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HUMAN PLATELET FACTOR 4 IS A DIRECT INHIBITOR OF HUMAN OSTEOBLAST-LIKE OSTEOSARCOMA CELL GROWTH

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Summary The effect of purified human platelet factor 4, a platelet α-granule protein, on the growth of the human osteoblastic osteosarcoma cell lines Saos-2 and G-292 was investigated. Platelet factor 4 (20 ng/ml to 2 μg/ml) caused a significant, dose-dependent inhibition of human osteoblast-like osteosarcoma cell proliferation. Platelet factor 4 exerted its inhibitory effect under all growth conditions tested: serum-free, serum-stimulated and thrombin-stimulated. The platelet factor 4-induced cell inhibition was not associated with a cytotoxic effect on the cells (assessed by lactate dehydrogenase release). The inhibitory effect of platelet factor 4 was not affected by the presence of indomethacin in the cultures, indicating that the effect was prostaglandin-independent. These results suggest that platelet factor 4 has direct antitumor effects and that it may be important in pathological and physiological processes of bone.

Human platelet factor 4 (PF4) is a 70 amino acid, 7.8 kDa basic protein found in megakaryocytes and platelet α-granules (1, 2). Stimulation of platelets results in the release of PF4 and other proteins stored in the α-granules, such as thrombospondin, platelet-derived growth factor, and β-thromboglobulin. PF4 was initially characterized as a heparin-binding protein able to neutralize the anticoagulant activity of heparin (3), and was subsequently found to be an inhibitor of human skin and granulocyte collagenase (4), a stimulator of granulocyte elastase (5), and a chemotactic factor for neutrophils (6), monocytes (6) and fibroblasts (7). Recent studies have shown that PF4 reverses concanavalin A-induced (8, 9) and antigen-induced (9, 10) immunosuppression. PF4 has also been found to inhibit megakaryocytopoiesis (11), angiogenesis (12), and the growth of human endothelial cells (12-14). Although PF4 inhibited the *in vivo* growth of melanoma and colon carcinoma, it did not affect the *in vitro* proliferation of such tumors (13, 14), suggesting an indirect pathway of PF4 action. Nevertheless, recent preliminary evidence suggests that PF4 may have *in vitro* growth inhibitory effects on Kaposi sarcoma cells (1, 15), a tumor of endothelial origin. The action of PF4 against endothelial and Kaposi sarcoma cells suggests that any direct antitumor effect of PF4 may be limited by tissue specificity.

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DNA, deoxyribonucleic acid; FCS, fetal calf serum; LDH, lactate dehydrogenase; PF4, platelet factor 4; PG, prostaglandin; SA, specific activity; TCA, trichloroacetic acid; U, NIH equivalent clotting units.

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PF4 inhibits *in vitro* bone resorption (16), a result suggesting that PF4 may have inhibitory effects on bone cells. This effect of PF4 on bone, along with the reported effects of PF4 on tumor growth, prompted the present study where two human osteoblast-like osteosarcoma cell lines were used to investigate the possible effects of PF4 on the *in vitro* growth of osteosarcoma cells.

Materials and Methods

Chemicals. Tissue culture media and media supplements were obtained from GIBCO (Grand Island, NY). Tissue culture disposable dishes and flasks were from Corning (Corning, NY). Highly purified human platelet factor 4 was from Calbiochem (San Diego, CA). Indomethacin and highly purified human plasma thrombin (SA: ~4,000.0 U/mg protein) were from Sigma Chemical Co. (St. Louis, MO). Alpha thrombin (17) was the kind gift of Dr. John W. Fenton, II (Albany Medical College, Albany, NY). ^{3}H-thymidine (SA: 7 Ci/mmol) was from ICN (Irvine, CA).

Cells and cell culture. The two human osteoblastic osteosarcoma cell lines Saos-2 (ATCC #HTB 85) and G-292 (ATCC #CRL 1423) were used. These cells have a well characterized osteoblast-like phenotype (18, 19). The cells were maintained under standard conditions. Cells were cultured in McCoy's 5a medium supplemented with 10% (G-292) or 15% (Saos-2) FCS, in a humidified, 5% CO_2, 37^{o}C incubator.

Cell proliferation. Cell proliferation was monitored by ^{3}H-thymidine incorporation. The method is a slight modification of the procedure described previously in detail (20). Briefly, cells were seeded in 24-well flat bottom polystyrene dishes ($0.9x10^6$ cells/ml; 0.5 ml of cell suspension/well). After a 24 h incubation period in McCoy's 5a medium supplemented with 10% FCS, the cells were washed twice with McCoy's supplemented with 1 mg/ml BSA. The cells were then cultured in McCoy's with BSA for 24 h. At the end of this 24 h period, the medium was removed and either McCoy's with BSA or McCoy's with BSA and thrombin or McCoy's with FCS was placed in the wells (0.5 ml/well). PF4 was then added to the wells (in all experiments reported herein PF4 was always added last, and at least 5 min after the addition of FCS or thrombin). The cells were then incubated for another 24-48 h. During the last three hours of this incubation period ^{3}H-thymidine (1 μCi/ml) was added to the medium. After the labeling period the cells were washed once with McCoy's 5a medium and then extracted with TCA. The acid-precipitable material was dissolved in KOH. The solution, after neutralization with HCl, was counted in Ecoscint A (National Diagnostics; Manville, NJ) scintillation fluid.

Cytotoxicity assay. Cytotoxicity (LDH release) assay kits, based on a published procedure (21), were obtained from Oxford Biomedical Research (Oxford, MI). For cytotoxicity experiments cells were seeded in 96-well flat bottom polystyrene dishes ($0.25x10^6$ cells/ml; 0.2 ml of cell suspension/well). After a 24 h growth period in McCoy's 5a medium supplemented with 10% FCS, the cells were washed twice with McCoy's supplemented with 1 mg/ml BSA. The cells were then cultured in McCoy's with BSA for 24 h. At the end of which the medium was removed and McCoy's with 2% FCS was placed in the wells (0.2 ml/well) with or without PF4 (six replicates/treatment group). The cells were then incubated for another 24 h. During the last three hours of this 24 h incubation period ^{3}H-thymidine (1 μCi/ml) was added to half the wells of each treatment group. The remaining three wells received lysing agent, in order to determine maximum LDH release values for each treatment group. After the 3 h period, a 100 μl aliquot was removed from each of the 6 wells and used in the LDH assay according to the manufacturer's instructions. The wells that had received ^{3}H-thymidine were then treated as above for proliferation.

Statistical Analysis. Data were analyzed using factorial ANOVA, and paired and unpaired Student's t test.

Results

PF4 caused a significant, dose-dependent decrease in DNA synthesis, as indicated by the decrease in ^{3}H-thymidine incorporation (Fig. 1, 2). Both G-292 (Fig. 1) and Saos-2 (Fig. 2) cells responded to PF4 in a similar manner. The PF4-induced inhibition of human osteoblast-like osteosarcoma cell proliferation was evident under all conditions tested. Cells cultured in the absence

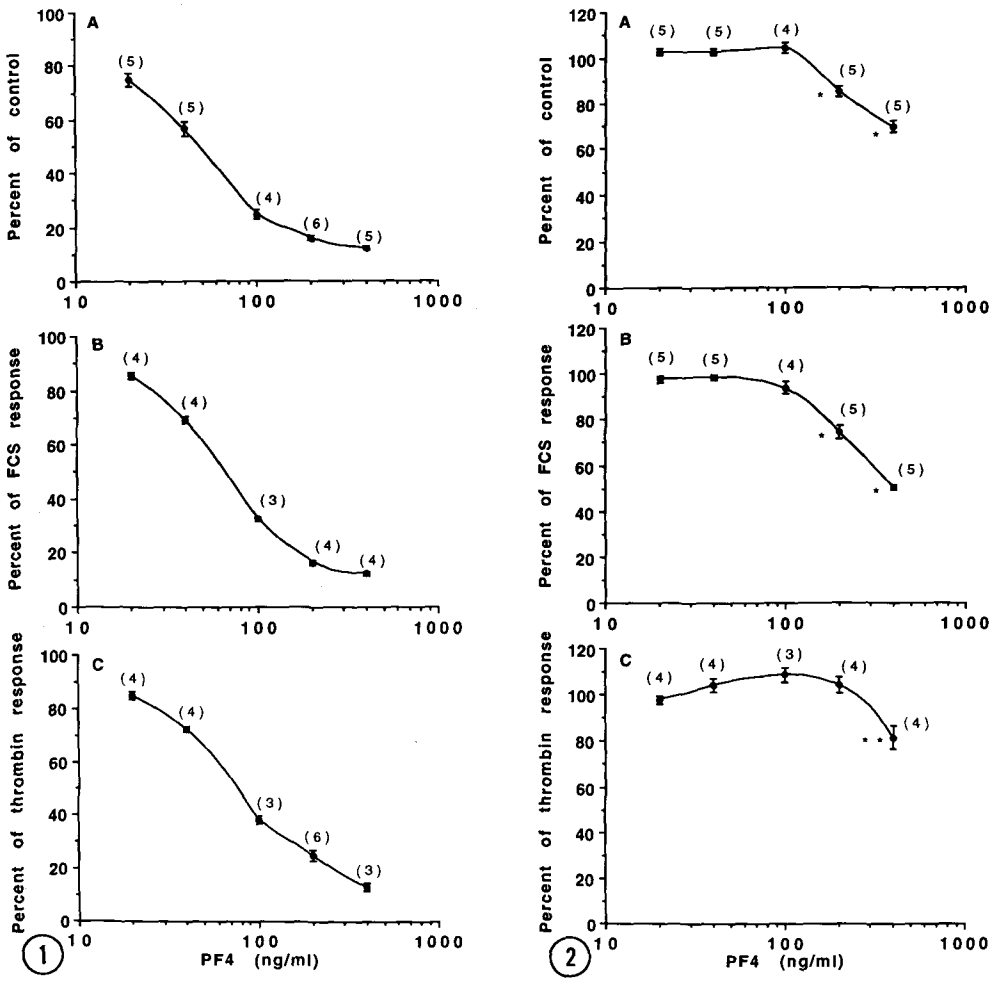

Fig. 1. PF4 inhibits proliferation of G-292 cells. A: Cells cultured in medium supplemented with 0.1% BSA. B: Cells cultured in medium supplemented with 2% FCS. C: Cells cultured in medium with 0.1% BSA and 0.5 U/ml human alpha thrombin. All PF4 doses were significantly different ($p<0.05$) from respective treatment without PF4. The average maximum response of the cells to 2% FCS and 0.5 U/ml thrombin was ~600% and ~750% of basal thymidine incorporation, respectively (see also Fig. 3B). Results are expressed as percentage of the maximum response under the culture condition tested and represent the mean ± S.E. of the mean. The number of experiments is given in parentheses above the specific points. In each experiment, points were tested in quadruplicates.

Fig. 2. PF4 inhibits proliferation of Saos-2 cells. A: Cells cultured in medium supplemented with 0.1% BSA. *$p<0.05$ from control. B: Cells cultured in medium supplemented with 2% FCS. *$p<0.05$ from FCS alone. C: Cells cultured in medium with 0.1% BSA and 1.0 U/ml human alpha thrombin. **$p<0.025$ from thrombin alone. The average maximum response of the cells to 2% FCS and 1.0 U/ml thrombin was ~1100% and ~370% of basal thymidine incorporation, respectively (see also Fig. 3A). Results are expressed as percentage of the maximum response under the culture condition tested and represent the mean ± S.E. of the mean. The number of experiments is given in parentheses above the specific points. In each experiment, points were tested in quadruplicates.

of growth factors (Fig. 1A, 2A), cells cultured in the presence of FCS (Fig. 1B, 2B) and cells stimulated by thrombin (Fig. 1C, 2C) were all inhibited by PF4 in a dose-dependent manner.

G-292 cells appeared to be much more susceptible to the action of PF4, compared to Saos-2 cells. A 100 ng/ml dose of PF4 caused a 75% inhibition of basal proliferation in G-292 cells (Fig.

Table 1
Effect of PF4 on osteosarcoma cell proliferation and viability

Treatment	^{3}H-thymidine incorporation (% of maximum)	Cell viability (% of maximum LDH release)
A. G-292 cells:		
2% FCS	100.0 ± 2.2	15.8 ± 3.4
FCS + PF4 2 μg/ml	8.3 ± 1.3[a]	17.7 ± 3.8
B. Saos-2 cells:		
2% FCS	100.0 ± 1.0	10.9 ± 0.6
FCS + PF4 2 μg/ml	68.4 ± 2.4[a]	8.8 ± 0.6

Results are the mean ± S.E. of the mean from two experiments, each performed in triplicates. [a] $p<0.001$ from FCS alone.

1A), while it had no effect on Saos-2 cells (Fig. 2A). Thrombin-stimulated cells (Fig. 1C, 2C) appeared to be the least affected by the inhibitory action of PF4, with the Saos-2 cells again being less susceptible. The degree of PF4-induced inhibition of FCS-stimulated cells (Fig. 1B, 2B) was close to the degree of inhibition under basal conditions (Fig. 1A, 2A).

The PF4-induced inhibition of osteosarcoma cell proliferation was not due to a cytotoxic effect of PF4 on these cells. PF4 doses that caused significant inhibition of DNA synthesis had no significant effect on LDH release by Saos-2 or G-292 cells (Table 1).

The inhibitory effect of PF4 on the human osteosarcoma cells was not affected by the presence of indomethacin (Fig. 3), suggesting that this effect is independent of any PF4-elicited prostaglandin synthesis.

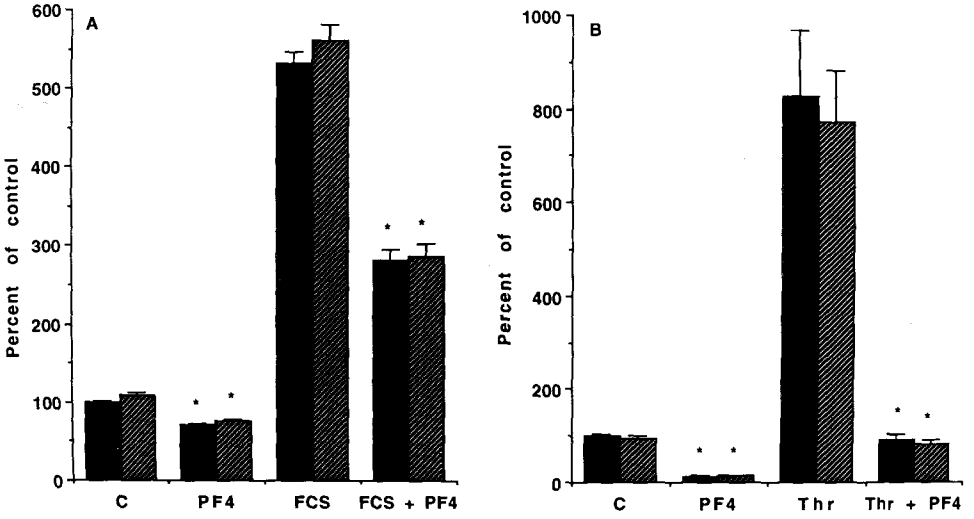

Fig. 3. Lack of indomethacin effect on PF4-induced inhibition of osteoblastic osteosarcoma cell proliferation. Cells were cultured in the presence of 1 μM indomethacin (hatched bars) or solvent control (ethanol, 0.1% final concentration; black bars). A: Saos-2 cells cultured in medium supplemented with 0.1% BSA (C) or 1% FCS (FCS), with or without 200 ng/ml PF4. B: G-292 cells cultured in medium supplemented with 0.1% BSA (C) or 0.5 U/ml human alpha thrombin (Thr), with or without 200 ng/ml PF4. *$p<0.01$ from respective treatment without PF4. Results are expressed as percentage of control (C) and represent the mean ± S.E. of the mean from two experiments. In each experiment, points were tested in quadruplicates.

Discussion

Sharpe *et al.* (13) and Maione *et al.* (14) have recently reported that PF4 inhibits the *in vivo* growth of melanoma and colon carcinoma tumors, without any direct *in vitro* effects on such tumor cells. These results, along with the reported inhibitory effect of PF4 on angiogenesis (12) and endothelial cell proliferation (13, 14), suggested that PF4 inhibits *in vivo* tumor growth by preventing tumor neovascularization. It has been reported in a recent abstract, however, that proliferation of cultured Kaposi sarcoma, a tumor of endothelial cell origin, is inhibited by PF4 (1, 15). The present study is the first full report that provides evidence for direct growth inhibitory effects of human PF4 on tumor cells, namely human osteoblastic osteosarcoma cells.

The present study also provides the first evidence for immediate effects of PF4, a platelet-specific secretory protein, on cells of bone origin. PF4 inhibited the growth of osteoblastic osteosarcoma cells under all culture conditions tested: serum-free, serum-stimulated and thrombin-stimulated. PF4 was effective against the human osteosarcoma cell line G-292 at doses between 20 ng/ml and 2 μg/ml, and against the Saos-2 cell line at doses over 100 ng/ml. These doses are lower than those required for stimulation of elastase activity (5), inhibition of collagenase (4), or inhibition of endothelial cell proliferation (12, 13), and comparable to the doses required for reversal of immunosuppression (8) and stimulation of monocyte (6) or fibroblast chemotaxis (7). The PF4 doses effective against the osteosarcoma cells fall in the high range of normal PF4 values in plasma (mean $\pm$ SD = 13.9 $\pm$ 6.1 ng/ml; [22]) and within the PF4 levels that are theoretically possible at sites of activation of the coagulation cascade and release of platelet contents (1, 23).

PF4 did not cause any cytotoxic effects on the osteosarcoma cells, as assessed by LDH release measurements, a finding that is in agreement with the reported lack of cytotoxic effects of PF4 on endothelial cells (12), and the lack of toxic effects when administered *in vivo* (14).

Thrombin is a potent stimulus of PF4 release from platelets (1, 2). Thrombin also stimulates osteosarcoma cell proliferation (20, 24, 25). Since human osteosarcoma cells secrete several proteins that activate the coagulation cascade (26-28) and PF4 levels at sites of activation of the coagulation cascade may reach the necessary doses (23), it is conceivable that inhibition of thrombin-stimulated osteosarcoma cells by PF4 could be a feedback mechanism likely to operate in *in vivo* conditions. It has been suggested that the procoagulant activity of osteosarcoma cells may be important for the pattern of osteosarcoma metastasis (29). Local PF4 release could be modulating this process.

The results presented here suggest that PF4 may have potent regulatory effects on the physiologic and pathologic metabolism of bone, in general, and osteoblastic cells, in particular. Consistent with this, Horton *et al.* (16) reported in 1980 that PF4 inhibits parathyroid hormone-stimulated bone resorption. Furthermore, PF4 was capable of inhibiting the minimal resorption that takes place in such bone organ culture systems in the absence of any hormonal stimulation (16). In this context, it should be noted that studies have shown that cytokine- and hormone-stimulated resorption requires osteoblastic cells as mediators of the resorptive signal, even though osteoclasts are the terminal effector cells (30). This, along with the results of the present study, suggests that the PF4-induced inhibition of resorption (16) may be the result of PF4 effects on osteoblastic cells.

The mechanism of PF4 action on osteosarcoma cells remains to be established. PF4 inhibited osteosarcoma cells grown either in the absence of any stimulus or in the presence of two differently

acting stimulants, FCS and thrombin, suggesting that PF4 has direct effects on intrinsic mechanisms regulating the proliferation of osteosarcoma cells. The most likely mechanism of PF4 action would be the binding to a specific membrane receptor. Scatchard analysis has shown that platelets have a single class of high affinity PF4 binding sites (31).

PF4 inhibited FCS- and thrombin- stimulated cells even though it was added five minutes after the addition of these agonists to the cells, suggesting that PF4 operates at a postreceptor stage of the stimulation process. The time element obtains particular significance when one considers that the thrombin-elicited intracellular signals occur within the first minute after addition of the enzyme to these cells (20, 24). PF4 inhibited thrombin-stimulated cells to a similar extent when it was added to the cells prior to the addition of thrombin (data not shown). Since the catalytic site of thrombin is required for osteoblastic cell response (24) and PF4 can inhibit collagenase (4), one could have considered that, under such experimental conditions, the PF4 effect was due to enzyme inhibition. Yet, PF4 does not alter the thrombin time of plasma (32) or the activity of thrombin against synthetic substrates (33). Furthermore, PF4 potentiates platelet aggregation and secretion induced by low doses of thrombin (31). These observations are consistent with tissue specific activities of PF4.

Prostaglandins (PGs) have direct effects on osteosarcoma cell proliferation (34) and cytokines modulate osteoblastic cell growth through stimulation of endogenous PG production (35). Indomethacin, a prostaglandin synthesis inhibitor, significantly inhibits the PF4-induced reversal of immunosuppression (1, 8), suggesting that this immunoregulatory activity of PF4 requires PG production. In the present study, however, indomethacin failed to alter the PF4-induced inhibition of osteoblast-like osteosarcoma cell growth. This suggests that the PF4 effect on osteosarcoma cells is PG-independent and that PF4 may act through different mechanisms in different systems.

The direct effect of PF4 against the osteosarcoma cells, the lack of PF4 toxic effects when given *in vivo*, and the immunoregulatory activities of PF4, all suggest that it could be worthwhile to explore the possible antitumor effects of PF4 against osteosarcoma *in vivo*.

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