

The chemotactic and mitogenic effects of platelet-derived growth factor-BB on rat aorta smooth muscle cells are inhibited by basic fibroblast growth factor

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SUMMARY

In response to endovascular injury, platelet-derived growth factor-BB (PDGF-BB) and basic fibroblast growth factor (bFGF) are released locally and modulate vascular smooth muscle cells (SMC) proliferation and migration within the vascular wall. The aim of the present *in vitro* study was to determine how rat aorta SMC respond to the simultaneous exposure to PDGF-BB and bFGF. In a modified Boyden chamber assay bFGF exhibited a dose-dependent effect to inhibit the chemotactic action of PDGF-BB. A comparable result was observed in proliferation assays. In contrast, MIP-1 β , epidermal growth factor (EGF), fibronectin and acidic FGF (aFGF) did not inhibit the chemotactic effect of PDGF-BB. Denatured bFGF did not exert an inhibitory effect and neutralizing antibodies either to bFGF or to bFGF-receptor abolished the inhibition observed in the

presence of bFGF. The role played by PDGF receptor α (PDGF-R α) was investigated in PDGF-R α -dominant negative-transfected SMC, by selectively blocking PDGF-BB-binding to PDGF-R α with neomycin, by neutralizing PDGF-R α with a monoclonal antibody and by selectively stimulating PDGF-R α with PDGF-AA; in all cases the effect of bFGF to inhibit PDGF-BB-directed SMC migration was abolished.

These *in vitro* studies show that bFGF significantly inhibits PDGF-BB-induced SMC migration and proliferation and that this effect is mediated by both PDGF-R α and bFGF receptor.

Key words: Platelet derived growth factor, Basic fibroblast growth factor, Smooth muscle cell, Migration, Proliferation

INTRODUCTION

A hallmark of many vascular diseases is the modified distribution of vascular smooth muscle cells (SMC) within the vessel wall, due to their altered proliferation and/or migration (Liu et al., 1989; Ross, 1986). Many growth factors modulate SMC function (Crowley et al., 1995) and several observations indicate that platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) play a key role in atherosclerosis and neointima accumulation at vascular injury sites (Ross et al., 1990; Akyurek et al., 1996; Uchida et al., 1996; Faries et al., 1996; Law et al., 1996). PDGF is produced by platelets, monocytes, endothelial cells, vascular SMC and has mitogenic and chemotactic activity on SMC (Bobik and Campbell, 1993). Three PDGF isoforms have been identified as disulfide-linked dimers, namely PDGF-AA, PDGF-BB and PDGF-AB. They interact with different affinity with two tyrosine-kinase receptors, PDGF-R α and PDGF-R β , both expressed on SMC (Majesky et al., 1990), leading to receptor dimerization and auto-phosphorylation (Claesson-Welsh, 1994). Upon ligand binding, receptors dimerize forming homodimers $\alpha\alpha$ or $\beta\beta$, or heterodimers $\alpha\beta$; PDGF-BB binds

$\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ dimers; PDGF-AA selectively binds $\alpha\alpha$ dimers and PDGF-AB binds $\alpha\alpha$ and $\alpha\beta$ (Claesson-Welsh, 1994).

SMC migration toward PDGF is modulated by its cooperation with other growth factors (Starksen et al., 1987; Gong and Pitas, 1995; Janat and Liau, 1992; Ishigami et al., 1998), as well as by the availability of PDGF-induced phosphorylated substrates (Rankin and Rozengurt, 1994) and by the selective activation of PDGF-R α and PDGF-R β receptors. In fact, PDGF can either promote or inhibit chemotaxis and cell growth (Heldin, 1997; Heldin et al., 1998; Clunn, 1997; Claesson-Welsh, 1994); more often PDGF-R β receptors mediate positive chemotactic and mitogenic signals while PDGF-R α may mediate either positive or negative signals, depending on the cell type (Siegbahn et al., 1990; Koyama et al., 1992, 1994; Yokote et al., 1996; Vassbotn et al., 1992).

Basic FGF is a mitogen and a chemotactic agent for SMC (Lauder et al., 1997; Kenagy et al., 1997), and has been shown to modulate PDGF-BB-induced migration. In fact, neutralizing endogenous bFGF reduces PDGF-BB-induced migration of SMC and CamKinase II phosphorylation (Sato et al., 1991; Bilato et al., 1995). Further, an enhanced response to PDGF-

BB has been reported in cells pretreated with bFGF for 12 to 48 hours and this effect was related to bFGF-induced up-regulation of PDGF-R α and integrins expression (Bonner et al., 1996; Schollmann et al., 1992; Pickering et al., 1997).

Since PDGF and bFGF may be released simultaneously in vitro and in vivo to modulate normal and pathological SMC proliferation and migration (Crowley et al., 1995; Sterpetti et al., 1994; Benzaquen et al., 1994; Kuwabara et al., 1995; Malek et al., 1993; Drubaix, 1998; Reidy et al., 1992), the present study examined rat aorta SMC chemotactic and mitogenic response to the simultaneous presence of PDGF-BB and bFGF (PDGF-BB/bFGF). Our results show that PDGF-BB/bFGF exerts a significantly reduced chemotactic and mitogenic activity on rat aorta SMC, as compared to PDGF-BB alone and the reduced response is mediated by PDGF-R α and bFGF receptor.

MATERIALS AND METHODS

Cell culture

Primary rat aorta SMC were obtained from six-month old male Wistar rats as previously described (Sterpetti et al., 1992), and were cultured at 37°C in a 5% CO₂ atmosphere, in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-I (Gibco), 2 mmol/l L-glutamine and 100 i.u./ml penicillin/streptomycin (Gibco), 10% heat-inactivated fetal bovine serum (FBS) (Gibco). SMC preparation purity was evaluated with LSAB-2 kit (Dako Corporation) and with DiI-Ac-LDL (Biomedical Technologies Inc.) and was consistently >97%. Cells from passage 3 to 7 were used in all experiments. Proliferating cells at 70% confluence were plated, allowed to adhere and cultured for 24 hours, and then used in migration and proliferation assays.

Migration assay

Migration assays were carried out in modified Boyden chambers (Costar Scientific Corporation) as described (Albini et al., 1995). Rat aorta SMC (2×10^5) were placed in the upper chamber of the Boyden apparatus mounted with 8 μ m pore polycarbonate filters (Costar Scientific Corporation) coated with 5 mg/l solution of porcine skin gelatin (Sigma). Human recombinant factors used as chemoattractants were: PDGF-BB and PDGF-AA (R&D Systems), acidic FGF (aFGF) and bFGF (Gibco), MIP-1 β (Gibco), epidermal growth factor (EGF) (Upstate Biotechnology) and fibronectin (Gibco). They were dissolved at the reported concentration in DMEM-0.1% BSA and placed in the lower chamber of the Boyden apparatus. The mixture containing PDGF-BB and bFGF (10 ng/ml each) and the mixture containing PDGF-BB and aFGF (10 ng/ml each) are here referred to as PDGF-BB/bFGF or PDGF-BB/aFGF, respectively.

Polyclonal anti-human PDGF-BB and anti-human bFGF neutralizing antibodies (R&D Systems) were placed in the lower chamber of the Boyden apparatus, at the final concentration of 1 μ g/ml and 20 μ g/ml, respectively, according to the manufacturer's instructions.

Neutralizing monoclonal antibodies anti-bFGF receptor (*flt* gene product) MAB125 (Chemicon International Inc.) and anti-PDGF-R α APA-5 (Pharmingen) were placed in the upper portion of the Boyden chambers, at 3 μ g/ml and 5 μ g/ml, respectively.

In some experiments, growth factors were mixed with heparin (Sigma) at 0.05 U/ml concentration.

Neomycin (Sigma) acts as specific inhibitor of PDGF-BB binding to PDGF-R α (Vassbotn et al., 1992; Fatatis and Miller, 1997). In our study it was used at 5 mM final concentration, by pre-treating SMC for 15 minutes, before starting the chemotaxis assay (Vassbotn et al., 1992).

Migration assays were all carried out at 37°C in 5% CO₂, for 5

hours, then filters were removed, fixed with absolute ethanol and stained with toluidine blue. Cells migrated were counted at $\times 400$ magnification in 15 fields for each filter and the average number of cells/field was reported. All experiments were performed at least 3 times in duplicate.

Proliferation assay

Rat aorta SMC plated in 6-well plates (1×10^5 cells/plate) were grown for 24 hours in DMEM supplemented with 10% FBS, at 37°C in 5% CO₂. Medium was then replaced with DMEM-0.1% BSA for 24 hours. Subsequently the medium was replaced with fresh medium containing either 0.1% BSA alone or 0.1% BSA with growth factors. Basic FGF heat-inactivation was performed at 100°C for 15 minutes, followed by fast refrigeration. Neutralizing antibody anti-bFGF (R&D Systems; 20 μ g/ml) and anti-bFGF receptor MAB125 (Chemicon International Inc.; 1 μ g/ml) and the control Ig-G (anti-goat from Santa Cruz; 1 μ g/ml) were also used. After treatment, cells were harvested and counted with hemacytometer. All experiments were carried out at least three times in duplicate.

Cell transfection with dominant negative PDGF-receptors constructs

SMC were transfected as previously reported (Palumbo, 2000) with either a dominant negative PDGF-R α vector (DN-PDGF-R α) or equal amount of dominant negative PDGF-R β vector (DN-PDGF-R β ; generous gifts from Dr C. H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden; Emaduddin et al., 1999), or pCDNA3 (Invitrogen) empty vector. Cells were co-transfected with pEGFP-N1 (Clontech) reporter vector (molar ratio 3:1). SMC (2×10^6 cells/100 mm diameter dish) were transfected with lipofectamine plus reagent (Gibco) for 5 hours at 37°C, 5% CO₂. Thereafter, medium was replaced with DMEM-10% FBS and after 48 hours chemotaxis assay was performed as reported above. Co-transfection with two vectors, at the reported ratio, results in the internalization of both plasmids by the same cell (Wigler et al., 1979). Therefore, migrated cells were counted under a UV light microscopy to evaluate only GFP positive cells, in order to overcome the potential limitations of low transfection efficiency. These experiments were carried out four times in duplicate.

Statistics

Data are expressed as the mean \pm s.e.m.. Student's two-tails paired *t*-test was performed and $P \leq 0.05$ was considered statistically significant.

RESULTS

Effect of bFGF on PDGF-BB-induced SMC migration

SMC migration in response to PDGF-BB, bFGF and PDGF-BB/bFGF was investigated in concentration-dependence experiments, with increasing concentrations of one factor and a fixed concentration of the other (Fig. 1). PDGF-BB alone showed a concentration-dependent bell-shaped effect on SMC migration (Fig. 1A). When a fixed concentration of bFGF (10 ng/ml) was added, the chemotactic activity of PDGF-BB was reduced by ~50% at PDGF-BB concentrations of 5 to 20 ng/ml. In additional experiments bFGF alone (0 to 20 ng/ml) exerted a weak chemotactic activity on SMC, and significantly inhibited the chemotactic effect of 10 ng/ml PDGF-BB (Fig. 1B). When MIP-1 β , a chemokine produced by SMC, or EGF were mixed with increasing concentration of PDGF-BB there was no modulation of PDGF-BB-induced SMC migration (Fig. 2A and B). Further, bFGF did not inhibit fibronectin-induced

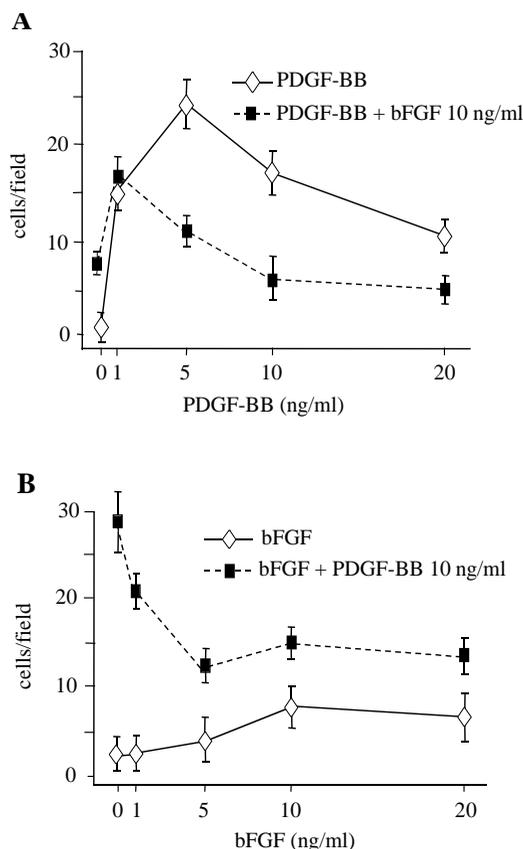


Fig. 1. Effect of PDGF-BB, bFGF and PDGF-BB/bFGF on SMC migration. (A) Cells were exposed to 0 to 20 ng/ml PDGF-BB alone or mixed with 10 ng/ml bFGF. PDGF-BB alone exerted a bell-shaped effect, which was inhibited up to ~50% by bFGF. Each point represents the average \pm s.e.m. of 5 experiments. (B) Cells were exposed to 0 to 20 ng/ml bFGF alone or mixed with 10 ng/ml PDGF-BB. Basic FGF alone showed a weak chemotactic effect, while at 5-20 ng/ml it reduced PDGF-BB effect by ~50%. Each point represents the average \pm s.e.m. of 3 experiments.

SMC migration (Fig. 2C). These data indicated that bFGF inhibitory effect was specifically directed toward PDGF-BB.

Specific neutralization of bFGF achieved by adding neutralizing anti-bFGF antibody (20 μ g/ml) to PDGF-BB/bFGF, restored the chemotactic action of PDGF-BB (Fig. 3), indicating that bFGF is unable to interfere with PDGF-BB chemotactic activity if it is functionally inactivated.

PDGF-BB and bFGF are both heparin-binding factors, therefore it was investigated whether heparin interferes with the observed inhibition. Under our experimental conditions, heparin (0.05 U/ml) did not affect the inhibitory activity of bFGF on PDGF-BB-induced migration (Fig. 4A).

Finally, increasing concentrations of aFGF (0 to 50 ng/ml) did not show any inhibition of PDGF-BB-induced migration (Fig. 4B). Rather, an increased migration in the presence of PDGF-BB/aFGF mixture was found, indicating that the inhibitory effect of bFGF (reported in Fig. 1) was a specific action of this FGF family member.

Effect of bFGF on PDGF-induced SMC proliferation

The effect of PDGF-BB on SMC proliferation was examined both in the absence and in the presence of bFGF. In time-course

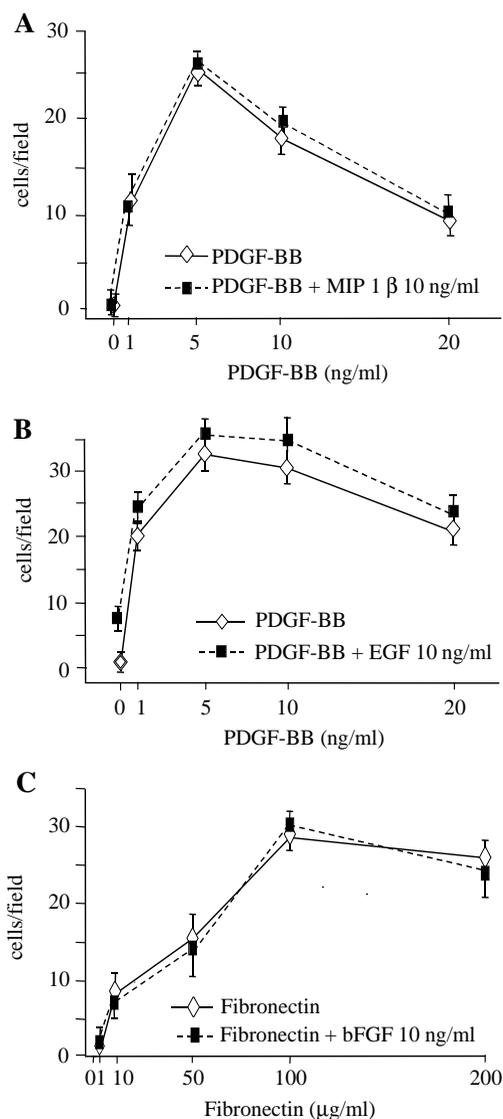


Fig. 2. Effect of different factors on PDGF-BB- and bFGF-induced SMC migration. (A) Cells were exposed to 0 to 20 ng/ml PDGF-BB alone or mixed with 10 ng/ml MIP-1 β . PDGF-BB activity was not affected by MIP-1 β at any concentration. Each point represents the average \pm s.e.m. of 3 experiments. (B) Cells were exposed to 0 to 20 ng/ml PDGF-BB alone or mixed with EGF 10 ng/ml. EGF did not modulate PDGF-BB activity at any concentration. Each point represents the average \pm s.e.m. of 3 experiments. (C) Cells were exposed to 0 to 200 μ g/ml fibronectin alone or mixed with bFGF 10 ng/ml. Basic FGF did not exhibit any significant effect on the chemotactic activity of fibronectin. Each point represents the average \pm s.e.m. of 3 experiments.

experiments the two growth factors were combined at the fixed concentration of 10 ng/ml each, and proliferation was significantly lower as compared to PDGF-BB alone, at all time-points (Fig. 5A). Additional proliferation experiments were carried out with increasing concentration of bFGF (0 to 50 ng/ml) combined with a fixed concentration of PDGF-BB (10 ng/ml). Basic FGF exerted a dose-dependent inhibitory effect, reaching the plateau at 10 ng/ml (Fig. 5B). In other proliferation assays heat-denatured bFGF mixed with PDGF-BB did not modulate the effect of PDGF-BB to increase SMC

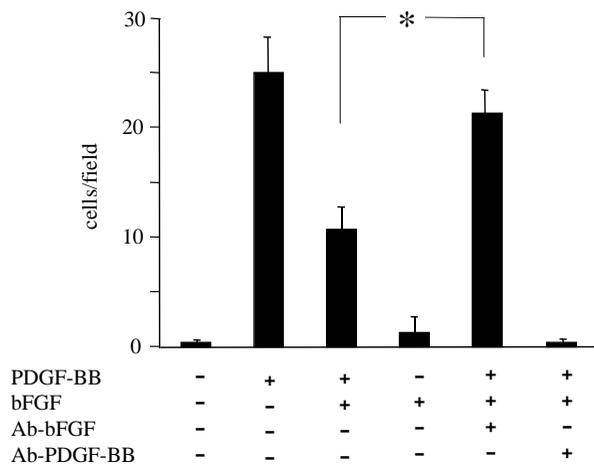


Fig. 3. Effect of neutralizing bFGF on PDGF-BB-induced SMC migration. SMC migration was evaluated in the presence of anti-bFGF (20 $\mu\text{g/ml}$) and anti-PDGF-BB (1 $\mu\text{g/ml}$) neutralizing antibodies added to PDGF-BB/bFGF in the lower chamber of the Boyden apparatus. When bFGF was neutralized, the inhibitory effect of bFGF was abolished. When PDGF-BB was neutralized, there was no migration, indicating that chemotaxis induced by PDGF-BB/bFGF was PDGF-BB-dependent. Asterisk indicates statistical significance ($P < 0.05$). Each bar represents the average \pm s.e.m. of 5 experiments.

number (Fig. 5C), indicating that native folding of bFGF was required to inhibit PDGF-BB mitogenic effect.

The effect of bFGF on PDGF-AA mitogenic activity was also investigated. PDGF-AA alone was a poor mitogen and the simultaneous presence of PDGF-AA and bFGF induced a mitogenic activity not significantly different from that of bFGF alone (Fig. 6). These results support the hypothesis of a specific inhibitory effect of bFGF on PDGF-BB-induced proliferation.

Role of bFGF- and PDGF-receptors

The mechanism of bFGF inhibition of PDGF-BB-induced SMC migration was then investigated by analyzing the role played by bFGF- and PDGF-receptors.

Neutralization of bFGF receptor with the monoclonal antibody MAB125 (3 $\mu\text{g/ml}$) did not modulate migration induced by PDGF-BB alone, while it significantly enhanced migration induced by PDGF-BB/bFGF, determining a complete recovery of PDGF-BB activity (Fig. 7A). A comparable result was obtained in proliferation assays; MAB125 (1 $\mu\text{g/ml}$) or anti-bFGF antibody (20 $\mu\text{g/ml}$) completely abolished the inhibitory effect of bFGF, while an aspecific antiserum did not interfere with bFGF inhibitory activity (Fig. 7B).

These results indicated that inhibition exerted by bFGF requires functioning bFGF receptor.

PDGF-BB binds both PDGF-R α and PDGF-R β , therefore PDGF-BB-induced SMC migration is the net result of positive chemotactic signals initiated by β receptors and negative chemotactic signals initiated by α receptors (Koyama et al., 1992). Thus, it was determined whether PDGF-R α modulated the effect of PDGF-BB and bFGF on SMC migration. The inhibitory effect of bFGF on PDGF-BB-induced migration was examined in the presence of PDGF-AA, which is a selective

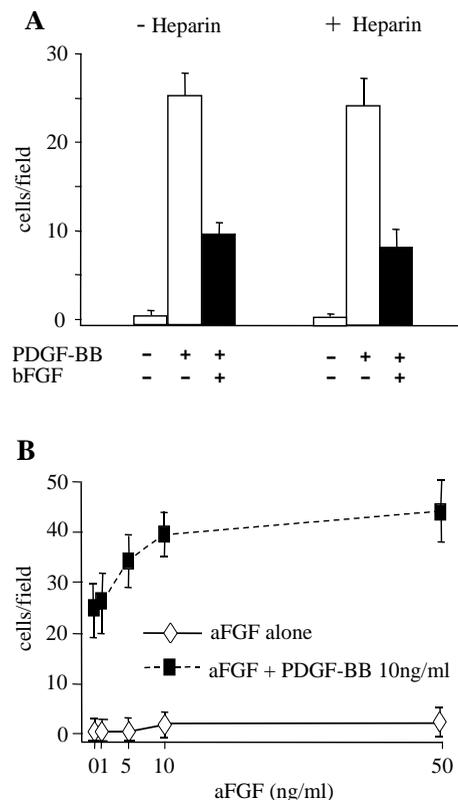


Fig. 4. Effect of heparin and aFGF on PDGF-BB-induced SMC migration. (A) Cells were exposed to PDGF-BB and PDGF-BB/bFGF in the absence or in the presence of heparin 0.05 U/ml. Heparin did not influence the inhibitory effect of bFGF. Each bar represents the average \pm s.e.m. of 3 experiments. (B) Cells were exposed to increasing concentration of aFGF (0 to 50 ng/ml) in the absence or in the presence of PDGF-BB (10 ng/ml). Acidic FGF showed a poor chemoattractant activity, and it did not inhibit, rather it enhanced, PDGF-BB-induced migration. Each point represents the average \pm s.e.m. of 3 experiments.

PDGF-R $\alpha\alpha$ homodimer agonist. Under these conditions PDGF-AA has been reported to inhibit PDGF-BB-induced migration (Koyama et al., 1992; Vassbotn et al., 1992; Heldin, 1996, 1997). Accordingly, PDGF-AA alone showed weak chemoattractant activity, but it inhibited PDGF-BB-induced migration (Fig. 8). Further, bFGF did not modulate PDGF-AA- and PDGF-BB/PDGF-AA-induced migration. Thus, bFGF and PDGF-AA did not exhibit an additive inhibitory effect on PDGF-BB-induced SMC migration. This result suggested a role of PDGF-R α in the inhibitory effect of bFGF. This hypothesis was further investigated by functionally inactivating PDGF-R α as reported in the following experiments. Three different strategies were followed. In some experiments SMC were transiently transfected with a vector coding for a dominant negative form of PDGF-R α (DN-PDGF-R α). It is expected that under these conditions, both $\alpha\alpha$ and $\alpha\beta$ receptor dimers will be inactivated and that PDGF-BB will function only through $\beta\beta$ receptor dimer. Cells transfected with the empty vector (pCDNA3) behaved similarly to un-transfected cells and the chemotactic response to PDGF-BB/bFGF was significantly lower than that induced by PDGF-BB alone (Fig. 9). In contrast, in cells transfected with DN-PDGF-R α the

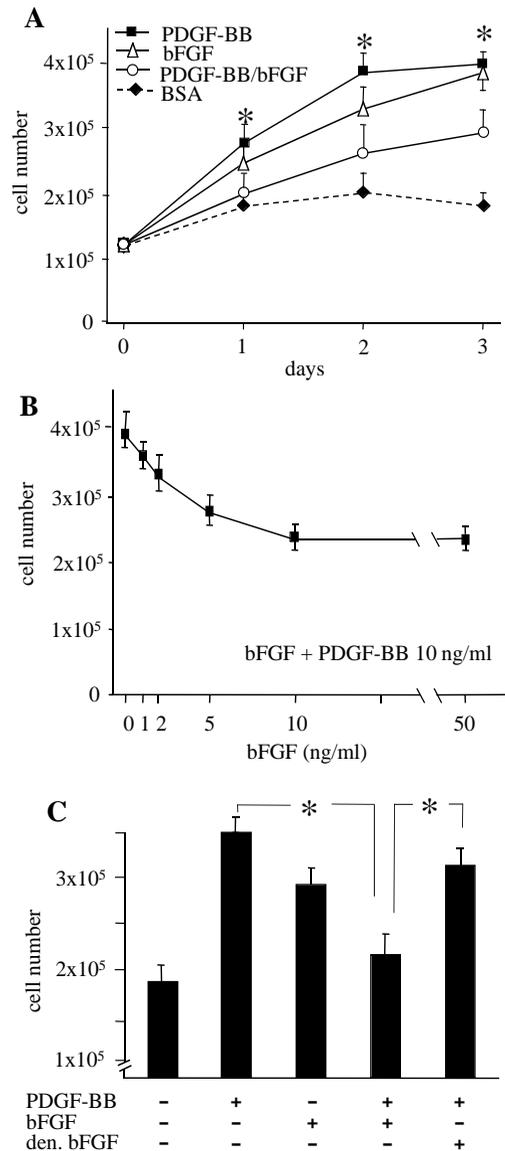


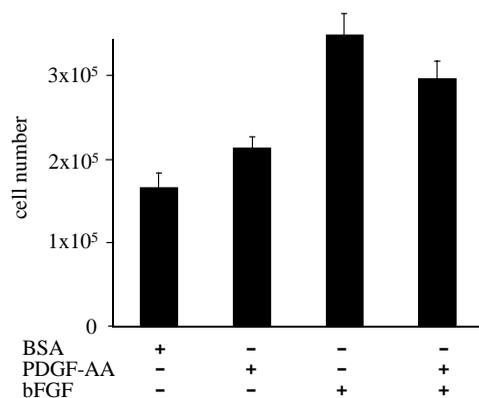
Fig. 5. Effect of bFGF on PDGF-BB-induced SMC proliferation. (A) Cells were grown in medium containing PDGF-BB (10 ng/ml), bFGF (10 ng/ml), or both (10 ng/ml of each). Cell number was determined at days 1, 2 and 3. The mitogenic effect of PDGF-BB/bFGF was significantly lower than that of PDGF-BB alone. Asterisks indicate statistical significance ($P < 0.05$). Each point represents the average \pm s.e.m. of 4 experiments. (B) Dose dependent effect of bFGF on PDGF-BB-induced SMC proliferation. SMC number was determined after 48 hours exposure to PDGF-BB (10 ng/ml) in the presence of bFGF. Increasing concentration of bFGF (0 to 50 ng/ml) showed a dose dependent inhibition of PDGF-BB induced proliferation, reaching the plateau at 10 ng/ml. (C) SMC proliferation was measured after 48 hours exposure to medium containing PDGF-BB, bFGF, PDGF-BB/bFGF, or PDGF-BB/denatured bFGF (10 ng/ml each). Native bFGF reduced significantly PDGF-BB-induced proliferation, while heat-denatured bFGF exerted a significantly weaker inhibitory effect. Asterisks indicate statistical significance ($P < 0.05$). Each bar represents the average \pm s.e.m. of 3 experiments.

pre-treatment did not reduce SMC migration in response to PDGF-BB (Vassbotn et al., 1992).

Finally, PDGF-R α was inactivated with the selective neutralizing APA-5 antibody (5 μ g/ml). APA-5 inhibited PDGF-BB-induced migration by approximately 60%, i.e. with a magnitude comparable to that of PDGF-BB/bFGF; further, APA-5 and bFGF did not show an additive inhibitory effect, supporting the role of PDGF-R α in bFGF-mediated inhibition (Fig. 11). Virtually identical results (data not shown) were obtained by blocking PDGF-R α with another selective neutralizing antibody (AF-307-NA from R&D).

It is noteworthy that SMC migration induced by PDGF-BB alone was inhibited in DN-PDGF-R α -transfected cells, as well as in SMC treated with PDGF-R α neutralizing antibodies, APA-5 and AF-307-NA. In contrast, there was no effect of neomycin on the chemotactic effect of PDGF-BB alone. The mechanism responsible for this difference is unclear, however it is possible that the reduced chemotactic action of PDGF-BB alone was due to functional inactivation of PDGF-R $\alpha\beta$ heterodimers occurred in DN-PDGF-R α -transfected cells as well as in cells treated with PDGF-R α neutralizing antibodies and not-occurred after exposure to neomycin. In fact, $\alpha\beta$ heterodimers have been reported to have a potent mitogenic and chemotactic effect (Ekman et al., 1999; Emaduddin et al., 1999; Rupp et al., 1994). Nevertheless, following $\alpha\alpha$ homodimer inactivation via different strategies, bFGF always failed to inhibit PDGF-BB-directed SMC migration.

Taken together, the experiments reported above indicate that



inhibitory effect of bFGF was abolished; rather bFGF potentiated PDGF-BB chemotactic activity. Finally, as expected, cells transfected with the DN-PDGF-R β showed almost no migration, since the positive chemotactic signals are only mediated by β receptors and in these cells only $\alpha\alpha$ dimers are expected to be functional.

In other experiments PDGF-R α was inactivated with neomycin, which has been shown to specifically inhibit PDGF-BB binding to $\alpha\alpha$ dimer without affecting its binding to $\beta\beta$ dimer (Vassbotn, 1992; Fatatis, 1997). In the presence of neomycin, the inhibitory effect of bFGF was completely abolished (Fig. 10), indicating that this effect requires PDGF-BB binding to α receptor. As previously reported, neomycin

Fig. 6. Effect of bFGF on PDGF-AA-induced SMC proliferation. SMC number was measured after 48 hours exposure to 10 ng/ml PDGF-AA alone or in combination with bFGF (10 ng/ml). PDGF-AA/bFGF-induced proliferation was not significantly different from that induced by bFGF alone. Each bar represents the average \pm s.e.m. of 3 experiments.

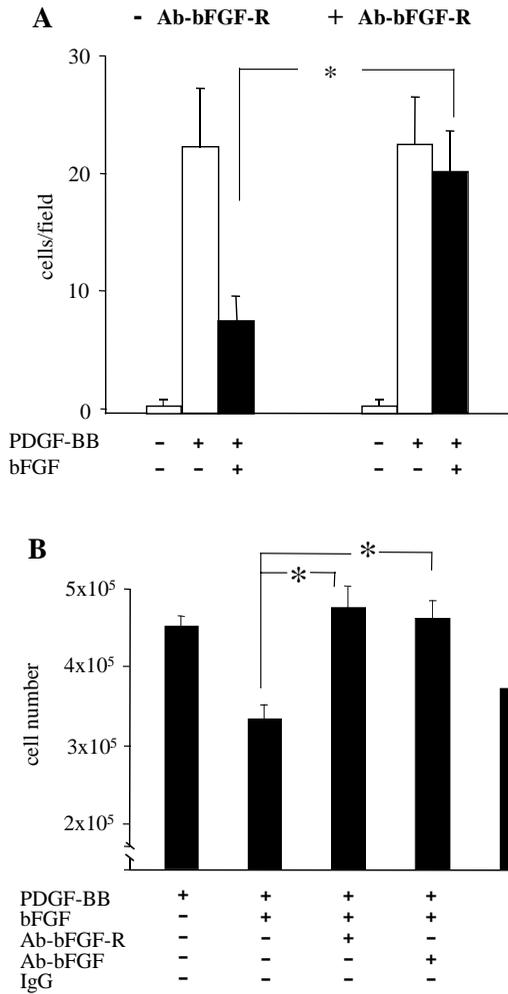


Fig. 7. Effect of neutralizing bFGF receptors on PDGF-BB-induced SMC migration and proliferation. (A) Neutralizing antibody anti-bFGF receptor (MAB125; 1 µg/ml) was added to the upper chamber of the Boyden apparatus in the presence of PDGF-BB or PDGF-BB/bFGF in the lower chamber. Basic FGF receptors neutralization abolished bFGF inhibitory effect on PDGF-BB-induced migration. Asterisk indicates statistical significance ($P < 0.05$). Each bar represents the average \pm s.e.m. of 4 experiments. (B) SMC proliferation was measured after 48 hours exposure to medium containing 10 ng/ml PDGF-BB alone or in combination with bFGF (10 ng/ml), and in the presence of antibody neutralizing bFGF (20 µg/ml) or antibody neutralizing bFGF receptor, MAB125 (1 µg/ml), or aspecific antibody (1 µg/ml). Neutralization of bFGF or neutralization of bFGF receptors abolished the inhibition observed in the presence of bFGF, while the aspecific antibody was ineffective. Asterisks indicate statistical significance ($P < 0.05$). Each bar represents the average \pm s.e.m. of 3 experiments.

the inhibitory effect of bFGF on PDGF-BB-directed SMC migration requires signaling via bFGF receptor and PDGF-R $\alpha\alpha$ dimer.

DISCUSSION

The present study shows, for the first time to our knowledge, that SMC exposed simultaneously to PDGF-BB and bFGF migrate and proliferate significantly less than in response to

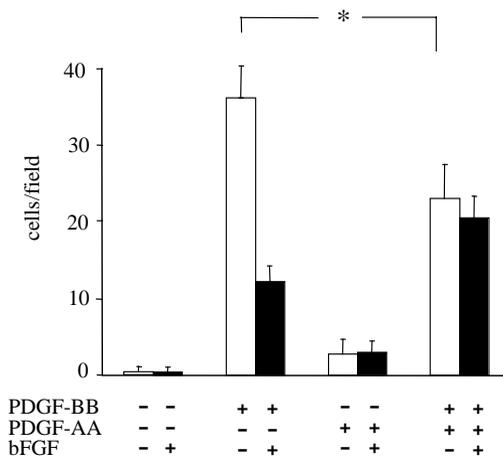


Fig. 8. Effect of bFGF on PDGF-BB-induced SMC migration, in the presence of PDGF-AA. Cells were exposed to PDGF-AA, PDGF-BB or PDGF-AA/PDGF-BB, either in the absence or in the presence of bFGF (10 ng/ml each). Basic FGF inhibited PDGF-BB-induced migration, but did not modulate PDGF-AA-induced and PDGF-AA/PDGF-BB-induced migration. Asterisk indicates a statistically significant difference ($P < 0.05$). Each bar represents the average \pm s.e.m. of 3 experiments.

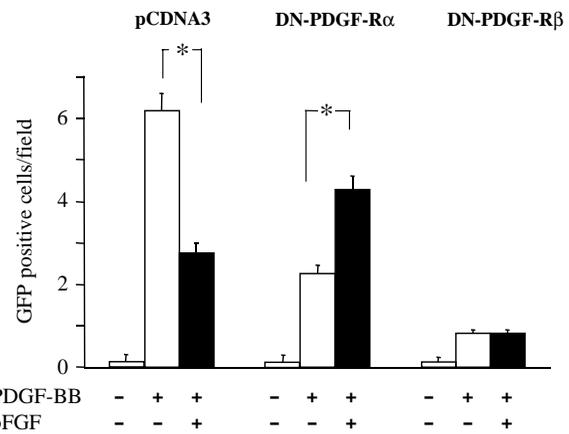


Fig. 9. Effect of bFGF on PDGF-BB induced migration in DN-PDGF-R α , PDGF-R β and pCDNA3 transfected SMC. Cells transfected with DN-PDGF-R α , DN-PDGF-R β and the pCDNA3 empty vector were exposed to medium containing PDGF-BB (10 ng/ml) alone or in combination with bFGF (10 ng/ml). When α receptors signaling was impaired, the inhibitory effect of bFGF was absent, rather bFGF potentiated PDGF-BB-induced migration. As reported in Materials and Methods, only GFP positive cells were counted, to evaluate only transfected cells. This explains the low number of cells/field counted. Asterisks indicate a statistically significant difference ($P < 0.05$). Each bar represents the average \pm s.e.m. of 4 experiments.

PDGF-BB alone. Thus, at least in vitro, simultaneous exposure to PDGF-BB and bFGF triggers inhibitory signals for SMC migration and proliferation.

Inhibition of PDGF-BB chemotactic activity by bFGF was specific, in fact it was not observed by combining PDGF-BB either with MIP-1 β , EGF, fibronectin or aFGF. Furthermore, the inhibitory effect was observed both in the absence and in

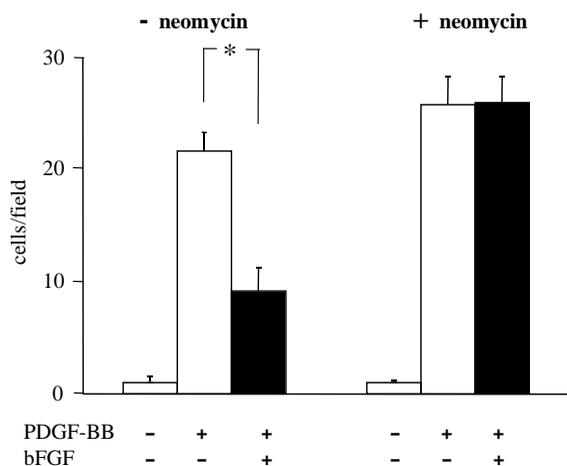


Fig. 10. Effect of bFGF on PDGF-BB-induced SMC migration in the presence of neomycin. SMC were pre-treated with 5 mM neomycin for 15 minutes before starting the chemotaxis assay. Neomycin acts as a specific inhibitor of PDGF-BB binding to PDGF-R α , while PDGF-BB binding to PDGF-R β is not affected. Basic FGF inhibitory effect on PDGF-BB-induced migration was abolished in neomycin-treated cells. Asterisk indicates a statistically significant difference ($P < 0.05$). Each bar represents the average \pm s.e.m. of 4 experiments.

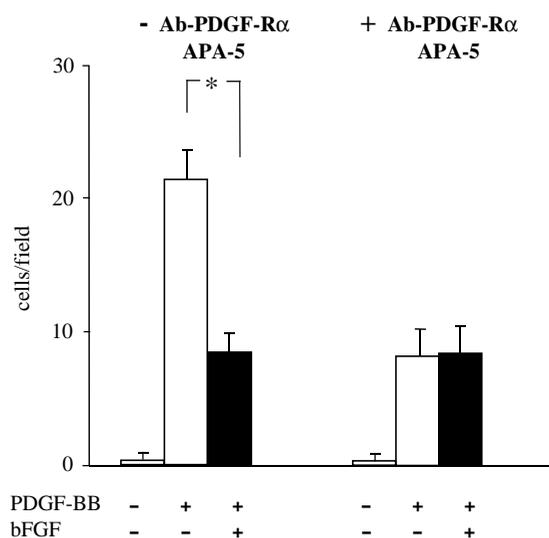


Fig. 11. Effect of bFGF on PDGF-BB-induced SMC migration in the presence of PDGF-R α neutralizing antibody APA-5. Neutralizing antibody anti-PDGF-R α (APA-5, 5 μ g/ml) was added to the upper chamber of the Boyden apparatus. Under these conditions SMC migration in response to PDGF-BB was not inhibited by bFGF (10 ng/ml). Asterisk indicates a statistically significant difference ($P < 0.05$). Each bar represents the average \pm s.e.m. of 3 experiments.

the presence of heparin. The active form of bFGF molecule was required to inhibit PDGF-BB-dependent migration and proliferation; in fact inhibition of proliferation was not observed when heat-denatured bFGF was mixed with PDGF-BB, and blocking bFGF with a specific neutralizing antibody abolished the inhibition in both chemotactic and mitogenic assays. These data indicate a specific and dose-dependent inhibitory effect of bFGF on the mitogenic and chemotactic action of PDGF-BB.

It has been previously shown (Rankin and Rozengurt, 1994; Clunn et al., 1997; Koyama et al., 1994), and confirmed in the present study, that PDGF-BB exhibits in vitro a concentration-dependent bell-shaped chemotactic effect with maximum activity between 5 and 10 ng/ml. The mechanism underlying the reduced chemotaxis at higher concentrations is still debated, and it was attributed to either PDGF-R α (Koyama et al., 1994) or PDGF-R β signaling (Clunn et al., 1997). Many reports in the literature focus their attention on the opposite effects of PDGF depending on the receptors expressed on the target cells and therefore on receptor dimerization events. (Koyama et al., 1994; Clunn et al., 1997; Claesson-Welsh, 1994; Heldin, 1997). It is currently accepted that PDGF-AA, whose signaling is only mediated by PDGF-R $\alpha\alpha$ homodimer, is not chemotactic on fibroblast and SMC, but it inhibits migration induced by PDGF-BB which binds PDGF-R $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ dimers (Siegbahn et al., 1990; Koyama et al., 1994). Other studies showed that $\alpha\alpha$ dimer has an intrinsic ability to initiate negative signals which suppress simultaneous positive migratory signals (Yokote et al., 1996; Claesson-Welsh, 1996). Therefore we hypothesized that $\alpha\alpha$ dimer may play a role in the inhibitory effect of bFGF on PDGF-BB-induced migration. The results of the present study indicate that bFGF is not able, at the tested concentrations, to sum its own inhibitory effect to the PDGF-AA inhibitory action on PDGF-BB-induced migration. Since the inhibitory effect of PDGF-AA depends on

negative signals initiated by $\alpha\alpha$ dimer, we argued that the inhibition exerted by PDGF-AA and bFGF might both require signaling via $\alpha\alpha$ dimer. This hypothesis was confirmed when PDGF-R $\alpha\alpha$ was functionally inactivated by using DN-PDGF-R α transfected cells, neomycin or PDGF-R α selective neutralizing antibodies. Under these conditions, the inhibitory effect of bFGF was abolished, indicating that the negative signals initiated by $\alpha\alpha$ dimer are required for the bFGF inhibitory effect to take place.

At the moment, different mechanisms may be proposed for the bFGF inhibitory effect of PDGF-BB action:

(1) it is possible that bFGF receptor phosphorylation activates a cascade of events which may amplify, at the intracellular level, the negative signals initiated by PDGF-R α . A cross talk between bFGF and PDGF signal transduction cascades has been previously shown (Chaudary and Avioli, 1998; Hayashi et al., 1999). They both activate c-myc (Coughlin et al., 1985; Sacca and Cochran, 1990; Barone and Courtneidge, 1995; Skaletz-Rorowski et al., 1999) whose simultaneous activation may lead to an imbalance of its pro-proliferative and pro-apoptotic action (Romashkova and Makarov, 1999). PDGF and bFGF signaling pathways also converge at the MAPK kinases level (Skaletz-Rorowski et al., 1999; Ahn et al., 1999) as well as CamKinase II (Bilato et al., 1995) and PKC (Heldin, 1998; Skaletz-Rorowski et al., 1999). According to these considerations, it is possible that the simultaneous presence of PDGF-BB and bFGF may affect the activation of common signal pathways;

(2) simultaneous binding of bFGF and PDGF-BB to the respective receptors may interfere with the appropriate receptor clustering and initiation of positive signals;

(3) simultaneous presence of PDGF-BB and bFGF in solution may interfere with their receptor binding properties. These hypotheses are not mutually exclusive and may explain,

at least in part, why SMC are quiescent in vivo despite the presence of potent growth factors (Liu et al., 1989) and may help explaining how PDGF can trigger both stimulatory and inhibitory signals (Heldin, 1997; Clunn et al., 1997; Koyama et al., 1992, 1994). Further, as recently pointed out (Nelson et al., 1997), the combined effect of different growth factors is not predictable from the action of the single factors alone, depending on the cross talk of their signaling pathways.

Data presented here may appear contradictory with previous studies showing that pre-exposing cells to bFGF increased chemotaxis toward PDGF by inducing integrin (Pickering et al., 1997) and PDGF-R α expression (Schollmann et al., 1992). In those studies cells were pre-treated for 12 to 48 hours with exogenous bFGF or simultaneously exposed to PDGF and bFGF at a molar ratio around 1:16. Therefore the experimental conditions are not comparable. In fact, in the present study SMC were *simultaneously* exposed to both growth factors, without any pre-treatment. In addition, inhibition of migration in the present study was observed after 5 hours treatment, which is a significantly shorter time as compared to 12 to 48 hours of the previous studies. Finally, in the present study most experiments were performed with PDGF-BB:bFGF molar ratios around 1:1, which is closer to the ratio observed in vivo (Law et al., 1996; Lindner et al., 1995; Drubaix, 1998).

In conclusion, data presented here indicate that SMC simultaneously exposed to PDGF-BB and bFGF exhibit diminished chemotaxis and proliferation than to PDGF-BB alone.

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