RAPID COMMUNICATION Jpn. J. Cancer Res. 84, 485–488, May 1993

Carboxyl-terminal Heparin-binding Fragments of Platelet Factor 4 Retain the Blocking Effect on the Receptor Binding of Basic Fibroblast Growth Factor

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Platelet factor 4 (PF-4) blocks the binding of basic fibroblast growth factor (bFGF) to its receptor. In the present study, we constructed carboxyl-terminal fragments, which represent the heparin-binding region of the PF-4 molecule, and examined whether these synthetic peptides retain the blocking effects on the receptor binding of bFGF. Synthetic peptides inhibited the receptor binding of bFGF. Furthermore, they inhibited the migration and tube formation of bovine capillary endothelial cells in culture (these phenomena are dependent on endogenous bFGF).

Key words: Platelet factor 4 — Heparin-binding domain — Basic fibroblast growth factor — Receptor binding

Platelet factor 4 (PF-4), a platelet α-granule protein, is a molecule that has a high affinity for heparin. PF-4 is reported to inhibit bone resorption,[1] megakaryocytopoiesis[2] and angiogenesis.[3] We previously reported that PF-4 blocks the binding of basic fibroblast growth factor (bFGF) to the receptor, and thereby inhibits migration, plasminogen activator (PA) synthesis[4] and tube formation of vascular endothelial cells,[5] which are all phenomena regulated by endogenous bFGF and required for angiogenesis.[5,6] However, the element of PF-4 responsible for blocking the receptor binding of bFGF was ill-defined. In the present study, we constructed carboxyl-terminal heparin-binding fragments of the PF-4 molecule and examined whether these synthetic peptides retained the blocking effect on the receptor binding of bFGF. We found that heparin-binding fragments blocked the binding of bFGF to the receptor.

Carboxyl-terminal fragments of 10, 11, 12, and 13 residues (C-10, K-K-I-I-K-K-L-L-E-S; C-11, Y-K-K-I-I-K-K-L-L-E-S; C-12, L-Y-K-K-I-I-K-K-L-L-E-S; C-13, P-L-Y-K-K-I-I-K-K-L-L-E-S) of PF-4 were synthesized manually by the solid-phase method based on 9-fluorenylmethyloxycarbonyl chemistry[7] using the benzotriazolyloxytris(dimethylamino)phosphonium hexafluorophosphate-1-hydroxybenzotriazole coupling precedure. After cleavage of the protecting groups and solid support with trifluoroacetic acid-phenol (95:5, v/w), the peptides were purified by gel filtration on Sephadex G-25. The homogeneity of the peptides was ascertained by high-performance thin-layer chromatography, analytical reverse-phase high-performance liquid chromatography, high-performance capillary electrophoresis, and amino acid analysis of the products after acid hydrolysis. Details of the synthesis have been described elsewhere.[8] The binding of ^{125}I-bFGF (Amersham, Buckinghamshire, England) to NIH 3T3 cells was examined as described previously.[4] Briefly, NIH 3T3 cells in 12-well plates were incubated with various concentrations of synthetic peptides or native PF-4 (Sigma, St. Louis, MO, USA) and 2 ng/ml of ^{125}I-bFGF for 3 h at 4°C in media containing 0.1% bovine serum albumin (BSA). After the incubation, cells were treated with 2 *M* sodium chloride containing 20 m*M* Hepes (pH 7.5) to recover the heparan sulfate bound fraction, and then treated with 2 *M* sodium chloride containing 20 m*M* sodium acetate (pH 4.0) to recover the receptor bound fraction. The migration of bovine capillary endothelial (BCE) cells isolated from adrenal glands was examined as previously described.[6] Briefly, confluent monolayers of BCE cells in 35 mm dishes were injured with a razor blade and incubated for 24 h with various concentrations of synthetic peptides in the media containing 0.1% BSA. Cells that migrated into the denuded area were counted. Tube formation of BCE cells in type 1 collagen gel (Nitta Gelatin, Osaka) was quantitated by the method described previously.[6] Briefly, BCE cells were plated between two layers of type 1 collagen gel, and incubated with various concentrations of synthetic peptides for 24 h at 37°C. After the incubation, network structures of BCE cells formed in the gel were measured by using a Cosmozone 1S Image Analyzer (Nikon, Tokyo).

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The binding of ^{125}I-bFGF to NIH 3T3 cells was determined in the presence of various concentrations of carboxyl-terminal fragments of PF-4. These synthetic peptides had no inhibitory effects on the binding of ^{125}I-bFGF to cell surface heparan sulfate within the concentration range tested, whereas they blocked the binding of ^{125}I-bFGF to the plasma membrane receptor in a dose-dependent manner (Fig. 1A and B). There were no significant differences in the blocking activities of ^{125}I-bFGF receptor binding between C-10, C-11, C-12 and C-13. The blocking activity of synthetic peptide (C-12) for ^{125}I-bFGF receptor binding was compared with that of unlabeled bFGF and native PF-4 (Fig. 2). The findings demonstrate that carboxyl-terminal heparin-binding fragments retain at least a part of the blocking effect of native PF-4 on the receptor binding of bFGF. However, native PF-4 and its carboxyl-terminal fragment (C-12) did not block the binding of ^{125}I-EGF, another growth factor, to its receptor (data not shown). We have previously reported that endogenous bFGF regulates migration, PA synthesis and tube formation of endothelial cells as an autocrine factor,[5,6] and PF-4 abrogates the effect of endogenous bFGF.[4,5] Therefore, we examined whether synthetic peptides affect those biological properties of endothelial cells. As shown in Fig. 3, C-10, C-11, C-12 and C-13 almost equally inhibited migration of BCE cells in a dose-dependent manner. The effect of synthetic peptides on tube formation of BCE cells in type 1 collagen gel was further examined. As shown in Fig. 4, at a concentration of 30 μM, the synthetic peptides aborted the tube formation of BCE cells in the gel. These findings correlate very well with our previous observations and indicate that carboxyl-terminal heparin-binding fragments of PF-4 may inhibit migration and tube formation of BCE cells by blocking endogenous bFGF binding to its receptor.

bFGF is a potent mitogen for a wide variety of cell types including vascular endothelial cells, and is known as an angiogenic growth factor.[9] Although, bFGF ex-

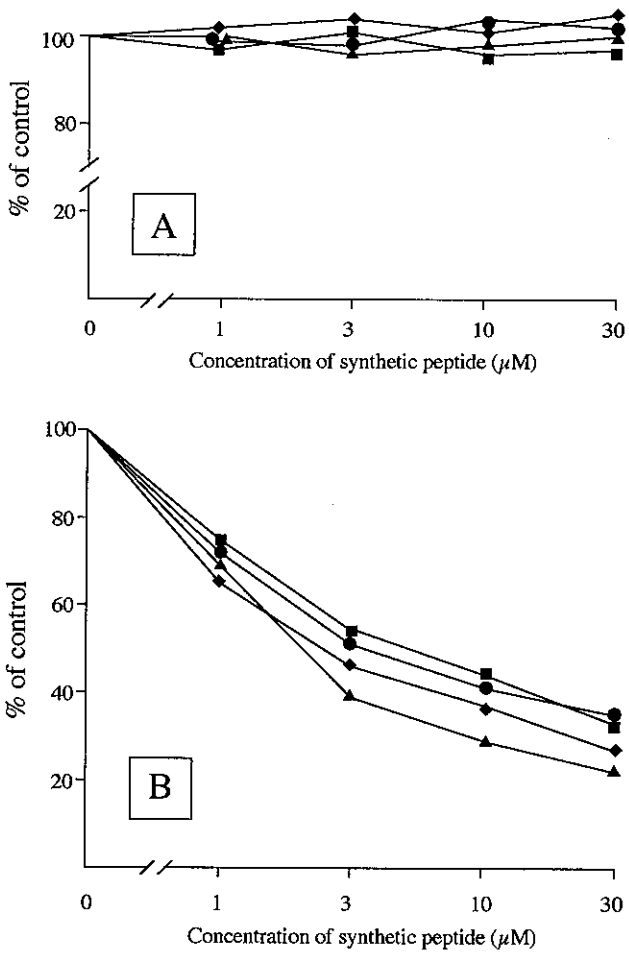

Fig. 1. Effects of synthetic peptides on the binding of ^{125}I-bFGF. (A) Heparan sulfate bound fraction: A monolayer of NIH 3T3 cells in a 12-well plate was incubated with 2 ng/ml of ^{125}I-bFGF and various concentrations of synthetic peptides (●: C-10, ■: C-11, ▲: C-12, ◆: C-13) as described. After the incubation, the monolayer was treated with 2 *M* sodium chloride containing 20 m*M* Hepes (pH 7.5) and the recovered radioactivity was determined. (B) Receptor bound fraction: The monolayer was subsequently treated with 2 *M* sodium chloride containing 20 m*M* sodium acetate (pH 4.0) and the recovered radioactivity was counted. Values are means of triplicate samples, expressed as % of control.

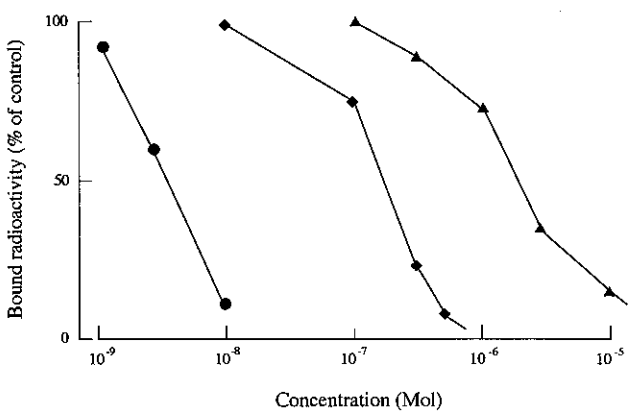

Fig. 2. Comparison between the effects of unlabeled bFGF, native PF-4 and synthetic peptide (C-12) on the binding of ^{125}I-bFGF to the receptor. A monolayer of NIH 3T3 cells in a 12-well plate was incubated with 2 ng/ml of ^{125}I-bFGF and various concentrations of unlabeled bFGF (●), native PF-4 (◆) and C-12 (▲). Receptor bound fraction was determined as described. Values are means of triplicate samples, expressed as % of control.

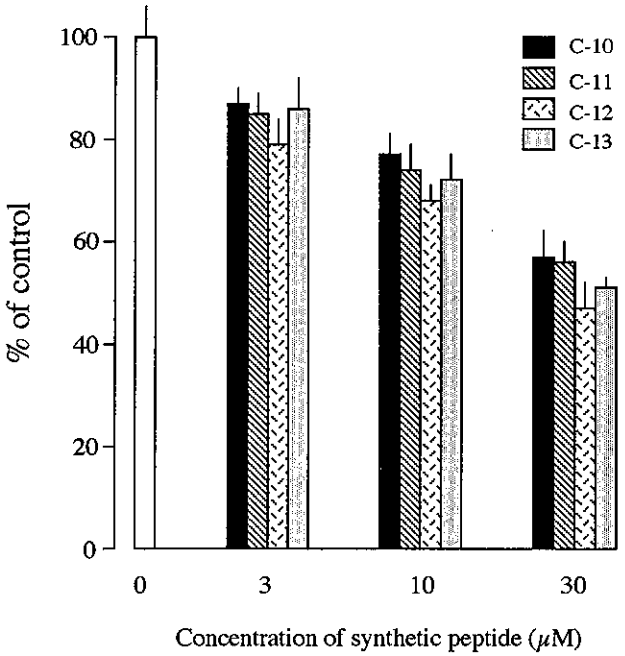

Fig. 3. Effects of synthetic peptides on the spontaneous migration of BCE cells. A monolayer of BCE cells was injured with a razor blade and incubated with various concentrations of synthetic peptides for 24 h. After the incubation, cells that had migrated from the edge of the wound were determined. Four random fields (×100 magnification) were counted. Values are expressed as mean and SD.

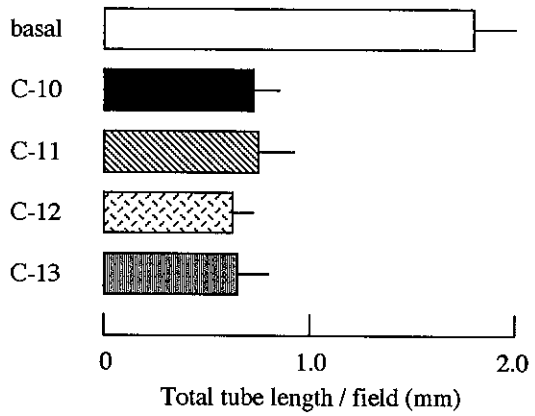

Fig. 4. Effects of synthetic peptides on tube formation of BCE cells. BCE cells (1×10^5) were plated onto type 1 collagen gel in a 35 mm dish, covered with another layer of the collagen gel, and incubated with 30 μM synthetic peptide for 24 h. After the incubation, the length of tube-like structures was determined. Eight random fields (×200 magnification) were measured. Values are expressed as mean and SD.

presses its effect through binding to the plasma membrane receptor, bFGF has a high affinity for heparin and binds to heparan sulfate glycosaminoglycan as well.[10] Moreover, cell surface heparan sulfate is indispensable for the binding of bFGF to the receptor[11] and for expression of its biological effects.[12] Therefore, we speculate that the heparin-binding domain of PF-4 binds to cell surface heparan sulfate, which is responsible for the receptor binding of bFGF, and blocks the binding of bFGF to the receptor. Whitson *et al.* have recently shown that PF-4 inhibits the binding of transforming growth factor (TGF)-β1 to the type 1 TGF-β receptor.[13] Since TGF-β1 was recently reported to have affinity for heparin,[14] the heparin-binding domain of PF-4 may retain the inhibitory activity of blocking the binding of TGF-β1 to the type 1 TGF-β receptor as well. In this context, it will be interesting to examine whether PF-4 blocks the binding of other members of the heparin-binding growth factor family.

This work was supported in part by a Grant-in-Aid (03670329) from the Japanese Ministry of Education, Science and Culture to Y.S. and by a grant from the Fukuoka Cancer Society to M.W.

(Received December 18, 1992/Accepted February 10, 1993)

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