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Structural Characteristics of Heparin-like Domain Required for Interaction of Midkine with Embryonic Neurons

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Midkine is a heparin binding growth/differentiation factor with neurite promoting activity. We examined inhibitory activities of various heparin derivatives toward interaction of midkine with neurons and elucidated the structural requirements of the heparin-like domain necessary for the interaction. All of the three sulfate groups in the heparin disaccharide unit (6-O-sulfate, 2-O-sulfate and N-sulfate) were necessary for full inhibitory activity. Among these, the N-sulfate group was critically important. The minimum size with inhibitory activity was approximately 7,000 Da. Thus, the highly sulfated region in cell surface heparan sulfate proteoglycan is required for neurons to interact with midkine.

Heparan sulfate, a heparin-related carbohydrate present in cell surface and extracellular matrices as protein-bound forms, plays important roles in regulation of cellular activities such as growth, differentiation and development (1). A group of growth factors known as heparin binding growth factors such as fibroblast growth factors (FGFs), hepatocyte growth factor (HGF) and platelet-derived growth factor bind to cell surface heparan sulfate (2). Binding to heparan sulfate is thought to be important for storage, release and stabilization of the factors. Furthermore, the heparan sulfate chain was found to function as a co-receptor of FGF: prior to binding to the receptor, FGF must bind to heparan sulfate (3). This binding leads to the dimerization of FGF, which is required for receptor activation (4).

Midkine (MK) and pleiotrophin (PTN, also known as HB-GAM) constitute a new family of heparin binding growth/differentiation factors called the MK family (5–9). They are 45 % identical to each other in amino acid sequence, and are structurally unrelated to other growth factor families. MK promotes neurite outgrowth and survival of embryonic neurons (10, 11), and enhances plasminogen activator activity in aortic endothelial cells (12). PTN also promotes neurite outgrowth (13), transforms NIH3T3 cells (14) and is involved in postsynaptic specification (15). The heparin binding properties of these molecules appear to be important for their biological functions. Especially in the case of PTN, N-syndecan, a cell surface heparan sulfate proteoglycan, has been proposed to be a receptor in PTN-dependent neurite outgrowth of embryonic neurons (16).

The heparan sulfate carbohydrate chain has microheterogeneity, and the structure may vary depending upon the cell type expressing it (1). The backbone structure is composed of repeated unit of glucuronic acid and N-acetylglucosamine residues. A portion of the chain is converted to heparin-like structure by changing glucuronic acid to iduronic acid, 2-O-sulfation of iduronic acid, and N- and 6-O-sulfations of the glucosamine residue. Using various derivatives of heparin and heparan sulfate, structural requirements of heparin-like domains for recognition by FGFs and HGF

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Abbreviations used: FGF, fibroblast growth factor; MK, midkine; PTN, pleiotrophin; HGF, hepatocyte growth factor; 6-O-DS-, 6-O-desulfated; 2-O-DS-, 2-O-desulfated; NDSNAc-, N-desulfated and N-acetylated.

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have recently been elucidated (17–24). However, nothing is known about the requirement for interaction with MK or PTN.

In this paper, we report the structural characteristics of the carbohydrate required for the interaction with MK on the MK-dependent nerve cell attachment and neurite outgrowth. Localization of MK in radial glial processes along which immature neurons migrate during the formation of cerebral cortex strongly suggests the physiological importance of MK in developing neurons (25).

MATERIALS AND METHODS

Materials and chemicals. Recombinant mouse MK was produced by the baculovirus expression system, and was purified to homogeneity on a heparin-Sepharose column (Hi-Trap Heparin, Pharmacia Biotech, Uppala, Sweden) as described elsewhere (26). Heparin from bovine intestine (LAOB, Brazil) were selectively O-desulfated as described previously (22, 23). Briefly, 2-O-desulfated (2-O-DS-) heparin was prepared by complete lyophilization of 1% heparin in 0.2 N NaOH. 6-O-desulfated (6-O-DS-) heparin was prepared by treatment of the pyridinium salt of heparin with N-methyltrimethyl silyltrifluoroacetamide in pyridine for 2 h at 95°C. The removal of sulfate groups from a specific site was confirmed by the disaccharide composition analysis as described (22). The 2-O- and 6-O-desulfations were 76.7% and 88.9% effective, respectively, without any detectable depolymerization and the other desulfations (data not shown). N-desulfated and N-acetylated (NDSNAc-) heparin prepared from porcine intestine was obtained from Seikagaku Corporation, in which N-desulfation was more than 99.8%. For the preparation of heparin-derived oligosaccharides, about 4g of heparin (LAOB, Brazil) was digested with 1.5 unit of *Flavobacterium heparinum* heparinase (Seikagaku Corporation, Tokyo) in 50 ml of 20 mM sodium acetate buffer, pH 7.0, containing 2mM calcium acetate, for 24 h at 37°C. The digest was subjected to gel-filtration on a column of Sephacryl S-100HR equilibrated with 0.2M NaCl. Each fraction was rechromatographed by the same gel-filtration column. Native heparin was size-fractionated by gel-filtration column of Sephadex G-150 equilibrated with 0.2M NaCl. Each fraction was desalted through a column of Cellulofine GCL-25, and lyophilized. The molecular weight of the size-fractionated heparin was estimated by gel-permeation HPLC connected a combined column of TSKgel 4000, 3000 and 2500PWXL (TOSO Co., Tokyo), using heparin standards as described previously (27, 28).

Nerve cell adhesion assay on patterned MK. Rat embryonic cerebral cortex (E17–18, post coitus) was isolated, minced and digested with 0.25% trypsin (Life Technologies Inc., MD) and 0.01% DNAse I (Sigma, St. Louis, MO). After incubation for 30 min at 37°C, single cells were prepared by gentle trituration in culture medium as described elsewhere (26). Patterned MK on plastic culture plates was prepared as described (26). Briefly, 24-well culture plates (Falcon 3057) were coated with purified MK (10 μg/ml) for 2 h at room temperature. After washing twice with H_2O, the plates were dried, metal electron microscopy grids (HDL 200, Veco, Amsterdam) were placed in the wells, and the plates were irradiated with ultraviolet light at 315 nm for 20 min in a UV chamber (GS Gene Linker, Bio-Rad, CA) to produce a grid pattern of MK. After removing the grid, wells were treated with Dulbecco's minimum essential medium containing 10 mg/ml of bovine serum albumin for 30 min at 37°C. After washing twice with H_2O, rat brain cell suspension was seeded into the plates (0.8×10^6 cells/well) and cultured for 24–48 h in the presence or absence of heparin or various heparin derivatives at 37°C under an atmosphere of 5% CO_2 and 95% air.

RESULTS

On culture dishes coated with patterned MK, nerve cells were attached to and extended their neurites along the track of MK (26, Fig. 1A). Extension of neurite along MK track was especially evident when cells were plated at low density, and most of attached cells were judged to be neurons from their morphology (Fig. 1B). This process of attachment and neurite outgrowth was completely inhibited by heparin, but not by other glycosaminoglycans (26). This experimental system provided an opportunity to clarify the aspects of the structure of heparin required for interaction with MK.

To investigate the contribution of each sulfate group of the heparin molecule to the inhibitory effect against MK-dependent neurite outgrowth, we used selectively desulfated heparins, namely 2-O-DS-, 6-O-DS, and NDSNAc-heparins. Intact heparin exhibited inhibition at the concentration of 0.6 μg/ml (Fig. 1C). With increasing concentration of heparin, the cells began to detach from the plate and aggregated to each other (Fig. 1D). Effects of the desulfated heparin derivatives at different concentrations are summarized in Table 1. At the concentration of 2.5 μg/ml, at which intact heparin showed marked inhibition, 2-O-DS-, 6-O-DS- and NDSNAc-heparins exhibited no inhibitory effect on nerve cells. When cultured at a lower cell density to examine neurite outgrowth, many neurites were still observe in the presence of 2.5 μg/ml of the derivatives, indicating no evident effect of the derivatives on neurite outgrowth in addition to cell attachment (data not

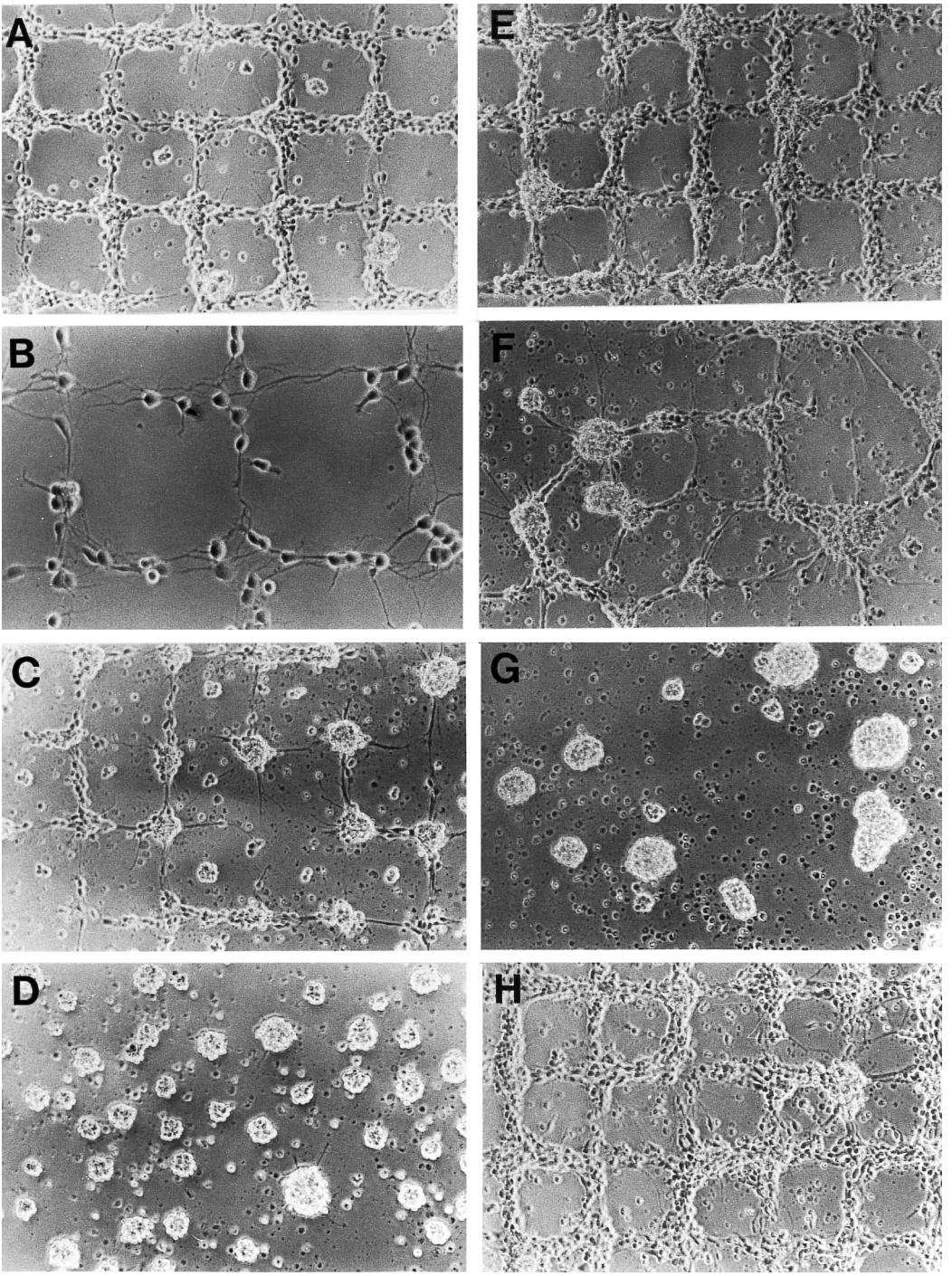

FIG. 1. Effects of heparin and desulfated heparin derivatives on rat embryonic nerve cells cultured on a patterned MK. Cells were cultured for 48 h, as described in Materials and Methods, and observed by phase contrast microscopy. A, control; B, control at low cell density (0.2×10^6 cells/well); C, native heparin, 0.6 μg/ml; D, native heparin, 2.5 μg/ml; E, 2-O-DS-heparin, 2.5 μg/ml; F, 2-O-DS-heparin, 10 μg/ml; G, 2-O-DS-heparin, 50 μg/ml; H, NDSNAc-heparin, 200 μg/ml. Pitch of grid = 125 μm.

TABLE 1
Effects of Selectively Desulfated Heparins on Rat Embryonic Nerve Cell Adhesion

		Desulfated heparin derivatives		
μg/ml	Heparin	2-O-Desulfated	6-O-Desulfated	N-Desulfated and N-acetylated
0.16	−	−	−	−
0.6	+	−	−	−
2.5	++	−	−	−
10	++	+	−	−
50	++	++	+	−
200	++	++	++	−

Rat embryonic nerve cells were cultured on plates coated with patterned MK (10 μg/ml). Cell adhesion was evaluated after 24 h. ++, Cells were detached from the plate and aggregated; +, Cells were attached to the plate, but no grid pattern was observed; −, No inhibition.

shown). It was noted that NDSNAc-heparin had almost no effect even at the highest concentration (200 μg/ml) added (Fig. 1H). These findings indicate that the tri-sulfate structure of heparin is required for maximum interaction between MK and nerve cells, and that the N-sulfate group of the glucosamine residue is essential for the inhibitory effect. 2-O-DS-heparin showed inhibition at 10 μg/ml or higher (Fig. 1E–G), while 6-O-DS-heparin was effective only at 50 μg/ml or higher. This may imply that the sulfate group bound at N-position is more important for the interaction than those at 2-O- and 6-O-positions. However, we should also take into account the effect of residual O-sulfate groups after O-desulfation reaction.

Table 2 shows the inhibitory effect of various size-fractionated native heparins and heparin-derived oligosaccharides with different chain lengths. Native heparins and heparin-derived oligosaccharides with an average molecular weight larger than 7,000 Da (approximately 22 monosaccharide units) showed inhibitory effects, while oligosaccharides with an average molecular weight of 5,600 Da or less had no inhibitory effect at the concentrations examined.

DISCUSSION

The inhibition study described here revealed the structural requirements of the heparin-like domain for the interaction with MK in nerve cell attachment and neurite outgrowth: the three sulfate groups in heparin (N- and 6-O-sulfate groups of glucosamine and 2-O-sulfate group of iduronic acid residues) were all required for the maximum interaction, and the minimum oligosaccharide with inhibitory activity was approximately 22 monosaccharide units.

TABLE 2
Effects of Size-Fractionated Native Heparins and Heparin-Derived Oligosaccharides with Different Chain Lengths on Rat Embryonic Nerve Cell Adhesion

	Average molecular weight (Da)								
μg/ml	16,000[a]	12,000[a]	9,000[a]	7,000[b]	5,600[b]	4,000[b] (12-mer)	2,600[b] (8-mer)	2,000[b] (6-mer)	1,300[b] (4-mer)
2.5	++	+	−	−	−	−	−	−	−
10	++	++	+	−	−	−	−	−	−
50	++	++	++	+	−	−	−	−	−

Rat embryonic nerve cells were cultured on plates coated with patterned MK (10 μg/ml). Cell adhesion was evaluated after 24 h. See the legend for Table 1.

[a] Size-fractionated native heparin.

[b] Heparinase-digested heparin-derived oligosaccharide.

FGF-2 (also known as basic FGF) requires N-sulfate and 2-O-sulfate groups, but does not require the 6-O-sulfate group for binding (17–20). The minimum oligosaccharide sequence with inhibitory activity has been elucidated (20). For promotion of FGF-2-mediated mitogenicity, the 6-O-sulfate group is also required; this sulfate group may interact with the FGF receptor (21, 22). On the other hand, FGF-1 (also known as acidic FGF) and FGF-4 (also Hst) require all three sulfate groups for binding (23). For binding to HGF, the 6-O-sulfate group of the glucosamine residue is the most important and other sulfate groups contribute only slightly (24).

Thus, the heparin-like structure required for MK interaction is distinct from those for FGF-2 or HGF, but is similar to those for FGF-1 and FGF-4. However, heparin-derived oligosaccharides with 10 monosaccharide units or longer promoted FGF-1 and FGF-4 activities (23). The difference in active oligosaccharide length between FGFs and MK suggests that MK requires a larger domain including fully sulfated region in heparin or heparan sulfate for interaction.

The present results are consistent with the view that in addition to specific receptors, defined structures in cell surface heparan sulfate could be responsible for the specificity of the responses of different cell types to various molecular species.

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