Rapid Communication

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Human Stem Cell Factor (SCF) is a Heparin-Binding Cytokine

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Binding affinities of chemically modified heparins for human stem cell factor (SCF) were examined using fragmin/protamine microparticles (F/P MPs) and an enzyme-linked immunosorbent assay (ELISA). The binding of SCF to F/P MP-coated plates was inhibited with high concentrations of heparin and fragmin, but not others. The binding of SCF was also inhibited with 0.55 M or higher concentrations of NaCl in the medium. These results suggested that a high content of all three sulfate groups in repeating disaccharide units is required for interaction with SCF. Furthermore, pre-immobilized SCF on F/P MP-coated plates significantly stimulated proliferation of a human erythroleukemia cell line.

Key words: enzyme-linked immunosorbent assay (ELISA), fragmin/protamine microparticles (F/P MPs), heparin-binding cytokine, heparinoids, human stem cell factor (SCF).

Abbreviations: SCF, human stem cell factor; F/P MPs, fragmin (low-molecular-weight heparin)/protamine microparticles; ELISA, enzyme-linked immunosorbent assay; TF-1 cells, human erythroleukemia cell line; N-DS/N-Ac-H, N-desulfated, N-acetylated heparin; 2-O-DS-H, 2-O-desulfated heparin; 6-O-DS-H, 6-O-desulfated heparin.

Hematopoietic stem cells proliferate and mature in a niche consisting of semi-solid media when stimulated by exogenous hematopoietic cell growth factors (HCGFs) such as stem cell factor (SCF), interleukin (IL)-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF), etc. In particular, SCF plays a key role in the regulation of hematopoiesis, acting as a regulator at various stages of this process. SCF promotes proliferation and early differentiation of cells at the level of multipotential stem cells. It has been suggested that SCF is essential for optimal production of various hematopoietic lineages, mainly because of its ability to prevent apoptosis when it co-stimulates with other cytokines (*1*). Hematopoietic stem cells also proliferate in association with bone marrow-derived stromal cells (*2*, *3*). In fact, it has been demonstrated that many natural and recombinant HCGFs such as IL-3, GM-CSF, etc. can be absorbed by heparinoids (heparin, heparan sulfate, modified heparin and other heparin-like molecules), which constitute the major sulfated glycosaminoglycans (GAGs) of bone marrow stroma (*2*, *3*). However, the heparin-binding character of SCF has not yet been elucidated.

We have prepared fragmin/protamine microparticles (F/P MPs) (about 1–0.5 μm in diameter) by mixing fragmin (low-molecular-weight heparin) as a heparinoid and protamine (*4*). Briefly, 0.3 ml of protamine solution (10 mg/ml; Mochida Pharmaceutical Co., Tokyo, Japan) was added drop by drop to 0.7 ml of fragmin solution (6.4 mg/ml; Kissei Pharmaceutical Co., Tokyo, Japan) with vortexing for 1 min. In order to maximize the production of microparticles, protamine and fragmin were mixed in a ratio of 3:7 (vol:vol) in this study. The F/P MPs, which constituted a milky solution, were then washed twice with phosphate-buffered saline (PBS) to remove non-reactants using centrifugation, and finally resuspended in 1 ml PBS. More than 7 mg of dry F/P MPs were obtained from 1 ml of F/P MPs milky solution. Although F/P MPs loosely bound to a plastic surface, the particles were easily rinsed away by washing. When the 24-well plate-bound F/P MPs were air-dried for 1 h on a clean bench, they were stably coated on the plastic surface.

IL-3 and GM-CSF are known to specifically bind to heparinoids (*5*), but it was previously unknown if SCF can bind to heparin. An enzyme-linked immunosorbent assay (ELISA) using cytokines (SCF, IL-3, and GM-CSF (R&D Systems Inc. Minneapolis, MN, USA)) and F/P MP-coated plates was performed to evaluate the adsorption of cytokines to the plates. Dulbecco's Modified Eagle's Medium (200 μl) (DMEM; Life Technologies Oriental, Tokyo, Japan) with indicated concentrations of those cytokines and 2% fetal bovine serum (FBS) was added to F/P MP-coated 48-well tissue culture plates (0.65 cm^2 of surface area) (Sumitomo Bakelite Corp., Tokyo, Japan), and the cytokines were bound to the plates at 37°C for 2 h. The plates were then washed

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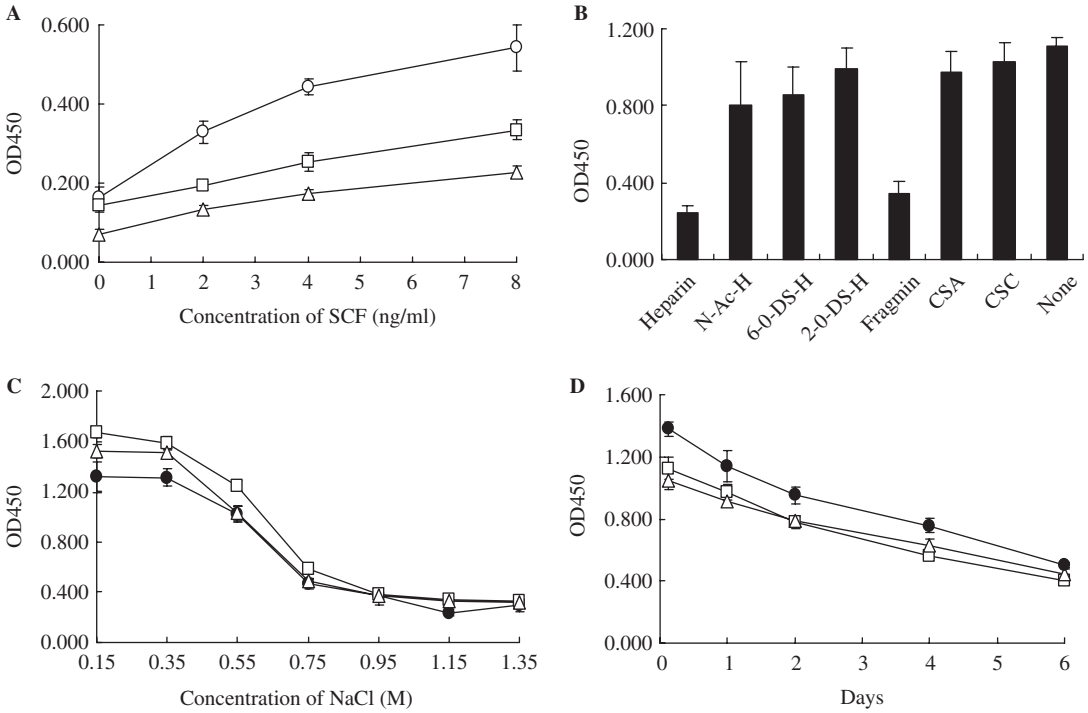

Fig. 1. **(A) Low FBS (2%) DMEM containing indicated concentrations of SCF was added to first F/P MP-coated plates (open circle) or non-coated plates (open triangle) and incubated at 37°C for 2 h.** The used medium was then added to second F/P MP-coated plates and incubated at 37°C for another 2 h (open square). (B) Various heparinoids and chondroitin sulfates (CSA and CSC) (200 μg/ml) were tested for their ability to competitively inhibit the binding of SCF to F/P MP-coated plates. C: Inhibitory effects of various concentrations of NaCl on the binding of SCF (filled circle), IL-3 (open square) and GM-CSF (open traingle) to F/P MP-coated plates. D: Release profile of SCF (filled circle), IL-3 (open square) and GM-CSF (open triangle) from pre-cytokine-immobilized F/P MP-coated plates. Amounts of immobilized cytokines were evaluated using an ELISA as described in text. Data represent mean ± SD of six determinations.

thoroughly with PBS/bovine serum albumin (0.1%) (BSA; Wako Pure Chemical Industries, Ltd., Osaka, Japan) three times. Diluted antibody (1:500 with PBS/BSA), anti-IL-3, anti-GM-CSF or anti-SCF (R&D Systems Inc.) was added to the plates, which were then incubated for 30 min at room temperature. Each well was again washed thoroughly with PBS/BSA and 200 μg/ml of anti-IgG horseradish peroxidase conjugate (diluted 1:1000 with PBS/BSA) (Bio-Rad Lab., Hercules, CA, USA) was added to the plates and incubated for 30 min at room temperature. Each well was again washed thoroughly with PBS/BSA, and the color was developed by adding 300 μl/ml of horseradish peroxidase substrate solution (Bio-Rad Lab.). The plates were mixed for 30 min and 50 μl of sulfuric acid (1 M) was finally added to each well to stop the reaction. The plates were read at 450 nm using a Mini Plate Reader (Nunc InterMed, Tokyo, Japan).

When 200 μl of various concentrations of SCF in DMEM with 2% FBS was added to F/P MP-coated 48-well tissue culture plates and incubated at 37°C for 2 h, SCF was detected on the plates in concentration-dependent manner by ELISA (Fig. 1A). Subsequently, the used culture medium was transferred into other F/P MP-coated plates and incubated once again at 37°C for 2 h. When the immobilized SCF in F/P MP-coated plates were measured by ELISA, the SCF levels of latter plates decreased to about 60% of the former plates. These results indicated that roughly 0.34 ng of SCF was immobilized onto the F/P MP-coated plates after incubating with 200 μl of 5 ng/ml SCF in DMEM with 2% FBS at 37°C for 2 h (Fig. 1A).

In order to measure the binding properties of SCF to heparinoid, various chemically modified heparins were evaluated for their ability to interact with SCF using ELISA and F/P MP-coated plates. Heparin and various chemically modified heparins which specifically bind to SCF should competitively inhibit the binding of SCF to the plates.

N-desulfated heparin was prepared according to the selective solvolytic method of Inoue and Nagasawa (*6*). The product was then converted to N-desulfated, N-acetylated heparin (N-DS/N-Ac-H) by N-acetylation procedures. Nitrous acid treatments at both pH 1.5 and pH 4 (*7*) did not cleave the product at all, indicating that N-desulfation and N-acetylation in those procedures were complete.

The 2-O-desulfated heparin (2-O-DS-H) (*8*) and 6-O-desulfated heparin (6-O-DS-H) (*9*) were prepared by methods reported previously. These procedures resulted in approximately 70% removal of 2-O-sulfates in 2-O-DS-H, 75% removal of 6-O-sulfates in 6-O-DS-H, and 95.4% removal of N-sulfates in N-DS/N-Ac-H (Table 1). Disaccharide compositional analyses of the chemically modified heparins were performed as described previously (*10*, *11*). Briefly, the polysaccharides (0.1 mg)

Table 1. **Disaccharide compositions of chemically modified heparins**

	Heparin (%)	Fragmin (%)	2-O-DS-H (%)	6-O-DS-H (%)	N-DS/N-Ac-H (%)
UA-GlcNAc	4.3	8.2	6.2	6.2	8.2
UA-GlcNS	3.8	7.2	20.5	5.3	0
UA-GlcAc(6-O-S)	0.7	0.5	2.9	0.3	8.7
UA(2-O-S)-GlcNAc	1.3	1.2	0	4.2	25.1
UA-GlcNS(6-O-S)	8.1	11.2	43.1	6.8	0.5
UA(2-O-S)-GlcNS	24.8	20.2	6.4	59.3	1.2
UA(2-O-S)- GlcNAc(6-O-S)	0	0	0	0.6	49.2
UA(2-O-S)- GlcNS(6-O-S)	52.6	47.2	14.4	12.7	2.9
Unknown	4.4	4.3	6.5	4.6	4.2

UA, uronate; GlcNAc, N-acetylglucosamine; GlcNS, N-sulfated glucosamine; UA(2-O-S), 2-O-sulfated uronate; GlcNAc(6-O-S), 6-O-sulfated N-acetylglucosamine; GlcNS(6-O-S), 6-O-sulfated N-sulfated glucosamine.

were treated with a mixture of heparinase (50 mU), heparitinase I (20 mU), and heparitinase II (20 mU) (Seikagaku Corp., Tokyo, Japan) in 220 μl of 2 mM calcium acetate and 20 mM sodium acetate (pH 7.0) at 37°C for 2 h. The completeness of the digestion was confirmed by gel-filtration chromatography with serially combined columns of TSK-Gel PW 4000, PW 3000 and PW 2500 (Tosoh Inc. Tokyo, Japan) using 0.2 M NaCl while monitoring absorbance at 230 nm and the refractive index for detection. The disaccharide composition was analyzed by ion-exchange chromatography of the reaction mixture with Dionex CarboPac PA-1 (4 × 250 nm). The gel-filtration chromatography showed no depolymerization for either 2-O-DS-H, 6-O-DS-H or N-DS/N-Ac-H.

As shown in Fig. 2B, heparin and fragmin at 200 μg/ml completely inhibited the binding of SCF to F/P MP-coated plates, while CSA, CSC, N-DS/N-Ac-H, 6-O-DS-H and 2-O-DS-H did not inhibit the binding even at concentrations of 400 μg/ml. We found that only a heparin–like molecule enriched in tri-sulfated disaccharide [UA(2-O-S)-GlcNS(6-O-S)] units (52.6% for heparin; 47.2% for fragmin) can competitively inhibit the binding of SCF to the F/P MP-coated plates; that is, only heparin-like molecules enriched in tri-sulfated disaccharide units (around 50%) can specifically interact with SCF. In contrast, chemically modified heparins enriched in di-sulfated disaccharide [UA-GlcNS(6-O-S), UA(2-O-S)-GlcNS and UA(2-O-S)-GlcNAc(6-O-S)] units have no inhibitory effect on binding. The inhibitory effects of heparin and fragmin for binding were concentration-dependent, and the half-inhibition concentrations were 25 and 35 μg/ml, respectively. These results suggest that SCF has affinity for heparin and fragmin, and that a high content of tri-sulfated (N-sulfate and 6-O-sulfate in GlcN residues and 2-O-sulfate in UA residues) disaccharide units is required for their interaction with SCF.

In the ELISA procedure, various concentrations of NaCl were tested for their ability to block the binding of the cytokines to F/P MP-coated plates. To inhibit the binding of SCF to the plates, 0.65 M NaCl was required. This requirement (0.65 M NaCl) is similar to that for GM-CSF (Fig. 1C). The binding of heparin-binding proteins to heparinoids can be blocked by high concentrations of NaCl, and the required concentration of NaCl is correlated with the affinity of the protein for heparinoid (*12*). To inhibit the binding of fibroblast growth factor (FGF)-1 and FGF-2 to F/P MP-coated plates, 1.05 M NaCl is required (*12*). On the other hand, the binding of hepatocyte growth factor (HGF) and IL-3 to the F/P MP-coated plates appears to be blocked with 0.75 M NaCl. Although the affinity of SCF for heparinoid is relatively low compared with other heparin-binding cytokines, the F/P MP-coated matrix efficiently adsorbed

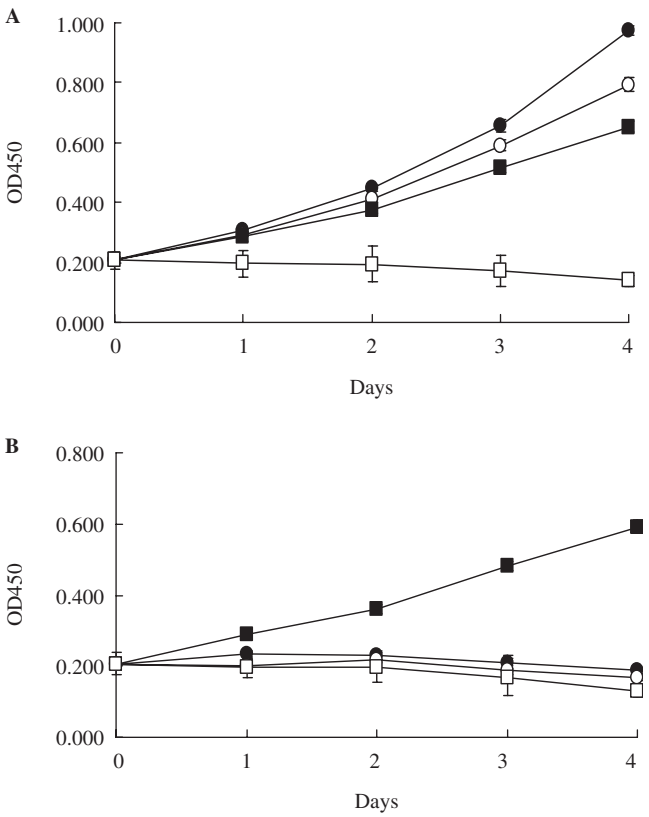

Fig. 2. **(A) TF-1 cells were cultured in low FBS (2%) DMEM with 5 ng/ml SCF either on F/P MP-coated plates (filled circle) or non-coated plates (open circle), and cultured in the same medium without SCF on pre-SCF-immobilized F/P MP-coated plate (filled square) or non-coated plates (open square).** (B) TF-1 cells were cultured in low FBS (2%) DMEM used to wash the pre-immobilized SCF for 2 days on F/P MP-coated plates (filled circle) or non-coated plates (open circle), and cultured in the same medium without SCF on the washed pre-SCF-immobilized F/P MP-coated plate (filled square) or non-coated plates (open square) for 2 days. Data represent mean ± SD of six determinations.

and stably retained SCF just as well as other heparin-binding cytokines.

It is recognized in polymer chemistry that positively and negatively charged polymers interact ionically. Basic protamine molecules complexed with acidic molecules (fragmin) form microparticles through ionic interactions (*4*). The F/P MPs are able to attach to polymeric surfaces such as plastic and glass, and they generate a stable paste-like coating upon complete drying as described in this study. It seems that SCF, once bound to F/P MP-coated plates, is gradually released from the coated surface *in vitro* with a half-life of 4–6 days (Fig. 1D). Furthermore, growth of TF-1 cells was stimulated both in low FBS (2%) DMEM (without SCF) on pre-SCF-immobilized F/P MP-coated plates and on non-coated plates using low FBS (2%) DMEM containing 5 ng/ml SCF (Fig. 2A). The medium to which the immobilized SCF was released for 2 days had little stimulatory effect on TF-1 cell growth (Fig. 2B). In contrast, TF-1 cell growth was significantly stimulated in low FBS (2%) DMEM (without SCF) on the pre-SCF-immobilized F/P MP-coated plates which were washed with low FBS (2%) DMEM (without SCF) for 2 days (Fig. 2B). Thus SCF immobilized onto F/P MP-coated plates appears to be bioactive for TF-1 growth. Our current study suggests that both the activity and stability of SCF are modified by the presence of heparin-like molecules which contain a high proportion of tri-sulfated disaccharide units. The molecular mechanism by which SCF is activated by interacting with F/P MP-coated plates is currently being investigated.

Heparinoids are known to bind various cytokines including FGFs, HGF, vascular endothelial growth factor, heparin-binding epidermal growth factor, platelet-derived growth factor, transforming growth factor-β, GM-CSF, interleukins (i.e. IL-1, IL-2, IL-3, IL-4, IL-6, IL-7 and IL-8), interferon γ and macrophage inflammatory protein-1, etc. (*13*, *14*). The present study suggests that SCF may be added to the list of heparin-binding cytokines.

CONFLICT OF INTEREST

None declared.

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