \(^{3}\)H-thymidine incorporation. These results demonstrated that heparin did not delay the starting time of DNA synthesis, suggesting that heparin does not inhibit PDGF-induced competence, but did inhibit progression in primary cultured SMC of rat aorta.

DISCUSSION

Cell growth proceeds from the competence phase to the progression phase (11). In the present study, PDGF shortens the lag time before the start of DNA synthesis, indicating that PDGF accelerates induction of competence in primary cultured SMC of rat aorta. We further confirmed that this PDGF-induced competence did not require IGF-I, a progression factor contained in FBS. PDGF induces expression of c-myc and c-fos, which are linked with the G0 to G1 transition (18, 19) through phospholipase C activation (20, 21). However, the relationship of the establishment of competence with the expression of these proto-oncogenes remains unknown.

Synchronized SMC initiated DNA synthesis 12 hr after 10% FBS stimulation, suggesting that the cells stayed in the Go phase like the serum-starved 3Y1 fibroblasts of rats (22) or quiescent Balb/c 3T3 cells (10). Following pretreatment with PDGF, hypophysectomized rat plasma does not stimulate DNA synthesis in Balb/c 3T3 cells (10) but does stimulate the synthesis in SMC of rat aorta. These results indicate that proliferation of primary cultured SMC of rat aorta may be regulated differently from the proliferation of Balb/c 3T3 cells. PDGF stimulated abnormally proliferation over confluence in SMC. This suggests that prolonged exposure to PDGF is involved in poor contact inhibition and deficiency in the normal regulation of cell proliferation.

Heparin blocks the progression phase at the mid-G1 phase because it inhibits the expression of c-myb and 2F1, which both increase in the G1 phase (14). However, the relationship between progression and expression of these proto-oncogenes remains unknown. When competence is inhibited, the start of DNA synthesis is delayed because interferon α/β inhibits the establishment of competence in the G0/S phase transition in Balb/c 3T3 cells, resulting in delayed DNA synthesis (23). Heparin may interfere with PDGF-receptor binding in human arterial smooth muscle (13). However, the present results with rPDGF showed that heparin did not inhibit PDGF-induced competence, but inhibits FBS-induced progression. The progression factor IGF-I stimulates Ca\(^{2+}\) influx (24) and induces protein modifications (25) in Balb/c 3T3 cells. Heparin may inhibit IGF-I-induced mechanisms.

In conclusion, we found that PDGF accelerated induction of competence in a concentration-dependent manner, but heparin did not inhibit PDGF-induced competence in primary cultured SMC of rat aorta. This study provided the fundamental criteria to distinguish competence-inhibitors or stimulators from those affecting progression of vascular SMC proliferation.

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