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# Enhancement of Neurite Outgrowth–Promoting Activity by Heparin Derivatives in Sodium Chlorate–Treated Explant Cultures of Rat Central Neurons

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**Periodate-oxidized/borohydride-reduced 2-*O*-desulfated heparin (OR2DSH) was prepared using intact heparin from pig intestine as the starting material. Successive treatments of the heparin by oxidation with sodium periodate and reduction with sodium borohydride yielded periodate-oxidized/borohydride-reduced heparin (OR-heparin). Subsequent 2-*O*-desulfation of OR-heparin, according to a previously established method, yielded OR2DSH. Digestion of OR2DSH with heparitinases generated unsaturated disaccharides, comprising 86.5% ΔDiHS-(6,N)S (ΔUA1→4GlcNS(6S)) and 13.5% ΔDiHS-NS (ΔUA1→4GlcNS), as well as undigested oligosaccharides in which uronate moieties were derivatized by the cleavage of the covalent bond between the C-2 and C-3 positions by periodate-oxidation. The molecular mass of OR2DSH was determined to be 11 kDa, which is almost the same as those of other heparin derivatives such as 2-*O*-desulfated heparin (2DSH), 6-*O*-desulfated heparin (6DSH) and *N*-desulfated *N*-reacetylated heparin (NDSNAc-heparin). The ability of OR2DSH to enhance neurite outgrowth-promoting activity was evaluated using the explant culture of neocortical tissue from rat embryo in which endogenous heparan sulfate at the cell surface lost substantial numbers of sulfate groups by the action of 40 μM sodium chlorate. The maximum activity of OR2DSH (29.7%) was achieved at 10 μg/ml, and those of OR-heparin (21.7%), 2DSH (18.7%) and intact heparin (16.3%) were 100 μg/ml, whereas that of NDSNAc-heparin (16.5%) was 1,000 μg/ml. Completely 6-*O*-desulfated heparin (100:6DSH) exhibited very weak activity (3.3%) at 1,000 μg/ml. These results suggest that the potency of OR2DSH to enhance neurite outgrowth–promoting activity is exerted synergetically by two different components in OR2DSH, i.*e.*, the IdoA α1→4GlcNS(6S) unit, which contains 6-*O*- and 2-*N*-sulfate groups, and the uronate moiety in which the covalent bond between C-2 and C-3 is cleaved, although the mode of action remains to be clarified.**

**Key words: neocortical tissue, neurite outgrowth, periodate-oxidized/borohydride-reduced 2-*O*-desulfated heparin, 6-*O*-desulfated heparin, sodium chlorate-treatment.**

Abbreviations: CDSNAc-heparin, completely desulfated *N*-reacetylated heparin; CDSNS-heparin, completely desulfated *N*-resulfated heparin; CS-C, chondroitin sulfate C; ΔDiHS-0S, 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyl uronic acid)-D-glucose; ΔDiHS-NS, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; ΔDiHS-6S, 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔDiHS-US, 2-acetamido-2-deoxy-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyl uronic acid)-D-glucose; ΔDiHS-(6, N)S, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔDiHS-(U, N)S, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; ΔDiHS-(U, 6)S, 2-acetamido-2-deoxy-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔDiHS-triS, 2-deoxy-2-sulfamino-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose; ΔUA, unsaturated uronic acid; DMEM, Dulbecco's modified Eagle's medium; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GAG, glycosaminoglycan; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GlcNS, *N*-sulfoglucosamine; HA, hyaluronic acid; HGF, hepatocyte growth factor; HPLC, high performance liquid chromatography; HSase, heparitinase; IdoA, iduronic acid; IGF, insulin-like growth factor; MAP-kinase, mitogen-activated protein kinase; MTSTFA, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide; NCAM, neural cell adhesion molecule; NDSNAc-heparin, *N*-desulfated *N*-reacetylated heparin; NGF, nerve growth factor; OR-heparin, periodate-oxidized/borohydride-reduced heparin; OR2DSH, periodate-oxidized/borohydride-reduced 2-*O*-desulfated heparin; 6DSH, 6-*O*-desulfated heparin; 100:6DSH, completely 6-*O*-desulfated heparin; 2DSH, 2-*O*-desulfated heparin.

Neurite outgrowth is a representative feature of morphological changes in neuronal tissue, resulting in the formation of synaptic connections during development (*1*). In this process, the interaction between a neuritogenic substance and its receptor on the surface of a neuronal

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cell triggers neurite outgrowth (*2*), followed by signal transduction through the mitogen-activated protein (MAP-) kinase pathway (*3–5*) and through the pathways of Rho family proteins such as Rho GTPases (*6–8*). The former pathway is involved in the reorganization of microtubules towards the future direction of neurite outgrowth (*1*), whereas the latter pathways are involved in the induction of actin polymerization, and so on (*9*). Several varieties of neuritogenic substances, including nerve growth factor (NGF) (*2*), a synthetic peptide ligand of the neural cell adhesion molecule (NCAM) (*10*), GM1 ganglioside in the plasma membrane (*11*), and a peptide corresponding to the homophilic binding site of the second Ig module for NCAM (*12*), *etc*, have been reported.

Although glycosaminoglycans (GAGs), such as heparin and heparan sulfate, have been shown to be involved in the growth and differentiation of cells comprising the nervous system (*13*, *14*), their neuritogenic functions are not fully understood from the viewpoint of physiological mechanism(s). In terms of structure, heparan sulfate is heterogenous depending on the source from which it is prepared. Heparan sulfate contains a linear polysaccharide structure in which the main repeating disaccharide unit is composed of glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc). The GlcA moieties are partially converted enzymatically to iduronic acid (IdoA) moieties by the action of C-5 epimerase (*15*), and the O-2 positions of IdoA moieties are partially sulfated by the action of 2-*O*-sulfotransferase (*16*), whereas GlcNAc moieties are derivatized to GlcNS(6S) by the actions of *N*-deacetylase/*N*-sulfotransferase (*17*) and 6-*O*-sulfotransferase (*18*) during the biosynthesis of heparan sulfate.

The biological functions of heparan sulfate are highly diverged, possibly because heparin and heparan sulfate possess similar active domain structures (*19*). With the accumulation of information concerning the biological roles of heparin and heparan sulfate, it has been revealed that their functions depend mostly upon interactions between polysaccharides and physiologically active molecules (*20–22*). In order to investigate the unique structure of heparin with the active molecules, various chemical modifications of heparin have been undertaken. Among such modification techniques, effective methods for the removal of specific sulfate groups of heparin, such as 2-*O*-sulfate (*23*, *24*), 6-*O*-sulfate (*25*, *26*), and 2-*N*-sulfate groups (*27–29*), have been established and a series of specific desulfated heparin derivatives have been prepared.

In the present study, heparin was modified successively by oxidation with sodium periodate (*30*), reduction with sodium borohydride, and 2-*O*-desulfation (*23*, *24*), yielding periodate-oxidized/borohydride-reduced 2-*O*-desulfated heparin (OR2DSH). We have examined the ability of OR2DSH to enhance neurite outgrowth using sodium chlorate-treated explant cultures of neocortical tissues from rat embryos (*31*), and compared the results with those of other heparin derivatives including periodate-oxidized/borohydride-reduced heparin (OR-heparin), 2-*O*-desulfated heparin (2DSH), 6-*O*-desulfated heparin (6DSH), and *N*-desulfated *N*-reacetylated heparin (NDSNAc-heparin). The present study shows that two different components in OR2DSH, the IdoA α1→4GlcNS(6S) unit, which contains 6-*O*- and 2-*N*-sulfate groups, and the uronate moiety in which a covalent bond between C-2 and C-3 is cleaved, exert synergetically potent activity to enhance neurite outgrowth in chlorate-treated neocortical explant culture.

## MATERIALS AND METHODS

*Materials*—Heparin sodium salt was purchased from Scientific Protein Laboratories (Waunakee, WI). Sodium chlorate and sodium periodate were purchased from Wako (Tokyo). Heparitinase I [EC 4.2.2.8], heparitinase II (no EC number), and heparinase (heparitinase III) [EC 4.2.2.7], and standard unsaturated disaccharides (H-Kit), and chemically modified heparin derivatives (CDSNAc-heparin, CDSNS-heparin, and NDSNAc-heparin) were obtained from Seikagaku Corporation (Tokyo). *N*-Methyl-*N*-(trimethylsilyl)trifluoro acetamide (MTSTFA) was purchased from Azmax (Chiba). 2-*O*-desulfated heparin (2DSH) was prepared according to the previously described method (*23*, *24*).

MEM amino acid solution (No. 11130-051) and MEM vitamin solution (No. 11120-0523) were purchased from Gibco (Paisley, Scotland). Sulfate-free Dulbecco's modified Eagle's medium (DMEM) was prepared in 2.5 liters of sterilized distilled water by adding 500 mg of anhydrous $CaCl_2$, 0.25 mg of $Fe(NO)_3/9H_2O$, 1.0 g of KCl, 193.3 mg of $MgCl_2$, 9.25 g of $NaHCO_3$, 312.5 mg of $NaH_2PO_4/H_2O$, 37.5 mg of Phenol Red, 275 mg of sodium pyrvate, 100 ml of MEM amino acid solution, 75 mg of L-glutamine, 105 mg of L-serine, 100 ml of MEM vitamin solution, 15.15 g of NaCl, and 11.25 g of D-glucose. The solution was then adjusted to pH 7.2.

A pregnant Wistar rat was purchased from SLC (Tokyo). Other reagents used were of analytical grade.

*Preparation of Periodate-Oxidized/Brohydride-Reduced 2-O-Desulfated Heparin*—The preparation of periodate-oxidized/borohydride-reduced 2-*O*-desulfated heparin (OR2DSH) was performed according to the following procedure. To 1.3 g of heparin sodium salt was added 50 ml of a reaction solution containing 50 mM sodium periodate and 50 mM sodium acetate (pH 5.0), and the oxidation reaction was carrried out at 4°C for 3 days. The reaction was terminated by the addition of sufficient glycerol (final concentration; 250 mM) to degrade the excess periodate. The reaction mixture was dialyzed against distilled water for 2 days and then lyophilized, yielding 1.2 g of periodate-oxidized heparin. The oxidized material was dissolved in 30 ml of 0.2 M sodium borohydride and allowed to stand at 4°C for 3 h in order to reduce the aldehyde groups generated during oxidation. After termination of the reaction, the reaction mixture was adjusted to pH 5.0 and allowed to stand at room temperature for 30 min, resulting in the degradation of excess sodium borohydride. The reaction mixture was again adjusted to pH 9.5 with 5 M NaOH, dialyzed against distilled water for 2 days and lyophilized, yielding 1.1 g of periodate-oxidized/borohydride-reduced heparin (OR-heparin) as the sodium salt. To 1.1 g of the OR-heparin sodium salt was added 20 ml of 50 mM NaOH, and the solution was allowed to stand at room temperature for 20 min. Then, the solution was lyophilized to remove selectively sulfate groups at C-2 of the IdoA and GlcA moieties in OR-heparin (*23*, *24*). The lyophilized powder obtained was

dissolved in 10 ml of 1 M NaOH, and the solution was adjusted to pH 9.0 with 20% acetic acid. The solution was allowed to stand at room temperature for 30 min, dialyzed against distilled water for 2 days, and lyophilized, yielding 0.8 g of OR2DSH as the sodium salt.

*Preparation of Partially and Completely 6-O-Desulfated Heparin*—Preparation of 6-*O*-desulfated heparin (6DSH) was performed according to the previously reported methods (*25*, *26*). Briefly, to 500 mg of heparin pyridinium salt was added 10 volumes (w/w) of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MTSTFA) and 100 volumes (v/w) of pyridine. This reaction mixture was heated at 110°C for 15, 30, 60, or 120 min. Each reaction mixture was concentrated 10-fold under reduced pressure, followed by the addition of 2 volumes of distilled water to degrade MTSTFA. The resultant whitish turbidity completely disappeared by treatment under reduced pressure at 35°C for 15 min. The product was then subjected to dialysis against running tap water for 3 days and further against distilled water overnight. The dialysate was applied to an Amberlite IR-120 column ($H^+$ form; $\phi 3 \times 13$ cm) equilibrated with distilled water, and elution was performed with distilled water. Acidic fractions were combined, the pH was adjusted to 9.5 with 1 M NaOH, and the combined materials were dialyzed against distilled water overnight. The final dialysate was subjected to lyophilization. The degree of 6-*O*-desulfation was estimated by chemical disaccharide analysis as described previously (*32*).

*Disaccharide Analysis*—Two hundred micrograms of each heparin derivative was digested with enzyme mixture [50 mU each of heparitinase I and II, and heparinase (heparitinase III)] in 30 μl of 2 mM calcium acetate and 20 mM sodium acetate (pH 7.0) at 37°C for 2 h. After the addition of 50 μl distilled water, the mixtures were heated at 100°C for 1 min to terminate the enzymatic reaction. A portion (15 μl) of the supernatant obtained by centrifugation at 3,000 ×*g* for 15 min was applied to a Tosoh CCPM HPLC equipped with serially combined TSK-gel G-4000, G-3000 and G-2,500$PW_{XL}$ ($\phi 7.5 \times 300$ mm each) columns and eluted with 0.2 M NaCl at 40°C and a flow rate of 0.6 ml/min, with monitoring by refractometry. Peak areas were measured by a Shimadzu C-R4A integrator. From the elution profiles obtained, the degree of enzymatic digestion was estimated.

The digests obtained as above were analyzed for unsaturated disaccharides on a Shimadzu LC-6AD HPLC equipped with a Dionex CarboPak PA-1 column ($\phi 7.5 \times 300$ mm), essentially according to the previously described method (*33*). HPLC was performed at 40°C and a flow rate of 1.0 ml/min, and the pumps were programmed to achieve a combination of five linear gradients of LiCl (from 51 mM to 2.2 M) over 25 min. The eluant was monitored at 230 nm and the area of each peak was calculated by a Hewlett-Packard 3390 integrator.

*Measurement of Molecular Mass*—Five molecular mass markers [HA of 104 kDa, CS-Cs of 52.2 kDa, 39.1 kDA, 8.05 kDa, and ΔDiHS-(U, 6)S of 458 Da] were applied to a Tosoh CCPM HPLC equipped with a series of TSK-gel G-4,000, G-3,000, and G-2,500 $PW_{XL}$ columns ($\phi 7.5 \times 300$ mm each) for gel permeation chromatography using 0.2 M NaCl at 40°C at a flow rate of 0.6 ml/min, with monitoring by refractometry. The molecular masses of the standard HA and CS-Cs were determined by light scattering (*34*). Peak-maximum retention times of GAG samples were measured and compared with the molecular mass standard curve in order to estimate the molecular masses of the GAG samples.

*Measurement of Neurite Outgrowth–Promoting Activity*—A fetus was aseptically excised from a Wistar rat on the 17th day of pregnancy. The brain was excised from the fetus, and the cerebral cortex was obtained by removing the cerebellum, mid-brain, inter-brain, and meninges in sulfate-free DMEM (*31*). Cerebral cortexes obtained from 10 animals were combined and finely sliced both longitudinally and crosswise with a safety razor in a 60 mm dish. To the sliced cerebral cortex tissue was added 10 ml of phosphate-buffered saline (PBS), and the dispersed tissues were collected by centrifugation at 500 ×*g* for 1 min. The explant pellets were dispersed in 5 ml of sulfate-free DMEM. To each 1 ml of explant suspension was added 11 ml of sulfate-free DMEM. The explant suspension was shaken gently and then uniformly divided into 500-μl portions. Each portion was put into a well of a 24-well microtiter plate that had been coated with 0.1% polyethylene imine. After 2-h culture, a 100-μl aliquot was withdrawn from the supernatant in each well and 50 μl of PBS containing the GAG samples to be tested was added along with 50 μl of sulfate-free DMEM containing 400 μM sodium chlorate. After 2-day culture at 37°C in 5% $CO_2$, 500 μl of PBS containing 1% glutaraldehyde was gently over-laid to fix the explant tissues at room temperature for 20 min. After removing the supernatant by suction, 0.5 ml of PBS was slowly over-laid and then immediately removed by suction. After the addition of potassium phosphate (pH 7.2) containing 20% Giemsa solution, the explant tissues were stained at room temperature for 2 h. After removing the supernatant by suction, 0.5 ml of PBS was added. By means of light microscopy at 40-fold magnification, the ratio (%) of the number of cerebral cortex nerve explants exhibiting neurite outgrowth to the number of explants in the whole visual field was calculated.

## RESULTS

*Characterization of Heparin and Its Derivatives*—For characterization, chemically modified heparin derivatives were subjected to Heparitinases (HSases-) digestion, followed by gel permeation HPLC analysis and strong anion exchange HPLC analysis. Although the degree of unsaturated disaccharide-production of OR2DSH, prepared as shown in Fig. 1, was *ca*. 60%, those of the other heparin derivatives were nearly 90%, which is almost the same as that of intact heparin (data not shown). The difference between these two values reflects the presence of undigestable disaccharide units in the OR2DSH backbone structure in which covalent bonds between the C-2 and C-3 positions of uronate residues are cleaved.

Table 1 shows the percentage distributions of unsaturated disaccharides generated from intact heparin and its derivatives. The HSases digest of intact heparin contained 64.2% ΔDiHS-(U, 6, N)S as the major component. The HSases digest of OR-heparin contained 84.4% ΔDiHS-(U, 6, N)S as the major component and 10.0%

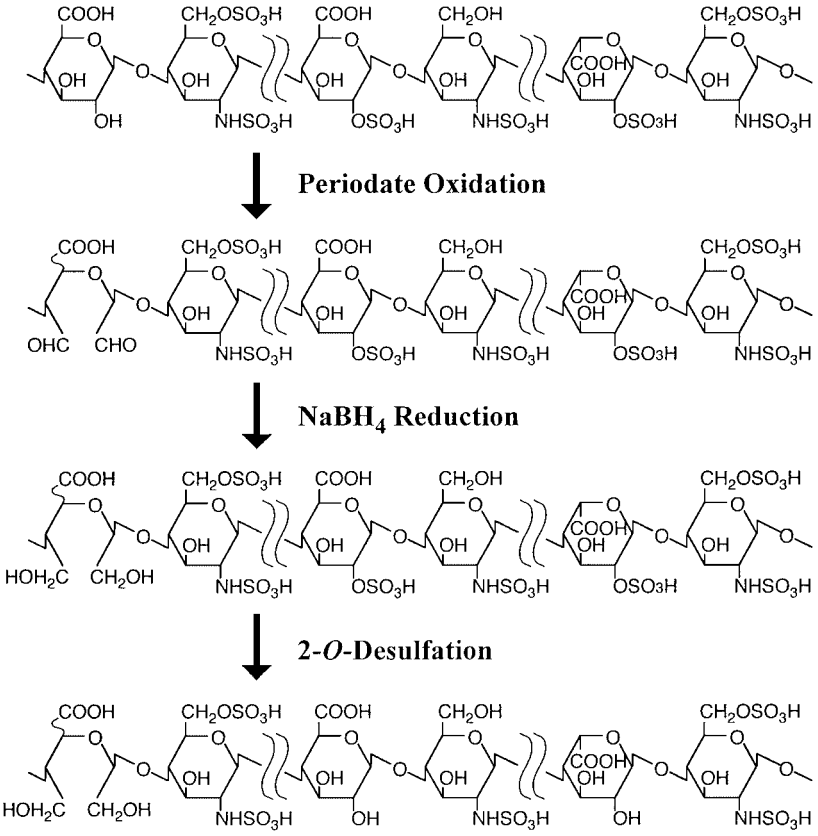


**Fig. 1. Schematic diagram of the preparation of periodate-oxidized/borohydride-reduced 2-*O*-desulfated heparin (OR2DSH).** Intact heparin sodium salt was modified successively by oxidation with sodium periodate, reduction with sodium borohydride, and 2-*O*-desulfation.

ΔDiHS-(U, N)S as a minor component, and no ΔDiHS-0S, ΔDiHS-NS, ΔDiHS-6S, or ΔDiHS-(6, N)S with reference to the unsaturated disaccharide composition of intact heparin, indicating that the periodate-oxidation/borohydride-reduction successfully cleaved the covalent bonds between nonsulfated C-2 and C-3 positions of uronate residues. The HSases digest of 2DSH contained 71.6% ΔDiHS-(6, N)S, and no ΔDiHS-US, ΔDiHS-(U, N)S, ΔDiHS-(U, 6)S, or ΔDiHS-(U, 6, N)S with reference to the unsaturated disaccharide composition of intact heparin, indicating that the 2-*O*-desulfation reaction proceeded completely. The HSases digest of OR2DSH contained 86.5% ΔDiHS-(6, N)S and 13.5% ΔDiHS-NS. This indicates that the former component was generated from ΔDiHS-(U, 6, N)S in the OR-heparin digest by the action of the 2-*O*-desulfation reaction, and the latter component was generated from ΔDiHS-(U, N)S in the OR-heparin digest. The HSases digest of CDSNAc-heparin contained 100% ΔDiHS-0S. The HSases digest of CDSNS-heparin contained 88.8% ΔDiHS-NS as the major component. The HSases digest of NDSNAc-heparin contained 61.6% ΔDiHS-(U, 6)S as the major component, and no ΔDiHS-

Table 1. **Percentage distributions of unsaturated disaccharides generated from various heparin derivatives (%).**

| Fraction | ΔDiHS- | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0S | NS | 6S | US | (6,N)S | (U,N)S | (U,6)S | (U,6,N)S |
| Heparin[a] | 4.1 | 3.4 | 3.7 | 2.6 | 12.7 | 7.6 | 1.7 | 64.2 |
| OR-heparin[b] | 0.0 | 0.0 | 0.0 | 3.4 | 0.0 | 10.0 | 2.2 | 84.4 |
| 2DSH[c] | 5.6 | 11.2 | 5.2 | 0.0 | 71.6 | 0.0 | 0.0 | 0.0 |
| OR2DSH[d] | 0.0 | 13.5 | 0.0 | 0.0 | 86.5 | 0.0 | 0.0 | 0.0 |
| 47:6DSH[e] | 7.5 | 5.2 | 0.0 | 3.8 | 10.1 | 39.4 | 0.0 | 34.0 |
| 75:6DSH[f] | 11.1 | 13.2 | 0.0 | 4.9 | 9.7 | 55.3 | 0.0 | 5.8 |
| 91:6DSH[g] | 11.4 | 17.1 | 0.0 | 5.7 | 7.1 | 58.6 | 0.0 | 0.1 |
| 100:6DSH[h] | 10.4 | 20.1 | 0.0 | 4.2 | 5.5 | 59.8 | 0.0 | 0.0 |
| CDSNAc[i] | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| CDSNS[j] | 11.2 | 88.8 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| NDSNAc[k] | 5.0 | 0.0 | 19.0 | 10.6 | 3.8 | 0.0 | 61.6 | 0.0 |

[a]Intact heparin sourced from pig intestine. [b]Periodate-oxidized/borohydride-reduced heparin. [c]2-*O*-desulfated heparin. [d]Periodate-oxidized/borohydride-reduced 2-*O*-desulfated heparin. [e]Partially (47%) 6-*O*-desulfated heparin. [f]Partially (75%) 6-*O*-desulfated heparin. [g]Partially (91%) 6-*O*-desulfated heparin. [h]Completely (100%) 6-*O*-desulfated heparin. [i]Completely desulfated *N*-reacetylated heparin. [j]Completely desulfated *N*-resulfated heparin. [k]*N*-desulfated *N*-reacetylated heparin.

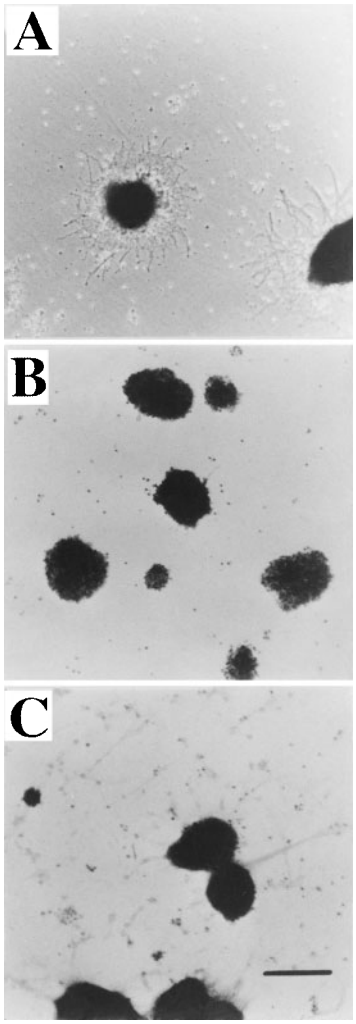


Fig. 2. **Inhibitory effect of sodium chlorate and the accelerative effect of OR2DSH on neurite outgrowth of a neocortical explant culture as observed by light microscopy.** The explants were incubated for 2 days in sulfate-free DMEM (A), followed by the addition of 40 μM sodium chlorate (B), or 40 μM sodium chlorate plus 10 μg/ml OR2DSH (C). The bar in (c) indicates 100 μm.

NS, ΔDiHS-(U, N)S or ΔDiHS-(U, 6, N)S with reference to the unsaturated disaccharide composition of intact heparin, indicating that the *N*-desulfation reaction proceeded almost completely. Unexpectedly, however, a small amount of ΔDiHS-(6, N)S remained.

The degrees of 6-*O*-desulfation of 6DSHs obtained in 15, 30, 60, and 120 min reactions, as determined by ion-pairing reversed phase HPLC analysis (*32*), were 47, 75, 91, and 100%, respectively. As shown in Table 1, the content of ΔDiHS-(U, N)S rose to 59.8% with the increasing degree of 6-*O*-desulfation. In contrast, ΔDiHS-(U, 6, N)S decreased with the increasing degree of 6-*O*-desulfation, and disappeared from the HSases digest of completely 6-*O*-desulfated heparin (100:6DSH), indicating that the 6-*O*-desulfation reaction proceeded completely. In addition, the HSases digest of 6DSHs differing in the degree of 6-*O*-desulfation contained ΔDiHS-NS as a minor component, the level of which rose to 20.1% with the increasing degree of 6-*O*-desulfation. This confirms that minor 2-*O*-desulfation occurred as a side reaction during the 6-*O*-desulfation reaction as reported previously (*26*). It appears that the peak identified as ΔDiHS-(6, N)S in the 100:6DSH digest should be reassigned as an oligosaccharide possessing the same retention time as that of ΔDiHS-(6, N)S, because 100:6DSH does not contain 6-*O*-sulfate groups as confirmed by ion-pairing reversed-phase HPLC and $^{13}$C-NMR techniques (*26*). In fact, the completion of 6-*O*-desulfation in our 100:6DSH has been confirmed by other researchers as well (*35*).

The molecular masses of chemically modified heparins were estimated by the gel permeation HPLC method. The molecular mass of OR2DSH was determined to be 11 kDa as compared with that (13 kDa) of intact heparin. The molecular masses of other heparin derivatives ranged between 10 and 12 kDa, indicating that the molecular masses of the present heparin derivatives do not differ so widely (data not shown).

*Neurite Outgrowth–Promoting Activity*—In order to optimize the conditions of sodium chlorate treatment, neocortical explants from rat embryos were cultured in the presence of various concentrations of sodium chlorate (0, 5, 10, 20, 40, 80, and 100 μM) in sulfate-free DMEM. In the absence of sodium chlorate, many neurites extended from the surface of neocortical explants (Fig. 2A). The degree of neurite outgrowth decreased in a chlorate dose-dependent manner (data not shown). Sodium chlorate concentrations above 40 μM completely inhibited neurite outgrowth (Fig. 2B), and the treatment with 40 μM sodium chlorate did not cause the explants to detach from the culture wells. Thus, 40 μM was found to be the optimum concentration of sodium chlorate to completely inhibit neurite outgrowth. As shown in Fig. 2C, exogenously added OR2DSH (10 μg/ml) restored neurite outgrowth even in the presence of 40 μM sodium chlorate, suggesting that this assay system can evaluate the ability of GAG samples to enhance neurite outgrowth without the influence of endogenous heparan sulfate located at the cell surface.

Since sodium chlorate suppresses the sulfation of cell surface GAGs such as heparan sulfate by reducing the activity of sulfotransferases (*36*), the degree of neurite outgrowth induced by the GAG samples added exogenously to the explant culture will depend on the specific array of sulfate groups linked to the GAG backbone. Chemically modified heparins were added to the explant culture in the presence of 40 μM sodium chlorate, in order to evaluate quantitatively their abilities to enhance neurite outgrowth at various concentrations. As shown in Fig. 3A, the profiles of the activities of OR2DSH, OR-heparin, and 2DSH were bell-shaped, whereas that of NDSNAc-heparin showed no activity below 10 μg/ml but minor activities were observed at 100 and 1,000 μg/ml. The profile of the activity of intact heparin was quite similar to but slightly lower than that of 2DSH. The maximum activity of OR2DSH (29.7%) was achieved at 10 μg/ml, and those of OR-heparin (21.7%), 2DSH (18.7%) and intact heparin (16.3%) were at 100 μg/ml, whereas the maximum activity of NDSNAc-heparin (16.5%) was at 1,000 μg/ml.

Figure 3B shows the neurite outgrowth enhancing profiles of 6DSHs differing in 6-*O*-sulfate content of the heparin backbone. Although the activities of 6DSHs

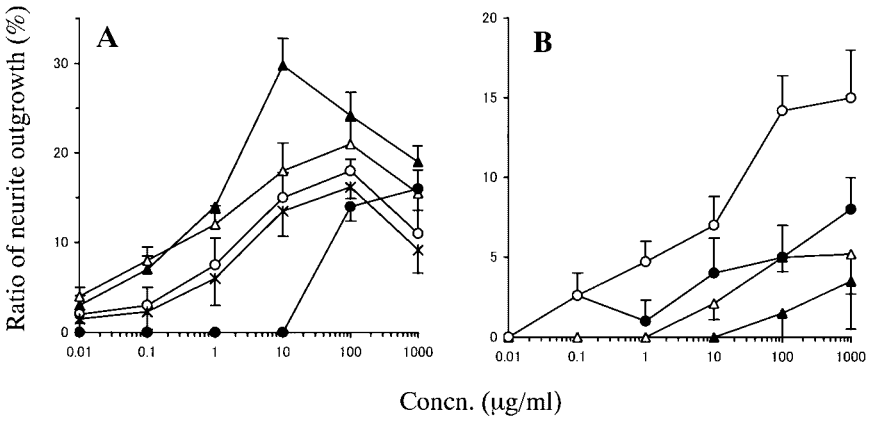


Fig. 3. **Concentration-dependency of the accelerative effects of OR2DSH (solid triangles), OR-heparin (open triangles), 2DSH (open circles) , intact heparin (*) and NDSNAc-heparin (solid circles) on neurite outgrowth from neocortical explant cultures (A).** Concentration-dependency of the accelerative effects of 6DSHs differing in the degree of 6-*O*-desulfation, 100% (solid triangles), 91% (open triangles), 75% (solid circles), and 47% (open circles), on neurite outgrowth from neocortical explant cultures (B).

increased with increasing concentration regardless of the degree of 6-*O*-desulfation, the maximum activities achieved at 1,000 μg/ml were reversely correlated with the degree of 6-*O*-desulfation. In particular, completely 6-*O*-desulfated heparin (100:6DSH) exhibited very weak activity (3.3%) at 1,000 μg/ml, indicating the importance of 6-*O*-sulfate groups for achieving neurite outgrowth. In addition, completely desulfated *N*-reacetylated heparin (CDSNAc-heparin) and completely desulfated *N*-resulfated heparin (CDSNS-heparin) both exhibited almost no activity at any concentration examined (data not shown). Thus, the data suggest that the presence of 6-*O*-sulfate groups on GlcNAc as well as 2-*N*-sulfate groups on GlcNS moieties in heparin derivatives such as OR2DSH are needed to achieve a potent capacity to enhance neurite outgrowth.

## DISCUSSION

In the present study, we prepared OR2DSH using intact heparin as the starting material by successive oxidization with sodium periodate, reduction with sodium borohydride, and 2-*O*-desulfation according to an established method (*23*, *24*), in order to evaluate its potential to induce neuritogenesis. The maximum activity of OR2DSH was at 10 μg/ml, whereas those of OR-heparin and 2DSH were at 100 μg/ml (Fig. 3A). This indicates that OR2DSH has a more potent activity than OR-heparin and 2DSH. Since OR2DSH was prepared from OR-heparin by the 2-*O*-desulfation reaction, the unsaturated disaccharide composition of OR2DSH resembles that of 2DSH (Table 1). In addition, OR2DSH carries a structure in which some of the uronate moieties are derivatized by cleavage of the covalent bond between the C-2 and C-3 positions by the action of periodate-oxidation. A sulfation inhibitor, sodium chlorate reduced the sulfation degree of endogenous heparan sulfate in explant cells. In the present study, we compared the neurite outgrowth-promoting activity of OR2DSH with those of other heparin derivatives including 6DSH and NDSNAc-heparin, using sodium chlorate-treated explant cultures. The modified heparin produced by the removal of 6-*O*-sulfate groups (Fig. 3B) or 2-*N*-sulfate groups (Fig. 3A) from the heparin backbone did not enhance neurite outgrowth-promoting activity in our explant culture system. In contrast, 2-*O*-desulfation and periodate-oxidization of heparin enhanced the neurite outgrowth-promoting activity. The potent activity of OR2DSH would be exerted synergistically by two components in OR2DSH, *i.e.*, the IdoA α1→4GlcNS(6S) unit, which contains 6-*O*- and 2-*N*-sulfate groups, and the uronate moiety, in which the covalent bond between C-2 and C-3 is cleaved. It is possible that C-2/C-3-cleaved uronate moieties in heparin may provide additional flexibility to the heparin backbone. Thus, the present study reveals that the specific sulfate substitution pattern in the heparin backbone and the presence of C-2/C-3-cleaved uronate residues within the backbone are both required for the enhancement of neurite outgrowth-promoting activity.

Heparin and heparan sulfate interact with numerous proteins, including enzymes, growth factors, cytokines, and extracellular matrix proteins (*37*). Furthermore, recent studies suggest that heparin and heparan sulfate have the significant potential to regulate the biological activities of all members of the FGF family (*38*, *39*). Although the physiological mechanism involved in the enhancement of neurite outgrowth by heparin and heparan sulfate is not fully understood, a group of heparin-binding growth factors appears to be involved in the neuritogenesis. Among such factors, basic fibroblast growth factor (bFGF) (*40*), hepatocyte growth factor (HGF) (*41*), nerve growth factor (NGF) (*2*), and insulin-like growth factor-I (IGF-I) (*42*) have been shown to possess potent neuritogenic activity. Damon *et al.* (*43*) reported that heparin alters the abilities of FGFs and NGF to enhance neurite outgrowth in PC12 cells. Hamanoue *et al.* (*41*) reported that HGF enhances neurite outgrowth in rat neocortical explant. Although it is still not clear whether such growth factors are involved in the ability of OR2DSH to enhance neurite outgrowth-promoting activity in our explant culture system, we believe that some growth factors are involved in the neuritogenic activity by OR2DSH.

In the case of NCAM-induced neuritogenesis, it has been shown that FGF receptors (FGFRs) are also involved (*40*, *44*, *45*). Green *et al.* (*44*) proposed that FGFRs might be activated directly by a much wider range of ligands, including heparan sulfate proteoglycan and NCAM, *i.e.*, FGFRs might be an even less specific family of receptor tyrosine kinases than is already appreciated. Brittis *et al.* (*45*) indicated that the activation of the FGFR signal cascade not only promotes the survival and proliferation of various cell types but also can mediate intraretinal axon guidance, which is closely related to

neuritogenesis. Archer *et al*. (*40*) demonstrated that the binding of bFGF and NCAM to nerve cell membranes promotes neurite outgrowth, and that signals from both bFGF and NCAM are transmitted through FGFR. In the case of FGFR-induced proliferation, it has been demonstrated that the formation of a complex consisting of FGF, heparin (or heparan sulfate) and FGFR is essential for signal transduction inducing proliferation (*46–48*). X-ray analyses of the crystal structures of biologically active FGF dimers have indicated that the two FGF molecules forming one dimer interact with the IgII and IgIII domains of FGFRs after inducing FGFR dimerization, irrespective of acidic and basic FGF species (*47*, *48*). According to this proposed model, the dimerization or even oligomerization of bFGF is mediated by heparin and/or heparan sulfate, confirming the previous results obtained by Kan *et al*. (*49*).

It is not clear whether heparan sulfate and FGFs are involved in the direct FGFR-NCAM interaction that induces neuritogenesis (*50*). Even if FGFR does not interact with NCAM directly, NCAM-induced neuritogenesis requires simultaneous bFGF signals through FGFR (*40*). In this process, it would be inevitable that signal transduction occurs through a complex comprising bFGF, heparin (or heparan sulfate) and FGFR after the induction of FGFR dimerization as described above (*47*, *48*). Thus, the importance and involvement of heparin, heparan sulfate and bFGF in NCAM-induced neuritogenesis should be taken into consideration in order to give a better understanding of the physiological mechanism(s) underlying this phenomenon. If it is assumed that NCAM and bFGF were released in our explant culture system in an autocrine manner, there is a possibility that the neurite outgrowth–promoting activity of OR2DSH was exhibited due to the formation of a complex comprising bFGF, OR2DSH and FGFR. However, bFGF binds preferentially to sequences in which the predominant disaccharide is IdoA(2S) $\alpha$1$\rightarrow$4GlcNS (*51*, *52*). Since the predominant disaccharide in OR2DSH is IdoA $\alpha$1$\rightarrow$4GlcNS(6S) (Table 1), it is unlikely that a complex consisting of bFGF, OR2DSH and FGFR was formed in our explant culture system. Alternatively, OR2DSH would have interacted with growth factors other than bFGF, resulting in the interaction with FGFR or other growth factor receptors. Further investigation of the physiological mechanism(s) underlying the ability of OR2DSH to enhance neuritogenic activity as well as exploring molecules that interact with OR2DSH are needed to provide a clue to disigning suitable heparin derivatives for the production of a drug useful for the treatment of disorders of the central nervous system.

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