Dermatan Sulfate as a Bifunctional Antithrombotic

Junichi Onaya, Mamoru Kyogashima, Tokiko Sakai, Mikio Arai, Satoshi Miyauchi, Takashi Sakamoto, and *Akikazu Takada

Seikagaku Corporation, Tokyo Research Institute, Tokyo 207-0021, Japan, and *Department of Physiology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan

Key Words: Dermatan sulfate—Glycosaminoglycans—Heparin cofactor II—Tissue plasminogen activator—Anticoagulation—Fibrinolysis

INTRODUCTION

Dermatan sulfate (DS) is one of the sulfated glycosaminoglycans (GAGs) that occur as GAG-chains of proteoglycans (PGs). DS is present in virtually all animals in a wide variety of tissues, such as dermis, tendon, skeletal muscle, vascular wall, cornea, bone, and cartilage. DS is believed to have important roles in cell-to-cell or cell-to-matrix interactions (7,8,10). For example, DS chains in the arterial walls are believed to provide flexibility to the blood vessels, and those in the corneal interstitium are believed to provide transparency to the cornea (6,16). Thus, DS is a naturally occurring molecule in many mammals, including humans, and birds and unlike many protein molecules, it generally exhibits no antigenicity. Another important physiological property of DS is its anticoagulant activity (5,7,12). DS increases the rate of inhibition of thrombin by approximately 1000-fold by binding to heparin cofactor II (HC II) (14,20,21). However, the anticoagulant activity of DS is milder than that of heparin or low molecular weight heparin (LMWH), both of which are other types of anticoagulant GAG and widely utilized in the clinical field as antithrombotics (2). In addition to anticoagulant activity, DS has been suggested to promote fibrinolytic activity. Abbadini et al. reported the release of tissue plasminogen activator (t-PA) from endothelial cells by DS (1). In collaboration with Dr. Krupinski, we recently demonstrated that DS enhances the thrombolysis of laser-induced thrombus in vivo (11). Thus, another important characteristic of DS is fibrinolytic activity.

To further characterize DS, we examined and demonstrated its fibrinolytic activity as well as its anticoagulant activity. In addition, to assess the usefulness of DS, pharmacological experiments were also carried out to compare the differences between DS and heparin or LMWH.
CHEMISTRY

DS is easily cleaved from the core protein (portion of PG) by β elimination with alkali treatment. DS is a linear polysaccharide, which consists of repeating units of N-acetyl-D-galactosamine (GalNAc) and uronic acid [mainly L-iduronic acid (IdoA) and some D-glucuronic acid]. These sugars are heterogeneously O-sulfated. The most commonly sulfated position was found to be the 4-position of GalNAc residues and occasionally the 6-position of GalNAc and the 2-position of IdoA residues (Fig. 1a) (8). Maimone and Tollefsen reported that the minimal essential structure of DS required for it to bind to HC II is specially arranged as a heptasaccharide sequence (Fig. 1b); in contrast, as 12–14 saccharides containing the sequence are required for HC II to exhibit antithrombin activity (13,14,21).

PHARMACOLOGY

Effect of DS on APTT and PT

The effect of DS on activated partial thromboplastin time (APTT) and prothrombin time (PT) of rat plasma was determined with an automatic coagulometer (Fig. 2). For com-

---

**FIG. 1a.** General chemical structure of DS. **b.** Structure in DS for binding to heparin cofactor II. Modified from ref. 14 with permission.
Comparison, heparin and low molecular weight heparin (LMWH, Fragmin®) were used. At concentrations higher than 50 μg/mL and at 100 μg/mL DS gradually prolonged APTT and PT, respectively, in a dose-dependent manner, which was probably due to the anti-thrombin activity of HC II that is accelerated by DS. On the other hand, at concentrations higher than 1 μg/mL heparin suddenly prolonged both parameters, and at concentrations higher than 5 μg/mL and at 10 μg/mL LMWH prolonged APTT and PT, respectively. Unlike the activity of DS, the anticoagulant activity of these two kinds of heparins is due mainly to antithrombin III binding (3,24).

**Effect of DS on ECLT**

Euglobulin clot lysis time (ECLT) is a test of fibrinolytic activity (25). The euglobulin fraction is generally believed not to contain inhibitors of fibrinolysis, such as α2 antiplasmin. The fraction of rat plasma was prepared by conventional methods (9). ECLT was carried out according to methods described by Urano et al. (22). One hundred microliters of euglobulin solution containing various amounts of DS, heparin, or LMWH was transferred to wells containing 20 μL of thrombin (10 unit/mL) in a 96-well microtiter plate. The decrease of absorbance by the decreased turbidity due to clot lysis was periodically monitored at 340 nm, and the time to minimum O.D. value was defined as euglobulin clot lysis time (ECLT). The results are shown in Fig. 3. Five μg/mL and 50 μg/mL of DS, both of which did not prolong or only slightly prolonged APTT and/or PT, significantly shortened ECLT in a dose-dependent manner. In contrast, 5 μg/mL of heparin or LMWH slightly or hardly shortened ECLT, whereas 5 μg/mL of heparin remarkably prolonged APTT and PT. The same dose of LMWH considerably prolonged APTT (Fig. 2). These results suggest that DS may be a well-balanced antithrombotic from the standpoint of anticoagulant as well as fibrinolytic activity.

**Effect of DS on the Activation of Plasminogen by t-PA**

To clarify the possible role of DS on acceleration of ECLT, the stimulatory effect of DS on the activation of plasminogen by t-PA was examined using a synthetic peptide as a substrate (23). The assay was carried out using a microtiter plate. Plasminogen (glu-plasminogen) was incubated with DS, t-PA (sct-PA), and synthetic peptide S-2251 (H-D-Val-Leu-Lys-p-nitroanilide or pNA). Absorbance at 405 nm was measured periodically and initial velocity was calculated from the slope of the plot of A405 vs. t². The ratio of initial velocity in the presence and in the absence of DS was defined as a potentiation factor. As shown in Fig. 4, DS dose-dependently potentiated plasminogen activity by t-PA.

**Effect of DS on PCLT by t-PA**

Plasma clot lysis time (PCLT), another test of fibrinolysis, was carried out to elucidate the effect of DS on the lysis of plasma containing exogeneous t-PA. One hundred microliters of human plasma were mixed in the wells of 96 well microtiter plates with the same volume of Tris-HCl buffer (pH 7.4) containing 6.8 U of single chain t-PA, 4 U of thrombin, and various amounts of DS. The PCLT was measured at 340 nm. The relationship between DS-PCLT/control-PCLT and DS concentration is plotted in Fig. 5.
FIG. 2. Effects of DS, LMWH and heparin on a) activated partial thromboplastin time (APTT), b) prothrombin time (PT) in rat plasma. Each value represents the mean ± SD from triplicate experiments. *P < 0.05, **P < 0.01, compared with corresponding to the control (Dunnett’s multiple comparison).
ratio decreased dose-dependently, and DS did not shorten the time without t-PA (data not shown), suggesting that DS can enhance plasma clot lysis in the presence of t-PA. These results, together with those in Fig. 3 and Fig. 4, suggest that fibrinolytic enhancement of DS may be due not only to the release of t-PA from endothelial cells (1) but also due to the direct enhancement of t-PA–mediated fibrinolysis.

Antithrombotic Effects of DS on the Rat Venous Stasis Model

A venous stasis model was prepared according to the method of Reyers et al. (18). Under anesthesia, the inferior vena cava of rat was ligated with a surgical thread below the left renal vein. For studies on preventing thrombus formation, DS or LMWH in physiological saline was administered to rats as a bolus shot through the tail vein 1 minute before ligation. In the control group, only vehicle was injected. After 3 hours of ligation, thrombi were extirpated. DS and LMWH inhibited the thrombus formation in a dose-dependent manner (Fig. 6a). Thrombus formation on administration of 3 mg/kg and 10 mg/kg of DS is comparable with that at 0.14 mg/kg and 1.4 mg/kg of LMWH, respectively. Studies on preformed thrombi were also performed. In this case, two control groups who were not administered any of the drugs were prepared. In the first group (preformed...
group), thrombi were extirpated from rats at 6 hours after the ligation. In the second group (untreated group), vehicle was injected at 6 hours after the ligation. After 2 more hours, thrombi were extirpated from the rats. As shown in Fig. 6b, thrombi developed during these 2 hours. When DS or LMWH was administered instead of vehicle, the development of thrombi was inhibited in a dose-dependent manner. It should be noted that although 10 mg/kg of DS and 1.4 mg/kg of LMWH exhibited comparable effects on prevention of thrombus formation, 10 mg/kg of DS more effectively reduced preformed thrombi than 1.4 mg/kg of LMWH. It was reported that venous occlusion elevated blood t-PA (22). Furthermore, DS itself was reported to release t-PA from endothelial cells (1). Studies on preformed thrombi suggest that DS might potentiate the t-PA which was induced by vein ligation and/or the administration of DS itself.

**Effects of DS or DS with t-PA on Rat Laser-Induced Thrombosis Model**

The effects of DS or DS with t-PA on rat laser-induced thrombosis were examined (11). Briefly, under anesthesia, vascular lesions were induced with argon laser. An intestinal loop exposed through a hypogastric incision was spread on a self-constructed object stage and mounted on the microscope table. The laser beam was directed on small mesenteric venules of diameter 20–30 mm in the mesentery. Drugs were injected from the tail vein 30 minutes before the application of the laser. Thrombus formation was evaluated by direct observation through the microscope. The lysis of the thrombus was measured as the time required for the recanalization of the occluded vessels. Fig. 7a shows the effects of DS on the lysis of a laser-induced clot. In the absence of DS, the clot lysed in 90 seconds and the injection of DS dose-dependently enhanced the lysis. Fig. 7b shows that the
injection of 0.1 mg/kg of DS together with 0.05 mg/kg of t-PA enhanced the lysis of the clot. When injected separately, neither 0.1 mg/kg of DS or 0.05 mg/kg of t-PA significantly shortened the lysis time. These results suggest that DS may stimulate the release of t-PA from endothelial cells and that DS actually enhances t-PA-mediated thrombolysis in vivo as is shown in the in vitro experiment in Figs. 3, 4, and 5.

Effects of DS on the Endotoxin-Induced Rat DIC Model

Endotoxin administered to animals rapidly stimulates monocytes and vascular endothelial cells which release various inflammatory cytokines or tissue factor, destroying the balance in the blood coagulation system. As a result, microthrombus formation occurs in many organs (disseminated intravascular coagulation, DIC), inducing multiple organ failure (15). An endotoxin-induced rat DIC model was prepared according to the method of Yoshikawa et al. (26). Under anesthesia, polyethylene catheters were retrogradely inserted into the abdominal vena cava by way of both femoral veins. Endotoxin was continuously infused at a rate of 2.5 mg/kg/h for 4 hours through the catheter in the left femoral vein using a multi holder-type syringe pump. DS or LMWH was infused simultaneously with the endotoxin for 4 hours through the catheter in the right femoral vein (17). After infusion, blood was withdrawn and the plasma and serum were prepared. As shown in Fig. 8, in all DS-treated groups the decrease of fibrinogen (Fbg) and the increase of fibrin-fibrinogen degradation products (FDP) were significantly ameliorated. DS inhibited also the decrease in glomerular fibrin deposition (%GFD) at the rates of 2.5 and 5 mg/kg/h for

![FIG. 5. Effects of DS on plasma clot lysis time in human plasma. Each value represents the mean ± SD from triplicate experiments.](image-url)
FIG. 6. Effects of DS and LMWH on the stasis-induced venous thrombus in the rats. a) Studies on prevention of thrombus formation. Each value represents the mean ± SEM from 6–14 rats. *P < 0.05, **P < 0.01, compared with the corresponding control group (Dunnett’s multiple comparison). b) Studies on preformed thrombi. Each value represents the mean ± SEM from 6–14 rats. *P < 0.05 compared with preformed group and untreated group (Student’s t test), and compared with untreated group and treated groups (Dunnett’s multiple comparison).
4 hours and the prolongation of thrombin clotting time (TCT) at rates of 1.25 and 2.5 mg/kg/h for 4 hours. LMWH significantly suppressed the decrease of Fbg and the increase of FDP at rates of 0.35 and 0.7 mg/kg/h for 4 hours. LMWH also completely inhibited the decrease of %GFD at the rate of 0.7 mg/kg/h for 4 hours, while it further increased the prolongation of TCT. DS and LMWH comparably ameliorated the prolongation of APTT, PT, TCT, the decrease of Fbg, the increase of FDP, and %GFD.

FIG. 7. Effects of DS or DS with t-PA on rat laser-induced thrombosis. a) Effects of DS on clot lysis time. *P < 0.05 compared with no DS (Student’s t test). b) Effects of DS and t-PA on clot lysis time. *P < 0.05 compared with no DS and no t-PA (Student’s t test). Each value indicates mean ± SD from 15 determinations in each test. Modified from ref. 11 with permission.
FIG. 8. Effects of DS and LMWH on various coagulation and fibrinolytic parameters in endotoxin-induced changes. With endotoxin, DS or LMWH were continuously infused for 4 hours. Each value represents the mean ± SEM from 7–20 rats. *P < 0.05, **P < 0.01, compared with the corresponding control group (Scheffe’s multiple comparison). Control indicates untreated group. Modified from ref. 17 with permission.

FIG. 9. Effects of DS and LMWH on transection bleeding time after continuous infusion for 4 hours. Each value represents the mean ± SEM from 5–6 rats. *P < 0.05, compared with the corresponding control group (Student’s t test).
Effects of DS on Transection Bleeding Time

To further characterize its safety, we compared DS with LMWH for the effect on bleeding time. DS or LMWH were continuously infused at a rate of 5 mg/kg/h and 0.7 mg/kg/h for 4 hours, respectively, which were chosen as the best and comparable therapeutic doses of DS and LMWH for the endotoxin-induced rat DIC model (Fig. 8). Bleeding time test was performed according to the transection model described by Dejena et al. (4). After the infusion, the tail was cut transversely and the tail was placed immediately in physiological saline (37 °C), and bleeding time was determined. DS prolonged bleeding time slightly, although not significantly, whereas LMWH prolonged it significantly (Fig. 9). These results together with other reported experiments indicate that DS compares favorably with LMWH in terms of therapeutic potential for thrombotic diseases and it is much safer than LMWH.

SUMMARY

We briefly reviewed DS as a unique antithrombotic. DS is a naturally occurring molecule, is one of the sulfated GAGs, and generally exhibits no antigenicity in mammals. DS bifunctionally inhibits thrombus formation: it enhances not only anticoagulant activity by binding to HC II to inhibit thrombin but also fibrinolytic activity by releasing t-PA from endothelial cells and potentiating t-PA mediated fibrinolysis (see Fig. 10).

Acknowledgments: We thank Dr. M. Waki for critical reading of this manuscript.

REFERENCES


