Preparation of Completely 6-O-Desulfated Heparin and Its Ability to Enhance Activity of Basic Fibroblast Growth Factor*

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Although regioselective removal of 6-O-sulfate groups of heparin has been undertaken by several researchers, complete 6-O-desulfation with little side reaction has not been attained successfully. In this work, a modified method with a certain silylating reagent, N-methyl-N-(trimethylsilyl)trifluoroacetamide, has been established to produce completely 6-O-desulfated heparin with few other chemical changes. The degrees of 6-O-desulfation were estimated by means of chemical disaccharide analyses and/or $^{13}$C NMR spectra. Although the completely 6-O-desulfated heparin lost about 20% of 2-O-sulfate groups, any other chemical changes and depolymerization were not detected. The completely 6-O-desulfated heparin displayed strong inhibition of COS-1 cell adhesion to basic fibroblast growth factor (bFGF)-coated heparin, and the interaction of heparin and/or HS necessary for binding with anti-thrombin III (3, 4), basic fibroblast growth factor (bFGF) (5–8), etc. Furthermore, minimum structures of heparin and/or HS necessary for binding with anti-thrombin III and/or bFGF have been determined (9, 12).

Chemical modification of heparin has been undertaken by several researchers, focusing on the elucidation of the mechanism underlying interaction between heparin and the physiologically active molecules as described above. Specific removal of major sulfate groups of heparin such as 2-O-sulfate, 6-O-sulfate, and N-sulfate groups would be useful in order to clarify the backbone structures of oligosaccharides bearing specific array of sulfate groups responsible for the interactions with physiologically active molecules. For instance, selective removal of O-sulfate groups from glucosamine residues of heparin is of great importance in order to evaluate the involvement of O-sulfate group(s) in the interaction between heparin, bFGF, and FGFRs (FGFRs) (13–29).

Although effective methods for 2-O- and N-desulfations have been established (30–34), methods for 6-O-desulfation have not. However, solvolysis and N-resultation reactions have been utilized to prepare 6-O-desulfated heparin as one of the available methods (32–34). Solvolysis of heparin affords complete removal of N-sulfate groups from GlcN residues, removal of a substantial part of O-sulfate groups from iduronic acid (IdoUA) residues, and incomplete removal of O-sulfate groups from GlcN residues, which occur even under the most optimized conditions (35). Accordingly, the heparin derivative prepared by the subsequent N-resultation contains significantly reduced amounts of 2-O-sulfate groups of IdoUA residues, resulting in the unsuccessful regioselectivity for desulfation and enhancement of the activity of basic fibroblast growth factor (bFGF) (20).
incomplete 6-O-desulfation. Use of such structurally undefined samples for molecular interaction analyses might cause alternative

Alternatively, 6-O-desulfation of heparin could be attained by the method with silylating reagents such as N,O-bis(trimethylsilyl)acetamide (BTSA) and N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MTSTFA), resulting in high regioselectivity for desulfation (36, 37). Although BTSA treatment of heparin causes incomplete 6-O-desulfation under mild conditions and complete 6-O-desulfation with concomitant N-desulfation under drastic conditions (38), our previous studies indicated that MTSTFA treatment yielded substantial 6-O-desulfated heparin with few side effects as was determined with \(^{13}C\) NMR spectra (38, 39).

In the present study, the conditions for 6-O-desulfation of heparin mediated by MTSTFA were precisely examined in order to optimize the reaction temperature and the reaction time for the complete 6-O-desulfation, with the aid of a new HPLC technique for disaccharide analyses of heparin and HS (40) and \(^{13}C\) NMR spectra. In addition, we refined the purification procedure of 6-O-desulfated heparin from the reaction mixture in order to obtain the final product susceptible to the enzymatic digestion prerequisite for the disaccharide composition analysis. Next, the present completely 6-O-desulfated heparin was examined for inhibition activity of COS-1 cell adhesion to bFGF-coated well and for its ability to modify mitogenic activity of bFGF by competitive cell adhesion assay and cell proliferation assay, respectively, in order to examine the involvement of 6-O-sulfate group(s) of heparin in the promotion of bFGF mitogenic activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Heparin from porcine intestine was obtained from Scientific Protein Laboratories. 2-O-Desulfated heparin was prepared according to the method of Piani et al. (30, 31). Solvolysis/N-refluxation-treated 6-O-desulfated heparin was prepared according to the previous method (32–34). MTSTFA was obtained from Azmac Co. PNp-hydrazine (reagent grade) was purchased from Wako Co. Heparitinase I (EC 4.2.2.8), heparitinase II (no EC number), and heparinase (EC 4.2.2.7) were obtained from Seikagaku Co. COS-1 cells (SV40-transformed African Green Monkey Kidney, ATCC CRL1650) were obtained from Dainihonseiyaku Co. A31 cells (BALB/c 3T3, clone A31) were obtained from Dainihonseiyaku Co. Basic fibroblast growth factor (bFGF) was obtained from Promega Co. All other reagents used were of analytical grade.

**6-O-Desulfation with MTSTFA under Different Reaction Temperatures**—To 500 mg of heparin pyridinium salt was added 10 volumes (v/w) of MTSTFA and 100 volumes (v/w) of pyridine. The mixture was subjected to stirring at room temperature until heparin pyridinium salt was thoroughly dissolved and then heated for 2 h at 80, 95, or 110 °C. During the reaction, 5-ml aliquots were withdrawn from the reaction mixture at due intervals and were quickly cooled in an ice-bath. To each aliquot sample was added 5 ml of distilled water (DW), affording the degradation of MTSTFA with the simultaneous appearance of whitish turbidity. In the next place, each aliquot with whitish turbidity was subjected to dialysis against running tap water for 3 days. After adjusting pH of the resultant dialysate to 9.5, the aliquot was further subjected to lyophilization. The 6-O-desulfation degree was estimated by the chemical disaccharide analysis as described below (40). Sample aliquots from 110 °C reaction were further analyzed with \(^{13}C\) NMR in order to estimate another 6-O-desulfation degree.

**6-O-Desulfation with MTSTFA at 110 °C under Different Reaction Times and Purification by the Modified Procedure**—To 6 g of heparin pyridinium salt was added 10 volumes (v/w) of MTSTFA and 100 volumes (v/w) of pyridine. This reaction mixture was subjected to stirring at room temperature until heparin pyridinium salt was thoroughly dissolved in the mixture. Then the reaction mixture was heated at 110 °C for 2.5 h, where another 30 min was necessary to attain the reaction to proceed compared with that of 0.5-g scale reaction (2 h). During the reaction, 220-ml aliquots were withdrawn from the reaction mixture at 1, 1.5, and 2.5 h, followed by cooling in an ice bath. Each aliquot sample was concentrated 10-fold with a rotary evaporator, followed by the addition of 2 volumes of DW to degrade MTSTFA. The resultant whitish turbidity completely disappeared by the 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 DW overnight. The final dialysate was subjected to lyophilization and its absorbance at 280 nm was measured by a modification of the procedure of Ishihara et al. (45). Briefly, each well of a 96-well tissue culture plate was coated with 40 μl of 10 μg/ml human recombinant bFGF overnight at 4 °C. The content of each well was aspirated, and the well was rinsed with 200 μl of PBS and blocked by incubation with PBS containing 5% (v/v) fetal bovine serum for 1 h at room temperature. A pellet of COS-1 cells was prepared by incubation with PBS containing 2 mM EDTA for 10 min at 37 °C, pipetting, and collection by centrifugation. The cells were then resuspended at a density of 1.2 × 10⁶ cells/ml with PBS containing 5% (v/v) fetal bovine serum (FBS). The 6-O-desulfated heparin was then added to the suspension. The cell suspension (100 μl) was immediately applied to the coated wells and incubated for 10 min at room temperature. Each well was washed twice with 200 μl of PBS, and then 20 μl of 5% SDS was added to lyse the bound cells. To each well was added 200 μl of Micro BCA protein assay reagent (Pierce), and the protein concentration of the lysate in each well was measured by a modification of the procedure of Phipps et al. (36, 37). The absorbance at 562 nm was measured by spectrophotometer. The estimation of absorbance was carried out using a standard curve. The absorbance of the blank was subtracted from that of the sample. The concentration of the protein in the lysate was then calculated from the standard curve. The test was performed in triplicate.
determined with bovine serum albumin as a standard by measuring the absorbance at 545 nm.

Cell Proliferation Assay—A31 cells were maintained in DMEM (Iwaki Co.) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO₂ in air and 100% relative humidity.

For the assay of the growth of non-chlorate-treated A31 cells, the cells were seeded at an initial density of 5000 cells/well in 96-well tissue culture plates and were grown for 3 days in 100 μl of DMEM supplemented with ITS+Ω (Life Technologies, Inc.; insulin (10 ng/ml), transferrin (5.5 μg/ml), selenium (5 ng/ml)), human recombinant bFGF (2 ng/ml), penicillin G (100 units/ml), streptomycin (100 μg/ml), and 6-O-desulfated heparin to be tested.

For testing the ability of the 6-O-desulfated heparin to restore the bFGF-induced proliferation of chlorate-treated A31 cells, cells were grown for 3 days in DMEM lacking sulfate but supplemented with ITS+Ω, bFGF (2 ng/ml), sodium chloride (20 mM), penicillin G (100 units/ml), and 6-O-desulfated heparin to be tested. After incubation, to each well was added 20 μl of MTS/PMS solution (cell titer 96 aqueous non-radioactive cell proliferation assay kit, Promega Co.). After incubation at 37 °C for 2 h, the number of living cells in each well was determined by measuring the absorbance at 492 nm.

For the assay of the growth of non-chlorate-treated A31 cells, the 6-O-desulfated heparin to be tested. After incubation at 37 °C for 2 h, the number of living cells in each well was determined by measuring the absorbance at 492 nm.

| Reaction time | Peak area | ISM | ISMS | D₁ | Signal intensity of C-6 | D₂ |
|---------------|-----------|-----|------|----|------------------------|----|
| min           |           |     |      |    |                        |    |
| 0             | 15.4      | 84.6| 0.0  | 10.2| 75.0                   | 0.0 |
| 15            | 55.9      | 44.1| 47.9 | 59.6| 34.5                   | 56.7|
| 30            | 76.0      | 24.0| 71.6 | 84.9| 19.0                   | 78.4|
| 60            | 92.2      | 7.8 | 90.8 | 80.1| 7.1                    | 90.5|
| 120           | 100.0     | 0.0 | 100.0| 93.2| 0.0                    | 100.0|

where

\[ D_{1} = \frac{A_{0} \times B_{0} - A_{1} \times B_{1}}{B_{0} \times (A_{1} + B_{1})} \times 100\% \]

RESULTS

Time Dependence of 6-O-Desulfation of Heparin by MTSTFA Treatment—Fig. 1 shows the IPRP-HPLC profiles for disaccharide compositions of the products obtained from 500-mg scale reaction in the presence of MTSTFA at 110 °C for 0 (intact heparin), 15, 30, 60, and 120 min. Since each profile was obtained by the application of the same amount of PNP-coupling disaccharides to IPRP-HPLC, comparison between mutual profiles could be valid. IPRP-HPLC profile of 0-min product is shown in Fig. 1a. The peaks prerequisite for the estimation of 6-O-desulfation degree are those assigned to be ISM (12 min) and ISMS (16 min). The area of the latter peak decreased with the increase of reaction time, whereas the area of the former peak increased with the increase of reaction time (Fig. 1, b–e), showing the steps of the 6-O-desulfation reaction. IPRP-HPLC profiles of 500-mg scale reactions at 80 and 95 °C represented similar but slower reaction time-dependent changes in 6-O-desulfation compared with that of 110 °C reaction (data not shown). Table I shows the peak areas of ISM and ISMS for the products from 500-mg scale 6-O-desulfation reaction at 110 °C for 0, 15, 30, 60, and 120 min. Fig. 2 shows the time dependence of 6-O-desulfation of heparin during 500-mg scale reaction in the presence of MTSTFA at three different reaction temperatures of 80, 95, and 110 °C. The 6-O-desulfation degree (D₁) was calculated with the IPRP-HPLC data by Equation 1, where A₀ indicates peak area of ISM of intact heparin; B₀ indicates peak area of ISMS of intact heparin; A₁ indicates peak area of ISM of 6-O-desulfated heparin; and B₁ indicates peak area of ISMS of 6-O-desulfated heparin.

As shown in Fig. 2, the 6-O-desulfation degree (D₁) increased with the increase of reaction time irrespective of reaction temperatures used, according to the kinetics of pseudo first-order reaction. When the temperature of 110 °C was adopted, the 6-O-desulfation reaction was essentially completed in 2 h. On the other hand, 80 and 95 °C reactions for 2 h yielded only 52 and 85% of 6-O-desulfation degrees, respectively. Accordingly, the 110 °C reaction was shown to superior to the 95 °C reaction adopted by previous study (39), in order to attain maximum 6-O-desulfation degree of MTSTFA-treated heparin.

Fig. 3 shows the 13C NMR spectra of 500-mg scale reaction products at 110 °C for 0 (intact heparin), 15, 30, 60, and 120 min. Since each spectrum was obtained by the use of the same amount of heparin derivative, comparison between mutual spectrum could be valid. 13C NMR spectrum of 0-min product is shown in Fig. 3a. The signals necessary for the estimation of 6-O-desulfation degree are those assigned as C-6 of GlcNS (57.0±15.4B)/(0.846±0.0102A±D) (%). The signals necessary for the estimation of 6-O-desulfation degree are those assigned as C-6 of GlcNS (15.4B)/(0.846±0.0102A±D) (%). The signals necessary for the estimation of 6-O-desulfation degree are those assigned as C-6 of GlcNS (0.0±100.0D) (%). The signals necessary for the estimation of 6-O-desulfation degree are those assigned as C-6 of GlcNS (15.4B)/(0.846±0.0102A±D) (%). The signals necessary for the estimation of 6-O-desulfation degree are those assigned as C-6 of GlcNS (0.0±100.0D) (%).
NMR data by Equation 2, desulfated heparin. D of reaction time, whose time dependence was associated with desulfation reaction. O (see Fig. 1) before and after each 6-degree was performed according to the E calculation of 6-O-desulfation degree (Eq. 2) at 110 °C (−desulfation E). Ml, 95 (under three different reaction temperatures, 500-mg scale in the presence of MTSTFA −desulfation degree ( ). Ml, 95 (−desulfation E).

The 6-O-desulfation degree of MTSTFA-treated heparin at 110 °C for 2 h was revealed to contain no 6-O-desulfated heparin; 95 (−desulfation E). Ml, 95 (−desulfation E). The 6-O-desulfation degree of MTSTFA-treated heparin at 110 °C for 2 h was confirmed to be essentially complete in 2 h (Table I). Fig. 2 shows the SAX-HPLC profiles of 80, 90 and 100% 6-O-desulfated heparins after enzymatic digestions with heparitinase and heparinase showed that the enzymatic digestion degrees were 93, 81, and 82% for 80, 90, and 100% 6-O-desulfated heparins, respectively, which were almost identical to the digestibility of intact heparin. The digestibilities of these 6-O-desulfated heparins purified by the modified procedure were greatly improved compared with that (less than 30%) of 6-O-desulfated heparin purified by the previous procedure (39). Fig. 4 shows the SAX-HPLC profiles of 80, 90, and 100% 6-O-desulfated heparins after digestions with heparitinase and heparinase. Major peaks observed were ΔDiHS-(U,N)S eluted at 16 min, ΔDiHS-NS eluted at 11 min, and ΔDiHS-0S eluted at 3 min for all of 80, 90, and 100% 6-O-desulfated heparin digests. The peak corresponding to ΔDiHS-(U,6,N)S eluted at 22 min was not found on the SAX-HPLC profile of 100% 6-O-desulfated heparin digest. This confirmed the occurrence of 6-O-desulfation by MTSTFA treatment of heparin. On the other hand, only a trace amount of ΔDiHS-(U,6,N)S was observed on the profiles for 80 and 90% 6-O-desulfated heparin digests.

Table II shows the percentage distributions of unsaturated disaccharides for the digests of MTSTFA-treated 80, 90, and 100% 6-O-desulfated heparins in addition to those of intact, solvolyysis/N-resultation-treated 100% 6-O-desulfated and 2-O-desulfated heparins. Since each percentage distribution of unsaturated disaccharides was calculated from each profile obtained after application of the same amount of sample to SAX-HPLC, comparison between mutual percentage distribution could be valid. The ΔDiHS-(U,6,N)S contents were decreased with the increase of 6-O-desulfation degree and 4.9, 0.1, and 0.0% for MTSTFA-treated 80, 90, and 100% 6-O-desulfated heparins. On the other hand, the ΔDiHS-(U,N)S contents were almost unchanged irrespective of 6-O-desulfation degree and 57.0, 57.6, and 53.4% for MTSTFA-treated 80, 90, and 100% 6-O-desulfated heparins, respectively. The ΔDiHS-NS contents were increased with 6-O-desulfation degree and 13.5, 20.1, and 25.9% for MTSTFA-treated 80, 90, and 100% 6-O-desulfated

Fig. 2. Time dependence of 6-O-desulfation degree of MTSTFA-treated heparin. 6-O-Desulfation reactions of heparin were performed at 500-mg scale in the presence of MTSTFA under three different reaction temperature conditions, at 80 (□), 95 (●), and 110 °C (○). Calculation of 6-O-desulfation degree was performed according to the change in peak area ratio of ISM to ISMS (see Fig. 1) before and after each 6-O-desulfation reaction.

where $P_0$ indicates signal intensity of 62.7 ppm of intact heparin; $Q_0$ indicates signal intensity of 69.1 ppm of intact heparin; $P_1$ indicates signal intensity of 62.7 ppm of 6-O-desulfated heparin; and $Q_1$ indicates signal intensity of 69.1 ppm of 6-O-desulfated heparin.

The 6-O-desulfation degree ($D_2$) increased with the increase of reaction time, whose time dependence was associated with the kinetics of pseudo first-order reaction. The 6-O-desulfation was again confirmed to be essentially complete in 2 h (Table I). The good coincidence between both 6-O-desulfation degrees of $D_1$ and $D_2$ indicates that the estimation of the degree of 6-O-desulfation is reproducible enough to confirm the completion of the 6-O-desulfation of MTSTFA-treated heparin at 110 °C for 2 h.

Purification with the Modified Procedure and Enzymatic Digestion of 6-O-Desulfated Heparin—The 6-O-desulfated heparin obtained from 500-mg scale reaction in the presence of MTSTFA at 110 °C for 2 h was revealed to contain no 6-O-sulfate group in the sugar backbone of the polysaccharide. Enzymatic digestion of this heparin derivative with heparitinase and heparinase, however, produced small amounts of unsaturated disaccharides which were less than 30% of total disaccharide units composing the polymer (data not shown). In the previous study (39), 6-O-desulfated heparin was purified without diminishing the whitish turbidity by treatment under reduced pressure. In this study, degradation of MTSTFA by the addition of distilled water was performed after 10-fold concentrations of the reaction mixture with a rotary evaporator under reduced pressure at 35 °C for 15 min to minimize a generation of whitish turbidity.

The final products obtained from 1-, 1.5-, and 2.5-h reactions at 6-g scale by the modified purification procedure were subsequently subjected to chemical disaccharide analyses. The 6-O-desulfation degrees of the final products obtained from 1-, 1.5-, and 2.5-h reactions were calculated to be ~80, 90, and 100%, respectively. Gel permeation chromatography-HPLC profiles of 80, 90 and 100% 6-O-desulfated heparins after enzymatic digestions with heparitinase and heparinase showed that the enzymatic digestion degrees were 93, 81, and 82% for 80, 90, and 100% 6-O-desulfated heparins, respectively, which were almost identical to the digestibility of intact heparin. The digestibilities of these 6-O-desulfated heparins purified by the modified procedure were greatly improved compared with that (less than 30%) of 6-O-desulfated heparin purified by the previous procedure (39). Fig. 4 shows the SAX-HPLC profiles of 80, 90, and 100% 6-O-desulfated heparins after digestions with heparitinase and heparinase. Major peaks observed were ΔDiHS-(U,N)S eluted at 16 min, ΔDiHS-NS eluted at 11 min, and ΔDiHS-0S eluted at 3 min for all of 80, 90, and 100% 6-O-desulfated heparin digests. The peak corresponding to ΔDiHS-(U,6,N)S eluted at 22 min was not found on the SAX-HPLC profile of 100% 6-O-desulfated heparin digest. This confirmed the occurrence of 6-O-desulfation by MTSTFA treatment of heparin. On the other hand, only a trace amount of ΔDiHS-(U,6,N)S was observed on the profiles for 80 and 90% 6-O-desulfated heparin digests.

Table II shows the percentage distributions of unsaturated disaccharides for the digests of MTSTFA-treated 80, 90, and 100% 6-O-desulfated heparins in addition to those of intact, solvolyysis/N-resultation-treated 100% 6-O-desulfated and 2-O-desulfated heparins. Since each percentage distribution of unsaturated disaccharides was calculated from each profile obtained after application of the same amount of sample to SAX-HPLC, comparison between mutual percentage distribution could be valid. The ΔDiHS-(U,6,N)S contents were decreased with the increase of 6-O-desulfation degree and 4.9, 0.1, and 0.0% for MTSTFA-treated 80, 90, and 100% 6-O-desulfated heparins. On the other hand, the ΔDiHS-(U,N)S contents were almost unchanged irrespective of 6-O-desulfation degree and 57.0, 57.6, and 53.4% for MTSTFA-treated 80, 90, and 100% 6-O-desulfated heparins, respectively. The ΔDiHS-NS contents were increased with 6-O-desulfation degree and 13.5, 20.1, and 25.9% for MTSTFA-treated 80, 90, and 100% 6-O-desulfated

Equation 2

$$D_2 = \frac{Q_0 \times P_1 - P_0 \times Q_1}{Q_0 \times (P_1 + Q_1)} \times 100(\%)$$
heparins, respectively. Furthermore, the $\Delta$DiHS-0S contents were almost unchanged irrespective of 6-O-desulfation degree and 10.1, 11.4, and 9.3% for MTSTFA-treated 80, 90, and 100% 6-O-desulfated heparins, respectively.

The contents of $\Delta$DiHS-(U,N)S and $\Delta$DiHS-NS in solvolysis/N-resulfation-treated 100% 6-O-desulfated heparin digests were 21.5 and 65.2%, respectively. On the other hand, the contents of $\Delta$DiHS-(U,N)S and $\Delta$DiHS-(U,6,N)S in MTSTFA-treated 100% 6-O-desulfated heparin digests were 53.4 and 25.9%, respectively. If 6-O-desulfation reaction occurs exclusively during MTSTFA treatment of heparin at 110 °C for 2.5 h without any side reaction, the contents of $\Delta$DiHS-(U,N)S and $\Delta$DiHS-NS should be 74.2 and 13.6%, respectively. If 6-O-desulfation reaction occurs exclusively during MTSTFA treatment of heparin at 110 °C for 2.5 h without any side reaction, the contents of $\Delta$DiHS-(U,N)S and $\Delta$DiHS-NS in MTSTFA-treated 100% 6-O-desulfated heparin digests were 21.5 and 65.2%, respectively. The former value is the sum of $\Delta$DiHS-(U,6,N)S and $\Delta$DiHS-(U,N)S contents in intact heparin digests, whereas the latter value is the sum of $\Delta$DiHS-(6,N)S and $\Delta$DiHS-NS contents in intact heparin digests (Table II). These results suggested that a partial 2-O-desulfation occurred during the MTSTFA-treated 6-O-desulfation as a side reaction, resulting in the partial additional conversion of $\Delta$DiHS-(U,N)S into $\Delta$DiHS-NS. Furthermore, the observation was ascertained by 13C NMR spectrum of 100% 6-O-desulfated heparin (data not shown). Accordingly, the degree of 2-O-desulfation was estimated as shown in Table III. The 2-O-desulfation degree ($D_2$) was calculated with the SAX-HPLC data by Equation 3,

$$D_2 = \frac{L_0 \times K_1 \times L_1}{L_0 \times (K_1 + L_1)} \times 100(\%)$$

where $L_0$ indicates sum of peak areas of $\Delta$DiHS-NS and $\Delta$DiHS-(6,N)S of intact heparin; $L_1$ indicates sum of peak areas of $\Delta$DiHS-(U,N)S and $\Delta$DiHS-(U,6,N)S of intact heparin; $K_1$ indicates peak area of $\Delta$DiHS-NS of 6-O-desulfated heparin;

![Fig. 3. Time-dependent changes in 13C NMR spectra of MTSTFA-treated 6-O-desulfated heparins prepared from various reaction times. 6-O-Desulfated heparins were generated by 500-mg scale reaction in the presence of MTSTFA at 110 °C for 0 (a), 15 (b), 30 (c), 60 (d), and 120 min (e). The signal indicated by 1 represents C-6 of GlcNS(6S), and the signal indicated by 2 represents C-6 of GlcNS, as seen in a.](http://www.jbc.org/)
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The 6-O-desulfation degrees were calculated to be 4.3, 12.3, and 20.3% for 80, 90, and 100% 6-O-desulfated heparins, respectively. The 2-O-desulfation degree gradually increased with the increase of 6-O-desulfation degree, indicating that 2-O-desulfation occurs during 6-O-desulfation reaction as a side effect. The regioselectivity of the present MTSTFA-treated 6-O-desulfation was accompanied by only 20% of 6-O-desulfation as a side reaction.

6-O-Desulfated Heparins as Inhibitors of COS-1 Cell Adhesion to bFGF-coated Wells—A cell-based assay that allows the evaluation of interactions between heparin derivatives and bFGF has been reported (44). We utilized a similar assay to evaluate the ability of the present 6-O-desulfated heparins selectively to inhibit the adhesion of COS-1 cell through its surface heparan sulfate proteoglycan to bFGF-coated plates.
during a 10-min incubation period, whereas no adhesion was observed to a non-coated plate in the same time interval (data not shown).

Fig. 5 shows the effects of intact 2-O-desulfated, MTSTFA-treated 6-O-desulfated heparins and solvolysis/N-resulfation-treated 6-O-desulfated heparins on the inhibition of COS-1 cell adhesion to bFGF-coated wells. The amount of COS-1 cell adhesion in the absence of intact and modified heparins was taken for maximum extent of cell adhesion (minimum inhibition) and was represented as 0% inhibition. All specimens used were shown to exhibit no cytotoxicity (data not shown). The inhibition (% of COS-1 cell adhesion increased with the increase of the concentration of intact and MTSTFA-treated 6-O-desulfated heparins, whereas it was not affected by the 2-O-desulfated heparin added. The inhibitory effect of solvolysis/N-resulfation-treated 6-O-desulfated heparin was rather smaller.

In order to evaluate the inhibition ability of intact, 2-O-desulfated, MTSTFA-treated 6-O-desulfated heparins and solvolysis/N-resulfation-treated 6-O-desulfated heparins, half-inhibition concentrations (IC$_{50}$) were measured from the above inhibition curves as shown in Table III. IC$_{50}$ values of intact and MTSTFA-treated 80, 90, and 100% 6-O-desulfated heparins were 2.26, and 2.14, 3.18, and 4.11 μg/ml, respectively, whereas IC$_{50}$ values of solvolysis/N-resulfation-treated 100% 6-O-desulfated and 2-O-desulfated heparins were both $>$200 μg/ml. These results indicate that COS-1 cells bind to bFGF-coated plates through a specific interaction between cell surface heparan sulfate proteoglycan and immobilized bFGF and that the exogenous heparin or heparin derivatives competitively inhibit the interactions (45, 46). In another words, 6-O-desulfated heparin was implied to possess strong affinity to bFGF. Furthermore, these results suggest that a high content of 2-O-sulfate groups in heparin polymer is required for specific binding of heparin and/or HS to bFGF, whereas that of 6-O-sulfate groups is not.

**Effect of 6-O-Desulfated Heparins on the bFGF-induced Proliferation of Non-chlorate-treated A31 Cells—**Fig. 6a shows the effect of 6-O-desulfated heparins on the bFGF-induced proliferation of non-chlorate-treated A31 cells. The bFGF-induced proliferation of A31 cells increased to almost 500% relative to control with the increase of concentration of heparin up to 20 μg/ml, whereas it did not change essentially at higher concentrations of heparin. The proliferations of A31 cells increased to 450–500% relative to control with the increase of concentrations of MTSTFA-treated 80, 90, and 100% 6-O-desulfated heparins up to 20 μg/ml (partial data not shown). Accordingly, it was suggested that MTSTFA-treated 6-O-desulfated heparins apparently possess high potential (more than 85% of that of intact heparin) to accelerate the bFGF-induced proliferation of the A31 cells, irrespective of 6-O-desulfation degree, because no proliferation occurred in the absence of bFGF even at higher concentrations of 6-O-desulfated heparin (data not shown). On the other hand, the bFGF-induced proliferation of A31 cells increased to almost 200% relative to control with the increase of concentration of solvolysis/N-resulfation-treated 100% 6-O-desulfated heparin up to 20 μg/ml. Thus, solvolysis/N-resulfation-treated 100% 6-O-desulfated heparin apparently possesses relatively low potential (25% that of intact heparin) to accelerate the bFGF-induced proliferation of the A31 cells. 2-O-Desulfated heparin showed no ability to accelerate the bFGF-induced proliferation of the A31 cells under the present conditions. As a whole, the manner of binding inhibition (Fig. 5) resembled that of fibroblast proliferation (Fig. 6a). This coincidence suggests that affinity of heparin derivative to bFGF is closely related to its accelerative capability of fibroblast proliferation under the present conditions adopted for assay.

**Effect of 6-O-Desulfated Heparins on the bFGF-induced Proliferation of Chlorate-treated A31 Cells—**Fig. 6b shows the effect of 6-O-desulfated heparins on the bFGF-induced proliferation of chlorate-treated A31 cells. The bFGF-induced proliferation of chlorate-treated A31 cells increased to almost 550% relative to control with the increase of concentration of heparin up to 1 μg/ml. The bFGF-induced proliferation of chlorate-treated A31 cells increased to $>$600% relative to control with the increase of concentrations of MTSTFA-treated 100% 6-O-desulfated heparin up to 1 μg/ml. It was shown that MTSTFA-treated 6-O-desulfated heparins possess a slightly higher potential (110% of that of intact heparin) to accelerate the bFGF-induced proliferation of the chlorate-treated A31 cells.

On the other hand, the bFGF-induced proliferation of A31 cells increased to almost 350% relative to control with the increase of concentration of solvolysis/N-resulfation-treated 100% 6-O-desulfated heparin up to 1 μg/ml. It was shown that solvolysis/N-resulfation-treated 100% 6-O-desulfated heparin possesses intermediate potential (55% of that of intact heparin) to accelerate the bFGF-induced proliferation of the present chlorate-treated A31 cells. 2-O-Desulfated heparin showed no ability to accelerate the bFGF-induced proliferation of the chlorate-treated A31 cells under the present conditions. These results strongly suggest that the bFGF-induced proliferation of
chlorate-treated A31 cells does not require 6-O-sulfate groups in the saccharide polymer of heparin derivative.

**DISCUSSION**

In the present study, intact and MTSTFA-treated heparin samples for IPRP-HPLC analyses were not subjected to hydrazinolysis before depolymerization and subsequent labeling reactions in order to obtain simplified HPLC patterns (not taking the disaccharide units bearing N-acetyl groups into consideration). Since only less than 20% of the disaccharide units are known to be N-acetylated, the remaining more than 80% of the disaccharide units bearing N-sulfate group could be susceptible to HONO-depolymerization. Accordingly, the resultant HPLC profile reflects approximate but almost reliable percentage distribution of disaccharide units initially bearing 6-O-sulfate group, because more than 90% of the disaccharide units initially bearing 6-O-sulfate group were additionally N-sulfated. Thus, reliability of the 6-O-desulfation degrees calculated from the IPRP-HPLC profiles would be enough high, as confirmed by $^{13}$C NMR spectra (Table I).

In the previous method, chemical treatment of heparin with a silylating reagent, MTSTFA, at 95 °C for 2 h generated incompletely 6-O-desulfated heparin unsusceptible to enzymatic digestion with the combination of heparitinase and heparinase (39). Next, we have found that MTSTFA treatment of heparin at 110 °C for 2 h enables specific and quantitative 6-O-desulfation, whereas the obtained product has not been well characterized enzymatically (38). In the present study, we examined the reaction conditions of MTSTFA-treated 6-O-desulfation to find that MTSTFA treatment of heparin at 110 °C for 2 h is optimum for complete 6-O-desulfation as was clarified by chemical disaccharide analyses (Table I and Figs. 1 and 2) and $^{13}$C NMR spectra (Table I and Fig. 3). Although the complete 6-O-desulfation was accompanied by about 20% of 2-O-desulfation (Table III) as a consequence of side reaction, the regioselectivity of the 6-O-desulfation reaction by the treat-
ment with MTSTFA was confirmed to be high enough to evaluate the involvement of 6-O-sulfate group(s) in the promotion of bFGF mitogenic activity with reference to that of intact heparin because more than 50% of the disaccharide units are sulfated at the O-2 position of IdoUA moiety.

The purification procedure of the 6-O-desulfated heparin from the reaction mixture was refined to improve its digestibility with heparitinase and heparinase. The 6-O-desulfated heparin purified according to the previous procedure (39) was not fully susceptible to the digestion with heparitinase and heparinase, attaining the partial digestibility less than 30%. The poor digestibility would be due to water-insoluble desulfated heparin derivative carrying residual trimethylsilyl groups that were not hydrolyzed unexpectedly during the dialysis step. In the modified procedure, degradation of MTSTFA was performed after 10-fold concentrations of the reaction mixture, whereas whitish turbidity was diminished by the treatment under reduced pressure at 35 °C for 15 min. This modified procedure overcame the disadvantage of the low digestibility of 6-O-desulfated heparin purified by the previous procedure, resulting in high enzymatic digestibility of the completely 6-O-desulfated heparins that were at the levels similar to that of intact heparin. The MTSTFA-treated 100% 6-O-desulfated heparin with clarified physicochemical properties is a useful tool for the examination of the involvement of 6-O-sulfate group(s) of heparin in the interaction between heparin, bFGF, and FGFRs (13–29), as well as the present 80 and 90% 6-O-desulfated heparins with reference to intact heparin.

The abilities of the MTSTFA-treated 100% 6-O-desulfated, solvolysis/N-resulfation-treated 6-O-desulfated and 2-O-desulfated heparins to accelerate the proliferation of non-chlorate-treated A31 cells were 88, 26, and 0% relative to that of intact heparin (100%) in the presence of bFGF, respectively (Fig. 6a). The ratios of disaccharide units containing both N- and 2-O-sulfate groups compared with total disaccharide units are 74, 53, 22, and 0% for intact, MTSTFA-treated 100% 6-O-desulfated, solvolysis/N-resulfation-treated 6-O-desulfated and 2-O-desulfated heparins, respectively (Table III). These results strongly indicate that the content of disaccharide units whose structures are IdoUA(2S)–4GlcNS irrespective of additional sulfation at O-6 of GlcNS moiety in heparin/heparan sulfate derivatives is well correlated to the accelerative capability of bFGF-induced A31 cell proliferation. In addition, the correlation between bFGF activation and the content of IdoUA(2S)–α1–4GlcNS irrespective of additional sulfation at O-6 of GlcNS moiety was also observed when chlorate-treated A31 cells were used for assay (Fig. 6b).

Guimond et al. (47) demonstrated that preferentially 6-O-desulfated heparin competes with native heparin for binding to bFGF but that it is unable to promote the mitogenic activity of bFGF. The former finding is comparable to our results that the present completely 6-O-desulfated heparin possesses high affinity to bFGF (IC50 = 4.11 μg/ml) which is almost similar to that of intact heparin (IC50 = 2.26 μg/ml) as was clarified by competitive cell adhesion assay (Table III and Fig. 5). Their latter finding, however, is contradictory to our results, since the present completely 6-O-desulfated heparin possesses high mitogenic activity as that of intact heparin at 1 μg/ml concentration using chlorate-treated 3T3 fibroblast for assay (Fig. 6b). This discrepancy would be due to the difference in preparation procedure and physicochemical properties of 6-O-desulfated heparin, because their 6-O-desulfated heparin was prepared by the combination of solvolysis and N-resulfation (48). Pye et al. (49) also reported that refined decasaccharide fractions were obtained from heparan sulfate and that the fractions contained certain ratios of 2-O- and N-sulfate groups but different ratios of 6-O-sulfate groups. Furthermore, they indicated that a positive correlation is observed between increasing 6-O-sulfate content and maximum bFGF activation for fibroblast proliferation when the above decasaccharide fractions were subjected to assay. Their findings are, however, contradictory to our results, since the present completely 6-O-desulfated heparin polymer possessed high mitogenic activity as that of intact heparin at 1 μg/ml concentration using chlorate-treated A31 cells for assay (Fig. 6b). This discrepancy would be due to the difference in the molecular size of heparin/heparan sulfate derivatives and/or cell species used which may express different types of FGFRs. In fact, four different isomers are known as FGFR (50–54).

Ornitz et al. (19) reported that chemically synthesized trisaccharides such as GlcUA β1→4GlcNAcα1→4GlcUA β1→OMe and IdoUA α1→4GlcNAcα1→4GlcUAβ1→OMe possess an enhanced ability for mitogenic activities of bFGF comparable to that of intact heparin. Furthermore, they concluded that signal transduction occurs due to the interaction between carboxylate backbone of heparin/HS bearing no sulfate group, bFGF and FGFRs when the F32 cell line, which express FGFR-1, was used for cell proliferation assay. This model is, however, contradictory to the previous findings that bFGF preferentially binds to sequences in which the predominant disaccharide is IdoUA(2S)α1→4GlcNS (11, 12).

X-ray analyses of crystal structures of biologically active FGF dimer indicated that both FGF molecules forming one dimer interact with the IgII and IgIII domains of FGFRs after inducing FGF/FGFR dimerization, irrespective of acidic and basic FGF species (28, 29). According to this proposed model, the dimerization or even oligomerization of bFGF is mediated by heparin and/or HS, confirming the present results obtained by Kan et al. (55).

Moy et al. (23) showed that a trans-oriented symmetric dimer of bFGF is formed in the presence of the heparin-derived tetrasaccharide, whereas two cis-oriented dimers are formed in the presence of the heparin-derived decasaccharide. Furthermore, they suggested that cis-oriented bFGF dimer is the minimal biologically active structural unit of bFGF. This theory is contradictory to the observation by Ornitz et al. (19) as quoted above, because the active trisaccharides can induce only trans-oriented bFGF dimer but not cis-oriented one due to lack of chain length.

Although there are some ambiguities in the recent studies as described above, a possibility to explain the present results would be provided. The completely 6-O-desulfated heparin may induce oligomerization of bFGF around the saccharide polymer of itself in preferentially cis-oriented manner more than solvolysis/N-resulfation-treated 6-O-desulfated heparin but slightly less than intact heparin due to the content of IdoUA(2S)–α1→4GlcNS irrespective of additional sulfation at O-6 of GlcNS moiety. This assumption would be related to the above positive correlation between bFGF activation and the content of IdoUA(2S)–α1→4GlcNS irrespective of additional sulfation at O-6 of GlcNS moiety. In other words, it is likely that the more highly specific bFGF is oligomerized in the presence of heparin derivative containing abundant IdoUA(2S)α1→4GlcNS units irrespective of additional sulfation at O-6 of GlcNS moiety, the more effectively the dimerization of FGFRs occurs, resulting in efficient signal transduction through the complex of the present heparin derivative, bFGF and FGFRs. However, more detailed understanding of the overall interactions of exogenous polysaccharides with bFGF in the presence of the receptors is required to explain the ability of MTSTFA-treated completely 6-O-desulfated heparin to enhance bFGF activity.

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REFERENCES

1. Shimada, K., Gill, P. J., Silbert, J. E., Douglas, W. H., and Fanburg, B. L. (1981) J. Clin. Invest. 68, 995–1002
2. Chong, P., Oosta, M. G., Bensadoun, A., and Rosenberg, R. D. (1981) J. Biol. Chem. 256, 12993–12998
3. Marcum, J. A., and Rosenberg, R. D. (1984) Biochemistry 23, 1730–1737
4. Marcum, J. A., and Rosenberg, R. D. (1985) Biochim. Biophys. Res. Commun. 126, 365–372
5. Flannemha, R., Moscatteli, D., and Rifkin, D. (1990) J. Cell Biol. 111, 1651–1659
6. Kifere, M. C., Stephens, J. C., Crawford, K., Okino, K., and Barr, P. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6985–6989
7. Rapraeger, A. C., Krufla, A., and Obin, B. B. (1991) Science 252, 1705–1708
8. Ishihara, M. (1993) Trends Glycosci. Glycotechnol. 5, 343–354
9. Cass, E., Oreste, P., Torri, G., Zoppetti, G., Chassy, J., Lormene, J. C., Petitou, M., and Sinay, P. (1981) Biochem. J. 197, 599–609
10. Thunberg, L., Backstrom, G., and Lindahl, U. (1982) Carbohydr. Res. 100, 393–410
11. Turnbull, J. E., Fernig, D. G., Ke, Y., Wilkinson, M. C., and Gallagher, J. T. (1992) J. Biol. Chem. 267, 10337–10341
12. Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K., and Kimata, K. (1992) Biochem. J. 285, 805–813
13. Aviezer, D., Levy, E., Safran, M., Svanhl, C., Budbecke, E., Schmidt, A., David, V., and Y von. A. (1994) J. Biol. Chem. 269, 114–121
14. Boghman, M., Mansukhani, A., Dell’Era, P., Zoppetti, G., Oreste, P., and Presta, M. (1994) Biochim. Biophys. Res. Commun. 203, 450–458
15. McKeehan, W. L., and Kan, M. (1994) Mol. Reprod. Dev. 39, 69–81
16. Ornitz, D., Herr, A. B., Nilsson, M., Westman, J., Svanhl, C. M., and Waksman, G. (1995) Science 268, 432–436
17. Richard, C., Liuzzo, J. P., and Moscatelli, D. (1995) J. Biol. Chem. 270, 24185–24191
18. Venkataraman, G., Sasisekharan, V., Herr, A. B., Ornitz, D. M., Waksman, G., Cooney, C. L., Langer, R., and Sasisekharan, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 845–850
19. Kan, M., Wang, F., To, B., Gabriel, J. L., and McKeehan, W. L. (1996) J. Biol. Chem. 271, 26143–26148
20. Moy, F. J., Safran, M., Sefd, A. P., Kitchen, D., Kohlen, P., Aviezer, D., Y von. A., and Powers, B. (1997) Biochemistry 36, 4752–4759
21. Wang, H., Toida, T., Kim, Y. S., Capila, I., Hileman, R. E., Bernfield, M., and Linhardt, R. J. (1997) Biochem. Biophys. Res. Commun. 235, 369–373
22. Herr, A. B., Ornitz, D. M., Sasisekharan, R., Venkataraman, G., and Waksman, G. (1997) J. Biol. Chem. 272, 16882–16889
23. Lam, K., Rao, V. S., and Qasba, P. K. (1998) J. Biomol. Struct. & Dyn. 15, 1009–1027
24. Ven der Begehe, L., Mortier, I., Zanibellato, C., Amalrie, F., Prats, H., and Bugler, B. (1994) Biochem. Biophys. Res. Commun. 209, 423–427
25. Rem, C., Caro, C., Marcum, J. A., and Rosenberg, R. D. (1991) Biochim. Biophys. Acta 1035, 71–77
26. Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P., and Gallagher, J. T. (1996) J. Biol. Chem. 271, 20286–20292
27. Yayon, A., Klagsbrun, M., Esko, J. D., Leder, P., and Ornitz, D. M. (1991) Cell 64, 641–648
28. Mansukhani, A., Dell’Era, P., Moscatelli, D., Kohlen, S., Hanafusa, H., and Basilico, C. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3305–3309
29. Ornitz, D. M., and Leder, P. (1997) J. Biol. Chem. 272, 16905–16911
30. Wang, J. K., Gao, G., and Goldfarb, M. (1994) Mol. Cell. Biol. 14, 183–188
31. Kan, M., Wang, F., Xu, J., and McKeehan, W. L. (1995) Science 258, 1918–1921
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