Inhibition of Endothelial Cell Proliferation by Platelet Factor-4 Involves a Unique Action on S Phase Progression

Shalley K. Gupta and Jai Pal Singh

Lilly Research Laboratories, Indianapolis, Indiana 46285

Abstract. Modulation of endothelial cell proliferation and cell cycle progression by the "chemokine" platelet factor-4 (PF-4) was investigated. PF-4 inhibited DNA synthesis, as well as proliferation of endothelial cells derived from large and small blood vessels. Inhibition by PF-4 was independent of the type and the concentration of stimuli used for the induction of endothelial cell proliferation. Inhibition of cell growth by PF-4 was reversible. The effects of PF-4 were antagonized by heparin. Cell cycle analysis using [³H]thymidine pulse labeling during traverse of synchronous cells from G0/G1 to S phase revealed that addition of PF-4 during G1 phase completely abolished the entry of cells into S phase. In addition, PF-4 also inhibited DNA synthesis in cells that were already in S phase. In exponentially growing cells, addition of PF-4 resulted in an accumulation of >70% of the cells in early S phase, as determined by FACS® (Becton-Dickinson Immunocytometry Systems, Mountain View, CA). In cells synchronized in S phase by hydroxyurea and then released, addition of PF-4 promptly blocked further progression of DNA synthesis. These results demonstrate that in G0/G1-arrested cells, PF-4 inhibited entry of endothelial cells into S phase. More strikingly, our studies have revealed a unique mode of endothelial cell growth inhibition whereby PF-4 effectively blocked cell cycle progression during S phase.

PLATELET factor-4 (PF-4)[1] is a member of a growing family of small, inducible proteins known as "chemokines" that mediate a variety of biological processes including inflammation, tissue repair, angiogenesis, and cell proliferation (21, 4, 31). To date, more than 16 members of the supergene family have been identified (21). The members are classified as α or β, based on the nature of a highly conserved cysteine motif present at the NH₂-terminal. The α gene family characterized by CXC motif (cysteines separated by an amino acid) includes PF-4, IL-8, human protooncogene Gro/melanocyte growth–stimulating activity, β-thromboglobulin, neutrophil-activating protein-2, neutrophil activating protein from epithelial cells (ENA-78), and interferon-inducing protein-10 (1, 15, 21, 24, 32, 34). The predominant activities associated with the CXC family of proteins are chemoattraction, and activation of neutrophils but not of monocytes (24). The β gene family characterized by CC motif (adjacent cysteines) includes monocyte chemotactic protein-1, RANTES, and macrophage inflammatory proteins-1α and -1β (MIP-1α and MIP-1β) (13, 20, 21, 27). The CC family of proteins stimulate chemotaxis in monocytes or T cells but not in neutrophils (13, 24, 33, 34). Other biological activities and physiological functions of the protein products of the chemokine supergene family are currently under investigation.

Human PF-4 is a 7.8-kD protein containing 70 amino acid residues (7). PF-4 shares 31%, 41%, and 51% homology with IL-8, human protooncogene Gro/melanocyte growth-stimulating activity, and β-thromboglobulin, respectively (21, 24, 31). Recent studies have revealed that PF-4 is an important modulator of endothelial cell proliferation (16) and angiogenesis (16, 17, 28). Purified recombinant human PF-4 blocked proliferation of human umbilical vein endothelial cells in a dose-dependent manner (16). Intralesional injection of recombinant PF-4 also reduced tumor growth in syngeneic C57BL/6J mice with B-16(F10) melanoma cell implants, as well as in semisyngeneic (CB/B6F1/J) athymic nude mice receiving primary cutaneous implants of HCT 116 colon carcinoma cells (16, 17, 28). Since PF-4 did not directly inhibit B-16(F10) or HTC-116 cells in culture, these studies have concluded that the inhibition of tumor development by recombinant PF-4 was caused by its direct effect on endothelial cells and angiogenesis. In this regard, the actions of PF-4 are unique as compared to the other endothelial cell growth inhibitors such as TGF-β and TNF. Both TGF-β and TNF inhibit endothelial cell proliferation in vitro (12, 22); however, these cytokines promote angiogenesis in vivo (9, 10, 26).

1. *Abbreviations used in this paper:* ECGF, endothelial cell growth factor; EGM, endothelial growth medium; FBHE cells, fetal bovine heart endothelial cells; MIP-1α, macrophage inflammatory protein-1α; PF-4, platelet factor-4.

The cell growth inhibitory actions of PF-4, particularly its cellular receptor and the mechanism of action on endothelial cells, have not been characterized. In the present study, we show that PF-4 exhibits a unique mode of action on cell growth cycle in endothelial cells. PF-4 inhibits endothelial cell proliferation by blocking cell cycle traverse from G1 to S phase. More importantly, our studies show that PF-4 inhibits progression of cells already in the DNA synthesis phase. The latter is a unique action of PF-4. An endogenous cytokine inhibiting cells in the S phase has not been previously described.

Materials and Methods

Cell Cultures

Endothelial Cells. Retinal capillary endothelial cell cultures were obtained from Dr. Larry Stramm (Eli Lilly and Company, Indianapolis, IN). Cultures were initiated from bovine eyes using a modification of the procedure of Buzney et al. (3). Dispersed cells were separated from contaminating cell types using a fluorescent cell sorter. Cell cultures were maintained in growth medium consisting of DME, 10% FBS, 2 mM, L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Human aorta and umbilical cord endothelial cells were obtained from Clonetics Corp. (San Diego, CA). These cells were maintained in endothelial growth medium (EGM) supplied by Clonetics Inc. Fetal bovine heart endothelial (FBHE) cells were obtained from (American Type Culture Collection, Rockville, MD). Stock cultures were propagated in medium containing 10% serum. Cultures were passaged once a week by trypsinization.

Vascular Smooth Muscle Cells. Smooth muscle cells were prepared from New Zealand white rabbits as described previously (2). Aorta were removed aseptically, adventitia was stripped off, and 1-2-mm explants were cultured in DME containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Smooth muscle cells were observed growing from the explants within 5–7 d. After 7–10 d, explants were removed, and cells were subcultured by trypsinization and propagated in the above medium.

Determination of DNA Synthesis

Endothelial cells were plated in 96-well plates at a starting density of 4 × 10³ in DME containing 10% FBS. The cultures reached confluence after 5 d, at which time the medium was replaced with fresh DME containing 10% FBS, 20 ng/ml basic FGF, 1 µCi/ml [³H]thymidine, and various concentrations of PF-4. For determination of entry into S phase, cells were pulsed with [³H]thymidine for 3-h intervals. At the indicated time points, cells were fixed in methanol, and radioactivity was determined by scintillation counting (30).

Determination of Cell Cycle Progression in Endothelial Cells

For determination of entry of cells into S phase, confluent cultures of endothelial cells were growth arrested by overnight incubation in DME containing 10% FBS without FGF. Cells were then stimulated with FGF (20 ng/ml, and PF-4 was added at various intervals thereafter. DNA synthesis was determined by pulse labeling with 1 µCi/ml [³H]thymidine for 3-h intervals. For determination of the effect of PF-4 on S phase, endothelial cells were plated at a cell density of 4 × 10³ in 96-well microtiter plates. After 4 d, when the cultures were still nonconfluent, cells were synchronized in S phase by incubation with 1 mM hydroxyurea for 24 h. Cell synchronicity in S phase was determined by (FACS®); (Becton Dickinson Immunocytometry Systems, Mountain View, CA). DNA synthesis after release from hydroxyurea arrest was determined by pulse labeling with [³H]thymidine at 1.5-h intervals during S phase.

FACS® Analysis

Fetal bovine heart endothelial cells were plated at a cell density of 5 × 10⁴ cells per well in six-well plates in DME containing 10% FBS. After 24 h, duplicate cultures were supplemented with fresh medium containing 10% serum, 20 ng/ml FGF with or without PF-4. The cultures were then in-

cubated for additional 40 h. Cells were harvested by trypsinization, washed in DME/10% serum, and then fixed for 2 minutes with 70% methanol. Cells were permeabilized by incubation in 0.1% Triton X-100 in phosphate-buffered saline for 15 min, and were then treated with RNAse (200 µg/ml). DNA was stained with propidium iodide (50 µg/ml) by overnight incubation at 4°C in the dark. DNA content was determined by flow cytometry. Results were analyzed by the Modfit cell cycle analysis program (Varity Software Inc., Topshan, NE).

Materials. FGF, PDGF, and endothelial cell growth factor (ECGF) were purchased from Genzyme Corp. (Cambridge, MA). PF-4 purified to homogeneity was from Sigma Immunochemicals (St. Louis, MO). [³H]thymidine (specific activity = 6.7 µCi/mmol) was from New England Nuclear/Dupont (Boston, MA).

Results

PF-4 Inhibits Proliferation of Various Types of Endothelial Cells

Endothelial cells from large vessels (human aorta, fetal bovine heart, and human umbilical cord) and microvessels (bovine retina) were cultured in DME containing 10% fetal bovine serum and basic FGF. Addition of PF-4 at the time of plating or 6–24 h thereafter produced a dose-dependent inhibition of cell proliferation during a 4-d incubation period (Fig. 1). The concentration of PF-4 required for 50% inhibition of endothelial cell growth was in the range of 1–3 µg/ml. An apparent higher concentration of PF-4 required for human aortic cells, as compared to the retinal or heart endothelial cells, is related to a significantly lower growth rate of aortic endothelial cells. For example, during the 4-d incubation period, human aortic endothelial cells underwent only about two doublings, an increasing in cell number from 1 × 10⁴ to 4.3 × 10⁴, whereas retinal and heart endothelial cells increased from 1×10⁴ to 11.2×10⁴ and 12.5 10⁴,
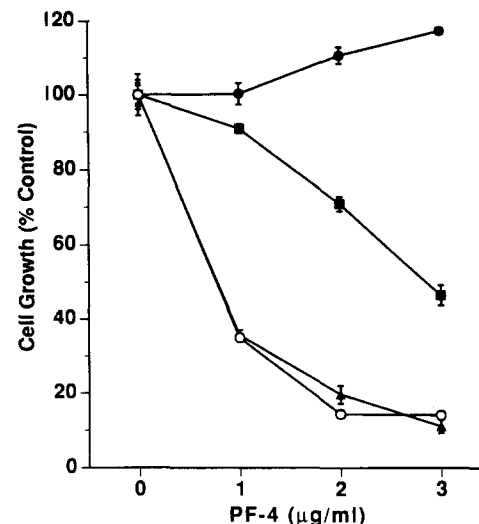

Figure 1. Inhibition of various types of endothelial cell proliferation by PF-4. Endothelial cells from human aorta (■), fetal bovine heart (O), bovine retina (▲), and vascular smooth muscle cells from rabbit aorta (●) were cultured in 12-well plates at a cell density of 1 × 10⁴ per well in growth medium containing 20 ng/ml FGF. The indicated concentration of homogeneously purified human PF-4 was added at the start of the incubation. Cells were then incubated for 4 d at 37°C under 5% CO₂/95% air. Cell growth was determined by cell counting using a Coulter counter.

respectively. Inhibition of endothelial cell prolfieration was a selective action of PF-4. Other members of the chemokine family (IL-8 and monocyte chemotactic protein-1) have not shown similar effects on endothelial cell proliferation (unpublished results). Fig. 1 also shows that under identical culture conditions, the growth of vascular smooth muscle cells was not inhibited by PF-4. These results show that PF-4 inhibited proliferation of endothelial cells derived from large and small vessels. Since the growth of FBHE cells was strictly dependent on the presence of FGF and, thus, a highly synchronous cultures of these cells is obtained by depriving them of FGF, we have used heart endothelial cells for a detailed characterization of the mechanism of growth inhibition by PF-4.

Kinetics of FBHE Cell Growth Inhibition by PF-4

Fig. 2 shows the rate of FBHE cell proliferation in the presence of various concentrations of PF-4. PF-4 produced a dose-dependent inhibition of cell proliferation that persisted throughout the 4-d incubation. PF-4 did not significantly alter the normal doubling time (18 ± 2 h) of endothelial cells, suggesting that the effect of PF-4 was not simply caused by delaying the cell division cycle. Instead, in an exponentially growing culture, PF-4 appeared to have reduced the fraction of cycling cells. These data are also supported by inhibition of DNA synthesis by PF-4 during extended incubation. Growth-arrested FBHE enter the DNA synthesis phase ~12 h after stimulation with FGF. As shown in Fig. 2, inset, PF-4 produced a dose-dependent inhibition of DNA synthesis, even ≤24 h after FGF stimulation. These data suggest that PF-4 reduces the number of cycling cells without affecting their doubling time.
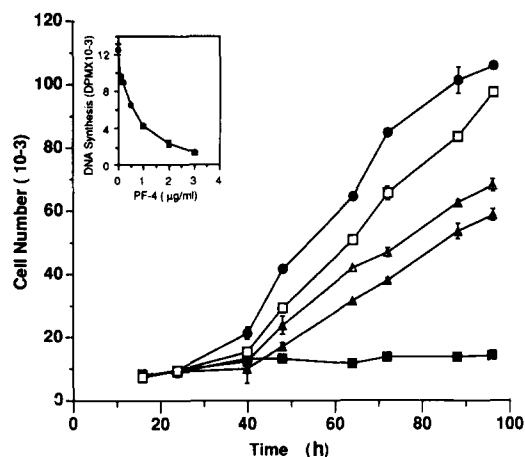
Figure 2. Kinetics of endothelial cell growth inhibition by PF-4. FBHE cells were plated in growth medium containing 20 ng/ml FGF in the absence (●) or presence of 0.5 μg/ml (◊), 1 μg/ml (△), or 2 μg/ml (▲) PF-4, as described in the legend to Fig. 1. Also shown here is the growth of FBHE cells in the absence of FGF (■). Cells were harvested at the indicated time points by trypsinization and then counted. (Inset) Dose-dependent inhibition of DNA synthesis in fetal bovine heart endothelial cells by PF-4. Growth-arrested quiescent cells in 96-well microtiter plates were transferred to fresh medium containing 20 ng/ml FGF and various concentrations of PF-4. Cumulative DNA synthesis at the end of 24-h incubation was determined by [³H]thymidine incorporation, as described under Materials and Methods.

Inhibition of Endothelial Cell Proliferation by PF-4 is Independent of the Nature of the Growth Stimuli

To obtain an initial insight into the cellular site of PF-4 action, and to rule out the potential effect of PF-4 on extracellular action of FGF, we determined the effect of PF-4 on endothelial cells stimulated with different growth stimuli, e.g., FGF, ECGF, or EGM, a medium containing a mixture of endothelial cell growth stimuli. Fig. 3 A shows that irrespective of the nature of the growth stimuli used, PF-4 effectively blocked endothelial cell proliferation. A slightly lower inhibition by PF-4 in cultures stimulated with ECGF was related to a slower growth of cells in medium containing ECFG, as compared to FGF or EGM. The results in Fig. 3 B show that increasing the FGF concentration while keeping the concentration of PF-4 constant did not reverse the growth inhibitory effect of PF-4, suggesting that the growth inhibitory effect of PF-4 was not simply by competition with FGF. For example, a PF-4 concentration that produced 50% inhibition of DNA synthesis in the presence of a suboptimal concentration (2 ng/ml) of FGF also produced similar inhibition in the presence of 100 times (200 ng/ml) excess FGF. It is important to note that the concentration of PF-4 used here (a concentration that produced 50% inhibition) was such that a direct competition by FGF is expected to reverse the PF-4 effect. Taken together, these data suggest that the effect of PF-4 is not caused by inhibition of FGF action at the cell surface. These conclusions are also supported by a lack of effect on FGF-mediated smooth muscle cell growth (Fig. 1) as well as other subsequent studies presented in a later section that show that the effect of PF-4 is on the events in the cell growth cycle.

Inhibition by PF-4 is Reversible

Inhibition of endothelial cell growth by PF-4 was not caused by cellular toxicity. Light microscopic examination of PF-4–treated cultures did not reveal any marked changes in cells
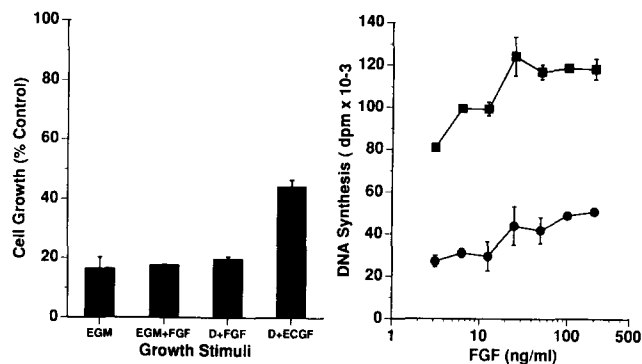
Figure 3. Inhibition of endothelial cell proliferation by PF-4 is not caused by competition with FGF: (A) Endothelial cells were plated in 12-well culture dishes, as described previously in the legend to Fig. 1, either in DME 10% serum (D) or EGM. Media were supplemented with 20 ng/ml of either FGF or ECGF. Cell growth was determined after 4 d by trypsinization and cell counting using Coulter counter. (B) Growth-arrested quiescent cells in 96-well microtiter plates were transferred to fresh medium and then stimulated with varying concentrations of FGF in the absence (■) or presence (●) of 2 μg/ml PF-4. DNA synthesis during a 24-h incubation was determined by [³H]thymidine incorporation.
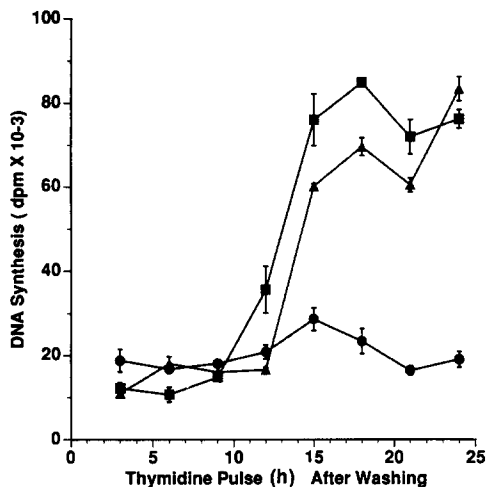
Figure 4. Reversibility of PF-4 inhibition of endothelial cells. Confluent cultures of FBHE cells were transferred to fresh DME containing 10% FBS, 20 ng/ml FGF, and 2 μg/ml PF-4. After 24 h, cells were washed and then further incubated in the presence (●) or absence (■) of 2 μg/ml PF-4 for 24 h. A control cells (▲) were without PF-4 throughout the 48-h incubation. DNA synthesis was then determined by pulse labeling with [³H]thymidine at 3-h intervals. The data are mean of triplicates ±SD.

morphology or detachment from the tissue culture plates. The lack of toxicity was further demonstrated by a reversibility of PF-4 actin (Fig. 4). Endothelial cultures were first treated with PF-4 for 24 h under the conditions described for the experiments in Figs. 1–3. After 4 h, the cultures were washed three times with phosphate-buffered saline to remove PF-4. The cells were then incubated in the presence or absence of PF-4. DNA synthesis and entry into S phase were determined by [³H]thymidine pulse labeling at 3-h intervals during the 4-h incubation. As shown in Fig. 4, incubation of endothelial cells with PF-4 produced both inhibition of DNA synthesis and entry of cells into S phase. Removal of PF-4 by washing and further incubation resulted in a complete restoration of DNA synthesis. A slightly higher DNA synthesis in washed cells could have been caused by a greater synchronization of cells previously incubated with PF-4. These results demonstrate that the effect of PF-4 on endothelial cell growth is reversible.

Site(s) of PF-4 Action in the Cell Growth Cycle

The site of PF-4–mediated cell cycle block was investigated by [³H]thymidine pulse-labeling studies. Endothelial cells were synchronized in G0/G1 phase by growing to confluence and by further incubation for 24 h in medium without FGF. The basal DNA synthesis in growth-arrested cells was only ~8% of that achieved after FGF stimulation. FGF treatment of growth-arrested cells resulted in entry into S phase after ~12 h and a 12-fold higher DNA synthesis than the controls after 18 h (Fig. 5). Addition of PF-4 during the cell cycle progression from G1 to S phase, i.e., at 0, 3, 6, and 9 h after FGF stimulation, resulted in >95% inhibition of DNA synthesis. PF-4 was able to block DNA synthesis when added 1–2 h before entry of cells into S phase. These results clearly showed that PF-4 inhibited entry of cells into S phase. An additional intriguing result from this experiment was the effect of PF-4 on S phase cells. First, we noticed that in these
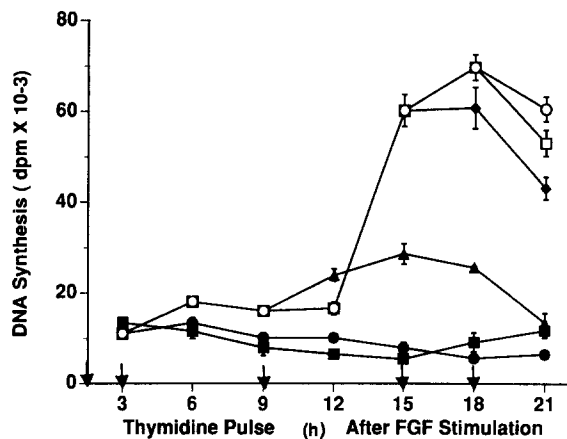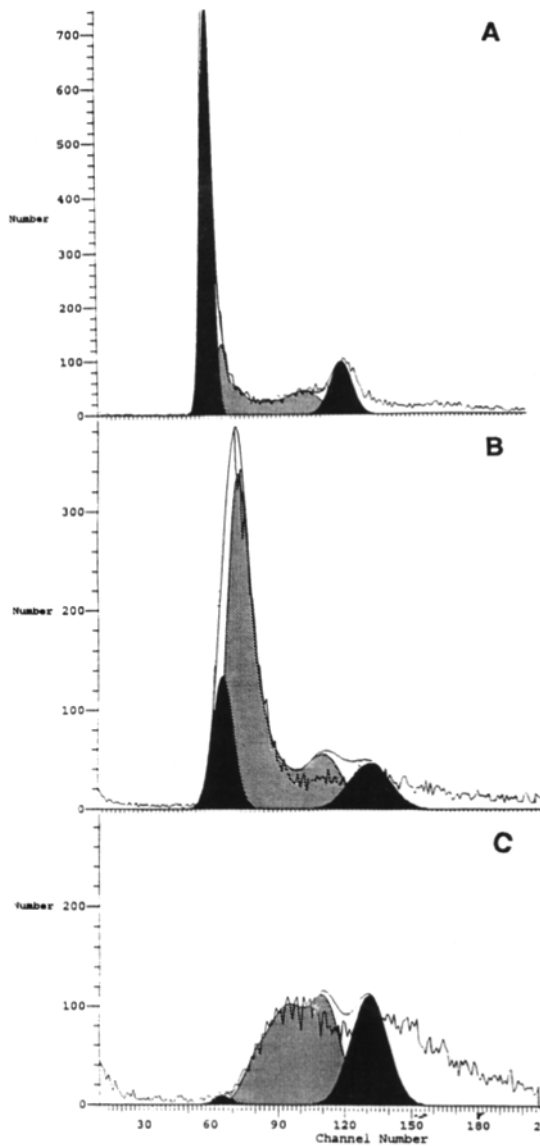

Figure 5. Effect of PF-4 on cell cycle traverse from G0/G1 to S FBHE cells in 96-well microtiter plates were growth arrested in G0/G1 by growing to confluence and then subjecting to further incubation in FGF-free growth medium for 24 h. The cells were then washed and stimulated with 20 μg/ml FGF. The effect of PF-4 on entry into S phase was determined by pulse labeling with [³H]thymidine for 3-h intervals in cells receiving PF-4 at 0 (●), 3 (■), 9 (▲), 15 (◆), and 18 (□) h. One set of cells were controls without PF-4 (o). The data are average of triplicates SD. Additional data collected but not shown here for clarity of presentation were for the time points when PF-4 was added at 6, 12, and 24 h.

cultures, PF-4 significantly (>50%) reduced the basal DNA synthesis in residual cycling cells (Fig. 5, PF-4 added at 0 h). Second, addition of PF-4 to cells already progressing in S phase (15 and 18 h after FGF stimulation) also reduced DNA synthesis. In both cases, the reduction in DNA synthesis occurred within 1–3 h after PF-4 treatment. These results suggested that PF-4 may affect cells actively undergoing DNA synthesis. However, the apparent inhibition of DNA synthesis by PF-4 when added during S phase could also result from the inhibition of the entry of residual cells in S phase and/or exit of some cells from S phase. Therefore, although PF-4 appeared to have inhibited DNA synthesis in S phase cells, further investigation was required to clearly distinguish a potentially direct effect of PF-4 on S phase cells from the reduction of DNA synthesis caused by influx and exit of cells from the S phase.

To further characterize the cell cycle block points, we performed cell cycle analysis using FACS®. Exponentially growing cultures were treated with PF-4 for 40 h. The control and treated cells were trypsinized, and DNA content was determined by FACS® analysis after propidium iodide staining (Fig. 6). The data in Table I show that PF-4 treatment produced a marked shift in the distribution of endothelial cells from G0/G1 to early S phase. The fraction of cells in G0/G1 were reduced from 56.74% to 17.04%, whereas fraction of cells in S phase increased from 28.64% to 71.17% upon PF-4 treatment. No significant change was observed in the G2/M fraction. Hydroxyurea used as an internal control produced an expected effect by blocking cells in S phase. These data suggested that in exponentially growing cultures of endothelial cells, PF-4 blocked cells in early S phase.

Effect of PF-4 on S Phase Cells

Further direct evidence for the effect of PF-4 on S phase

	G0/G1	S	G2/M	CV
Control	56.74	28.64	14.62	±4.13
Hydroxyurea	1.45	63.71	34.84	±6.24
PF-4	17.04	71.17	11.79	±6.92

Distribution of cells in G0/G1, S, and G2/M was obtained from the data presented in Fig. 5 using the Modfit program. CV, coefficient of variance.
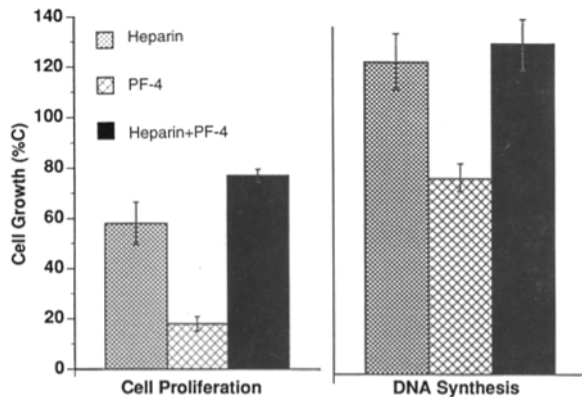
PF-4 immediately after the removal of hydroxyurea produced a complete inhibition of DNA synthesis. Fig. 7 also shows that treatment of cells with PF-4 at various times after hydroxyurea removal inhibited DNA synthesis within 1 h of its addition. For example, in control cultures, DNA synthesis reached ~3.5-fold at 4.5 h after removal of hydroxyurea. Addition of PF-4 at this point resulted in a 277% lower DNA synthesis, as compared to the untreated controls within 1.5 h. DNA synthesis was reduced to basal level within 3 h after PF-4 addition. These data clearly demonstrate that PF-4 treatment directly inhibits DNA synthesis, as well as progression of cells through S phase.

Effect of Heparin on PF-4 Activity

Heparin is an important modulator of endothelial cell proliferation and binds PF-4 with high affinity. The effect of heparin on the cellular actions of PF-4 is not yet fully understood. We have examined the effect of heparin on PF-4–mediated inhibition of endothelial cell proliferation, as well as DNA synthesis in S phase cells. FBHE cells were incubated with 1 U/ml of heparin in the absence or presence of PF-4. Cell growth or DNA synthesis in cells synchronized in S phase by hydroxyurea was then determined. As before, PF-4 treatment inhibited cell proliferation and DNA synthesis in S phase–synchronized cells (Fig. 8). Addition of heparin alone produced ~40% reduction in cell proliferation.

Figure 6. PF-4 induces accumulation of endothelial cells in S phase. FBHE cells were plated as triplicates at a cell density of 5 × 10⁴ cells per well in six-well tissue culture dishes in DMEM containing 10% serum and 20 ng/ml FGF. Duplicate wells were treated with (A) none, (B) 2 μg/ml PF-4, or (C) 1 mM hydroxyurea. After 40 h, cells were processed for propidium iodide staining and FACS® analysis. Cell distribution of G0/G1, S, and G2/M was determined using the Varity Modfit program. From left to right, the areas under the first dark peak, the gray peaks, and the second dark peak correspond to G0/G1, S, and G2/M, respectively.

Figure 7. Inhibition of DNA synthesis in S phase cells by PF-4. Sparse cultures of FBHE cells were synchronized in S phase by treatment with hydroxyurea, as described in the legend to Fig. 6. After 24 h, hydroxyurea was removed by washing twice in DME containing 10% serum. PF-4 (2 μg/ml) was then added at 0 (▲), 3 (○), 4.5 (▼), and 6 (□) h after washing. Also shown are the control cells without removal of hydroxyurea (——) and washed cells without PF-4 treatment (●). DNA synthesis was determined by [³H]thymidine pulse labeling for 1.5-h intervals.

progression was obtained by using endothelial cells synchronized in S phase. As shown previously in Fig. 6, incubation of endothelial cells in the presence of hydroxyurea resulted in an accumulation of cells in S phase. Under these conditions, >62% of the cells in culture were accumulated in S phase. More importantly, only <1.5% cells were in the G0/G1 phase (Table I), therefore enabling us to dissect the effect of PF-4 on G1 and S phases. Removal of hydroxyurea by washing resulted in a prompt resumption of DNA synthesis leading to a five- to sixfold increase in DNA synthesis, as compared to the unwashed controls (Fig. 7). Addition of

Figure 8. Effect of heparin on PF-4 inhibition. (*A*) Effect on cell proliferation was determined by incubation of FBHE cells in DME containing 10% FBS, 20 ng FGF, and 2 μg/ml PF-4 in the presence or absence of 1 U/ml heparin. Cell growth was determined after 4 d. (*B*) Effect of heparin on DNA synthesis in S phase cells was determined by incubation of cells in 1 mM hydroxyurea, as described in the legend to Fig. 7. Hydroxyurea was then removed by washing, and the cells were incubated with 2 μg/ml PF-4 in the presence or absence of 1 U/ml heparin. DNA synthesis was determined by [^{3}H]thymidine incorporation at the end of a 24-h incubation. The controls were without PF-4 or heparin.

Addition of heparin to PF-4–treated cells resulted in a reversal of inhibition of cell proliferation by PF4. Similarly, heparin treatment antagonized the inhibition of DNA synthesis by PF-4 in cells synchronized in S phase. These results demonstrate that heparin antagonizes the effect of PF-4 on cell proliferation and DNA synthesis.

Discussion

Recent studies have demonstrated that PF-4, an archetype of "chemokine" superfamily of genes, is an important modulator of endothelial cell proliferation (16, 17, 28). Unlike TGF-β or TNF, which inhibit endothelial cell proliferation in vitro but stimulate angiogenesis in vivo, PF-4 is a unique endogenous modulator that inhibits endothelial cell growth in vitro, as well as angiogenesis in vivo (16, 17, 28). However, at the present time, very little is known about the mechanism of action of PF-4 on endothelial cells. Our studies, for the first time, have revealed a unique mode of action of PF-4 on cell cycle progression in endothelial cells. We have shown that PF-4 inhibited proliferation of endothelial cells derived from large and microvessels. Growth inhibition by PF-4 occurred without altering the doubling time of the exponentially growing endothelial cell population, suggesting that the effect of PF-4 was not simply caused by delaying of cell division. Growth inhibition by PF-4 was not caused by cytotoxicity. During an extended incubation of endothelial cells, smooth muscle cells, or fibroblasts with PF-4, no discernible morphological change or detachment of cell monolayer was observed. Furthermore, cell growth inhibition by PF-4 was reversed upon washing the cultures. For vascular cells, growth inhibition by PF-4 appeared selective for endothelial cells. Growth of vascular smooth muscle cells was not affected by PF-4.

Our initial evidence suggested that cell growth inhibition by PF-4 is mediated by blocking intracellular cell cycle events. For example, endothelial cell growth inhibition was independent of the nature of the growth stimuli used. Growth inhibition of a FGF-dependent endothelial cell line was not abrogated by excess FGF. Growth of vascular smooth muscle cells stimulated with FGF (growth factor used for endothelial cells) was not inhibited by PF-4. Finally, PF-4 was equally effective when added 8–10 h after FGF stimulation of endothelial cells.

A most striking action of PF-4 is its effect on cell cycle progression from G1 to S and on S-phase cells. By [^{3}H]-thymidine pulse-labeling studies in G0/G1-synchronized confluent cultures, we have demonstrated that PF-4 blocked entry of cells into S phase when added during the traverse of endothelial cells from G1 to S. In addition, PF-4 reduced DNA synthesis in cells that were already in S phase. Inhibition of endothelial cells in S phase was further confirmed by flow cytometry. In exponentially growing cultures of endothelial cells, treatment with PF-4 resulted in accumulation of >70% cells in early S phase. Further direct confirmation of the inhibition of S phase progression by PF-4 was obtained from cells synchronized in S phase by hydroxyurea treatment. Incubation of endothelial cells with hydroxyurea produced inhibition of DNA synthesis, as well as accumulation of cells in S phase. Removal of hydroxyurea from S phase–arrested cells by washing resulted in an immediate resumption of DNA synthesis. Treatment with PF-4 after hydroxyurea removal blocked resumption of DNA synthesis. Furthermore, PF-4 also inhibited DNA synthesis in cells progressing in S phase after release from hydroxyurea arrest.

Heparin has been shown to enhance endothelial cell proliferation (25), whereas it inhibits vascular smooth muscle cell proliferation (4). The COOH-terminal α helix region of PF-4 contains a lysine-rich domain that has been implicated in heparin binding (14). An analogue of recombinant PF-4 lacking a lysine residue and the heparin-binding activity was found equally effective as an inhibitor of endothelial cell proliferation (17), suggesting that heparin binding is not essential for PF-4 activity on endothelial cells. Our results show that addition of heparin to the culture medium antagonizes the effect of PF-4 on cell proliferation, as well as DNA synthesis in S phase cells.

The effect of PF-4 on S phase progression is unique as compared to the mode of action of the previously known cytokines inhibiting endothelial cell proliferation. TNF-mediated cell growth inhibition occurs in the G2 phase of the cell growth cycle (6). TGF-β, another potent inhibitor of endothelial cell proliferation in vitro, blocks cells in late G1 phase (12, 29). Also, unlike PF-4, the activity of TNF and TGF-β is not restricted to endothelial cells. Both of these cytokines inhibit fibroblasts, smooth muscle cells, and a variety of tumor cells. Furthermore, in contrast to PF-4, TNF and TGF-β promote angiogenesis in vivo (9, 26). The site of cell cycle block by another endothelial cell growth inhibitor protein known as SPARC is also in G1 phase (11). The characterization of the biological functions and mode of action of the chemokine family of peptides are currently under intense investigation. Furthermore, since the cell growth–modulating activity of the chemokines such as PF-4, IL-8, and MIP-α is only a recent finding, it remains to be seen whether S phase inhibition is specific for PF-4 or it represents a unique mode of action of the chemokine supergene family. In this regard, a recent observation suggesting an effect of MIP-α on S phase progenitor cells is very intriguing (18). The mode of PF-4–mediated cells growth inhibition

at G1/S transition and in S phase is also intriguing with respect to the potential molecular targets affected by PF-4. It was recently shown that cyclin E (7, 23) and cyclin-dependent kinase cdc-2 (5, 8, 19) play important role in progression of cells from G1 to S, as well as in S phase. Further studies will examine the molecular mechanism of PF-4 action, including its effects on cyclin/cyclin kinases regulating the G1 and S phases of endothelial cells.

Formation of new blood vessels is a highly controlled process. Under physiological conditions, new blood vessels are formed during embryonic development, leutinization, or wound healing. However, neovascularization is widely associated with a variety of pathologies, including neoplasia, atherosclerosis, diabetic retinopathy, psoriasis, rheumatoid arthritis, inflammation, and accelerated vascular disease after angioplasty or coronary artery bypass graft. A very important aspect of PF-4 activity is the inhibition of endothelial cell proliferation in vitro, as well as angiogenesis in vivo (16, 17). This unique activity of PF-4 has shown promise for the treatment of angiogenic diseases. In preclinical mice models, injections of recombinant PF-4 to primary cutaneous implants of B-16(F10) melanoma and HTC-116 colon carcinoma have shown dramatic reduction in tumor size that was accompanied by significant increase in survival rate (16, 17). The unique effect of PF-4 on cell cycle progression and further understanding of the mechanism of action on endothelial cells could ultimately lead to the development of novel targets for antiangiogenic therapies in the future.

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