Effects of Dermatan Sulfate, a Heparin Cofactor II Mediated Thrombin Inhibitor, on the Endotoxin-Induced Disseminated Intravascular Coagulation Model in the Rat: Comparison With Low-Molecular Weight Heparin, Nafamostat Mesilate and Argathroban

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ABSTRACT—Effects of dermatan sulfate (DS) on the endotoxin-induced disseminated intravascular coagulation (DIC) rat model were compared with those of low-molecular weight heparin (LMWH), nafamostat mesilate (NM) and argathroban (AR). At doses of 5, 10 or 20 mg/kg/4 hr, DS significantly ameliorated the decrease of fibrinogen (Fbg), the increase of fibrin-fibrinogen degradation products (FDP) and except at the highest dose (20 mg/kg/4 hr), the prolongation of thrombin clotting time (TCT). It also decreased the glomerular fibrin deposits (%GFD) at doses of 10 or 20 mg/kg/4 hr. LMWH suppressed the decrease of Fbg and the increase of FDP at doses of 1.4 or 2.8 mg/kg/4 hr. Only the highest dose of LMWH suppressed the decrease of the platelet count (PL), the prolongation of prothrombin time, and improved the %GFD. AR suppressed the decrease of PL and improved the %GFD. At the dose required to improve the %GFD, DS (5, 10 mg/kg/4 hr) significantly suppressed the prolongation of TCT, which is related to the bleeding frequency, while LMWH and AR further increased the prolongation of the TCT. These results suggest that DS has potential as a therapeutic drug with a lower hemorrhagic risk as compared with LMWH and AR in the treatment of DIC.

Keywords: Endotoxin, Dermatan sulfate, Disseminated intravascular coagulation, Hemorrhagic tendency, Thrombin clotting time

DS is one of the glycosaminoglycans that has been reported to occur in a wide variety of tissues such as dermis, tendon, skeletal muscle, vascular wall, bone and cartilage in virtually all animals. It is a sulfated polymer consisting of three kinds of mono-saccharides: *N*-acetyl-D-galactosamine and L-iduronic acid or D-glucuronic acid (1). One of its physiological actions is based on the specific inhibition of thrombin, mediated by heparin cofactor II (2, 3). DS has been demonstrated to be an effective anti-thrombotic agent with a lower hemorrhagic risk than standard heparin in experimental animal models (4–6). In addition, DS has been reported to activate the fibrinolytic system by inducing the release of tissue plasminogen activator, and it shows anti-inflammatory effects in vitro (7, 8). Therefore, DS has been expected to be a new drug for the treatment of DIC syndrome, deep vein thrombosis, or the prevention of coagulation in hemodialysis for chronic renal failure (9–11).

DIC is a syndrome that is induced by a variety of serious diseases. During progression of the syndrome, DIC induces multiple organ failure by formation of fibrin thrombi and a bleeding tendency caused by the loss of platelets and/or coagulant factors (12). The former is a consequence of septicemia, and the latter is caused by pre-myeloblastic leukemia. A representative clinically used drug is standard heparin. However, because standard heparin has marked side effects accompanied by bleeding, many drugs that cause less bleeding and have anti-thrombotic action have been developed (e.g., LMWH, NM, AR) or are currently under development.

Abbreviations used are: DS, dermatan sulfate; DIC, disseminated intravascular coagulation; LMWH, low-molecular weight heparin; NM, nafamostat mesilate; AR, argathroban; FDP, fibrin-fibrinogen degradation products; Fbg, fibrinogen; PL, platelet count; APTT, activated partial thromboplastin time; PT, prothrombin time; TCT, thrombin clotting time; GFD, glomerular fibrin deposits.

The endotoxin-induced DIC model is a representational model caused by the depletions of platelet and coagulant factors. There is a good possibility that DS with its anti-thrombotic effect could inhibit a disorder of the blood coagulation system induced by endotoxin. In this study, we examined the effect of DS and other anti-coagulant agents using the endotoxin-induced DIC model in rats and discussed the action of these agents in this model.

MATERIALS AND METHODS

Animals

Six-week-old male Sprague-Dawley rats were obtained from Charles River (Atsugi). After a resting period of 7 days, rats with body weights between 182–224 g were used for the experiments.

Test substances and reagents

DS from chicken comb was prepared at Seikagaku Corporation (Tokyo); it had a mean molecular weight of 36,000 as determined through gel filtration analysis with high performance liquid chromatograpy (13). LMWH, NM and AR were obtained from Kabi Pharmacia (Stockholm, Sweden), Torii Yakuhin Co. (Tokyo) and Daiichi-Seiyaku Co. (Tokyo), respectively. For the experiments, DS or NM was dissolved 0.9% NaCl solution to the appropriate concentration. LMWH or AR solution was diluted in 0.9% NaCl solution to the appropriate concentration. Endotoxin (lipopolysaccharide from *E. coli*, 055:B5) was obtained from Difco (Detroit, MI, USA); it was dissolved in 0.9% NaCl solution immediately before use. The following reagents and assay kits were also used in this study: actin and thromboplastin (Baxter Diagnostics, Miami, FL, USA); human thrombin (Midori-Juji Co., Tokyo), FDP assay kit (Teikoku-zōki Co., Tokyo) and Fbg assay kit (Nitto-bōseki Co., Tokyo).

General procedures

Two types of experiments were performed: A) normal rats that were infused with each test substance alone, and B) endotoxin-induced DIC rats that were infused with each test substance. In experiment A, under anesthesia with sodium pentobarbital (50 mg/kg, i.p.), polyethylene catheters were retrogradingly inserted 4 cm into the abdominal vena cava by way of the left femoral vein of the rats. The catheter was filled with 0.9% NaCl solution, exteriorized at the nape of the neck and sealed until use. After the rat recovered from the anesthesia, test substances were continuously infused at a flow rate of 1.5 ml/hr for 4 hr through the catheter in the left femoral vein using a multi holder-type syringe pump (Model-22M; Harvard Apparatus, South Natick, MA, USA). In experiment B, the endotoxin-induced DIC model was produced according to the method of Yoshikawa et al. (14). As in experiment A, polyethylene catheters were retrogradingly inserted 4 cm into the abdominal vena cava by way of both femoral veins of the rats. Endotoxin (2.5 mg/kg/hr) was continuously infused at a flow rate of 0.75 ml/hr for 4 hr through the catheter in the left femoral vein using an multi holder-type syringe pump. Each test substance was infused simultaneously with the endotoxin at a flow rate of 0.75 ml/hr for 4 hr through the catheter in the right femoral vein. Rats in the normal control group were given 0.9% NaCl solution at a flow rate of 1.5 ml/hr for 4 hr through the catheters in both femoral veins. After the 4 hr, blood was withdrawn from the abdominal vena cava of the rats under anesthesia using a syringe with or without 3.2% sodium citrate, and the plasma and serum (only for measurement of FDP) were prepared. The kidneys were rapidly removed and fixed with 10% neutrally buffered formalin.

Parameters

The severity of DIC was determined with the following parameters: PL, APTT, PT, TCT, plasma Fbg, FDP and %GFD. PL was counted by an automatic cell counter (MEK-4500; Nihon Kohden, Co., Tokyo). For the measurement of APTT, plasma (100 μl) was mixed with 100 μl of actin and preincubated for 2 min at 37℃. After adding 0.02 M $CaCl_2$ (100 μl) into the mixture, the coagulation time was measured by a coagulometer (KC-10A; Amelung, Lemgo, Germany). For the measurement of PT, plasma (100 μl) was preincubated for 2 min at 37℃, and after adding 200 μl of thromboplastin, the coagulation time was measured. For the measurement of TCT, plasma (100 μl) was preincubated for 2 min at 37℃, and after adding 100 μl of human thrombin (10 units/ml), the coagulation time was measured. Plasma Fbg was quantified by determining the clotting time with a commercial kit that used the thrombin coagulation method. Serum FDP was quantitatively determined by evaluating the dilution magnification of agglutinated sera with a kit that used the latex agglutination reaction. The left kidneys of all rats were examined histologically by staining with a phosphotungstic acid hematoxylin. All glomeruli in each kidney were examined, and the number of the glomeruli showing any degree of fibrin-deposition was counted; the results were expressed as percentage of GFD (%GFD).

Statistical analyses

All data are expressed as the mean±S.E.M. All results obtained from the experiments were analyzed with Scheffe's multiple comparison test after the analysis of variances. Statistical significance was set at $P<0.05$.

RESULTS

Effects of DS and the other anti-DIC agents on normal rat

As shown in Table 1, 4-hr continuous injection with DS prolonged the APTT in a dose-dependent manner, and DS produced significant prolongation at doses of 5, 10 and 20 mg/kg/4 hr. It also prolonged the TCT at a dose of 20 mg/kg/4 hr. However, at doses of 1.25 and 2.5 mg /kg/4 hr, it had no effect on any parameter. LMWH significantly prolonged the APTT, PT and TCT at a dose of 2.8 mg/kg/4 hr, but at a dose of 0.7 mg/kg/4 hr, it had no effect on any parameter. AR significantly prolonged the PT at a dose of 1.25 mg/kg/4 hr and prolonged the APTT, PT, TCT at a dose of 5 mg/kg/4 hr. NM had no effect on any parameter. Because NM showed no anticoagulation activity at a concentration of 0.4 mg/kg/4 hr, lower concentrations were not evaluated.

Therapeutical effects of DS and the other anti-DIC agents on the DIC rat

In the endotoxin-treated rats (control group), all parameters deviated significantly from the normal control group: decrease of PL ($21\pm1\times10^4/\mu l$ in the control group vs $70\pm2\times10^4/\mu l$ in normal group); prolongations of APTT (80.8±4.7 sec vs 18.1±0.2 sec), PT (24.1±1.2 sec vs 14.7±0.1 sec) and TCT (25.4±1.6 sec vs 10.1±0.1 sec); decrease of Fbg (40±4 mg/dl vs 233±3 mg/dl); and increases of FDP (34.5±2.8 μg/ml vs <2.5± 0.0 μg/ml) and %GFD (42.9±4.4% vs not detected).

In the DS group, significant ameliorations were observed in the prolongation of TCT at doses of 5 and 10 mg/kg/4 hr (Fig. 1). It also inhibited the decrease of Fbg and the increase of FDP at doses of 5, 10 and 20 mg /kg/4 hr and inhibited the decrease in %GFD at doses of 10 and 20 mg/kg/4 hr. At doses of 1.25 and 2.5 mg/kg/4 hr, it had no effect on any parameter (Fig. 2).

LMWH significantly suppressed the decrease of Fbg and the increase of FDP at doses of 1.4 and 2.8 mg/kg /4 hr. The highest dose of LMWH also suppressed the decrease of PL, the prolongation of PT and the increase of %GFD, but further increased the prolongation of TCT. At doses of 0.35 and 0.7 mg/kg/4 hr, it had no effect on any parameter. NM showed no effect on any parameter. AR significantly suppressed the decrease of PL at doses of 1.25, 2.5 and 5 mg/kg/4 hr, while the increase of %GFD was suppressed at a dose of 5 mg/kg /4 hr. However, AR, similar to the action of LMWH, further increased the prolongation of TCT at doses of 2.5 and 5 mg/kg/4 hr and increased the prolongation of PT at a dose of 2.5 mg/kg/4 hr.

In the control group, massive fibrin deposits were observed in the glomerular capillaries. The highest dose (20 mg/kg/4 hr) of DS clearly inhibited the glomerular fibrin formation in the DIC rats (Fig. 3). LMWH completely inhibited the deposition of fibrin in the glomeruli at a dose of 2.8 mg/kg/4 hr. NM had no effect on the %GFD. In animals treated with AR (5 mg/kg/4 hr) glomerular fibrin deposits could be histologically observed although the drug statistically inhibited the increases of %GFD.

Table 1. Effects of DS and the other anti-DIC agents on blood parameters of normal rats

Test substance	Dose (mg/kg/4 hr)	N	Blood parameters					
			PL ($\times10^4/\mu l$)	APTT (sec)	PT (sec)	TCT (sec)	Fbg (mg/dl)	FDP (μg/ml)
Normal	—	38	70±2	18.1±0.2	14.7±0.1	10.1±0.1	233± 3	<2.5
DS	1.25	4	72±7	18.7±0.6	14.5±0.2	10.2±0.2	232±10	<2.5
	2.5	4	69±5	21.1±1.0	14.9±0.2	9.6±0.4	238± 9	<2.5
	5.0	4	74±3	24.4±1.5*	14.3±0.3	10.5±0.3	224± 4	<2.5
	10.0	4	72±3	33.8±0.5**	15.1±0.3	11.9±0.9	229± 7	<2.5
	20.0	3	78±6	41.2±1.2**	15.0±0.2	20.1±3.2*	211±17	<2.5
LMWH	0.7	4	65±4	19.6±0.4	14.7±0.1	10.5±0.3	229±10	<2.5
	2.8	4	71±5	27.6±0.4**	15.4±0.1*	69.0±7.2**	236±23	<2.5
NM	0.4	4	67±3	18.1±0.3	14.8±0.2	9.8±0.6	239±14	<2.5
AR	1.25	4	67±5	19.1±1.2	15.7±0.2*	13.5±1.9	221±11	<2.5
	5.0	4	75±8	25.0±1.0**	18.5±0.5**	39.0±5.1**	220± 4	<2.5

Each test substance was continuously infused for 4 hr. Each value represents the mean±S.E.M. *P<0.05, **P<0.01, compared to the corresponding normal group. DS: dermatan sulfate, DIC: disseminated intravascular coagulation, PL: platelet counts, APTT: activated partial thromboplastin time, PT: prothrombin time, TCT: thrombin clotting time, Fbg: fibrinogen, FDP: fibrin-fibrinogen degradation products, LMWH: low-molecular weight heparin, NM: nafamostat mesilate, AR: argathroban.

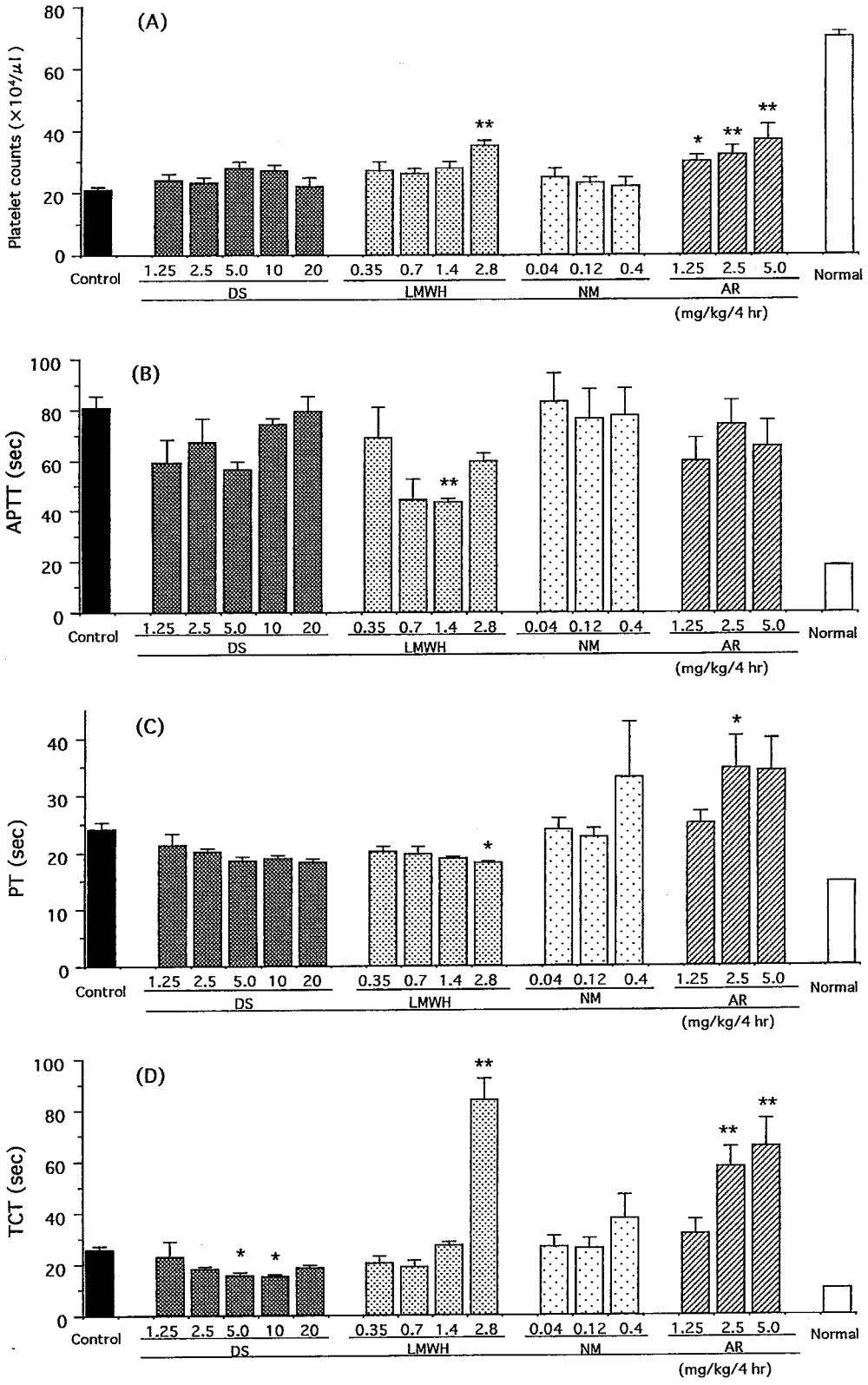

Fig. 1. Effects of DS and the other anti-DIC agents on the endotoxin-induced changes in platelet counts (A), APTT (B), PT (C) and TCT (D). Each value represents the mean±S.E.M. of 7–38 rats. *$P<0.05$, **$P<0.01$, compared to the corresponding control group. Each test substance was infused simultaneously with the endotoxin for 4 hr. DS: dermatan sulfate, DIC: disseminated intravascular coagulation, APTT: activated partial thromboplastin time, PT: prothrombin time, TCT: thrombin clotting time, LMWH: low-molecular weight heparin, NM: nafamostat mesilate, AR: argathroban.

DISCUSSION

When endotoxin, tissue thromboplastin or thrombin are continuously infused into various animals, they show human-DIC-like symptoms. Therefore, animals receiving the infusion of these factors have been established as an experimental model for DIC. The endotoxin-induced model is an especially representational DIC model that has been employed by many researchers (14–17). Endotoxin administered to rats rapidly stimulates monocytes and vascular endothelial cells. Cells activated by endotoxin release various inflammatory factors (interleukin-1,

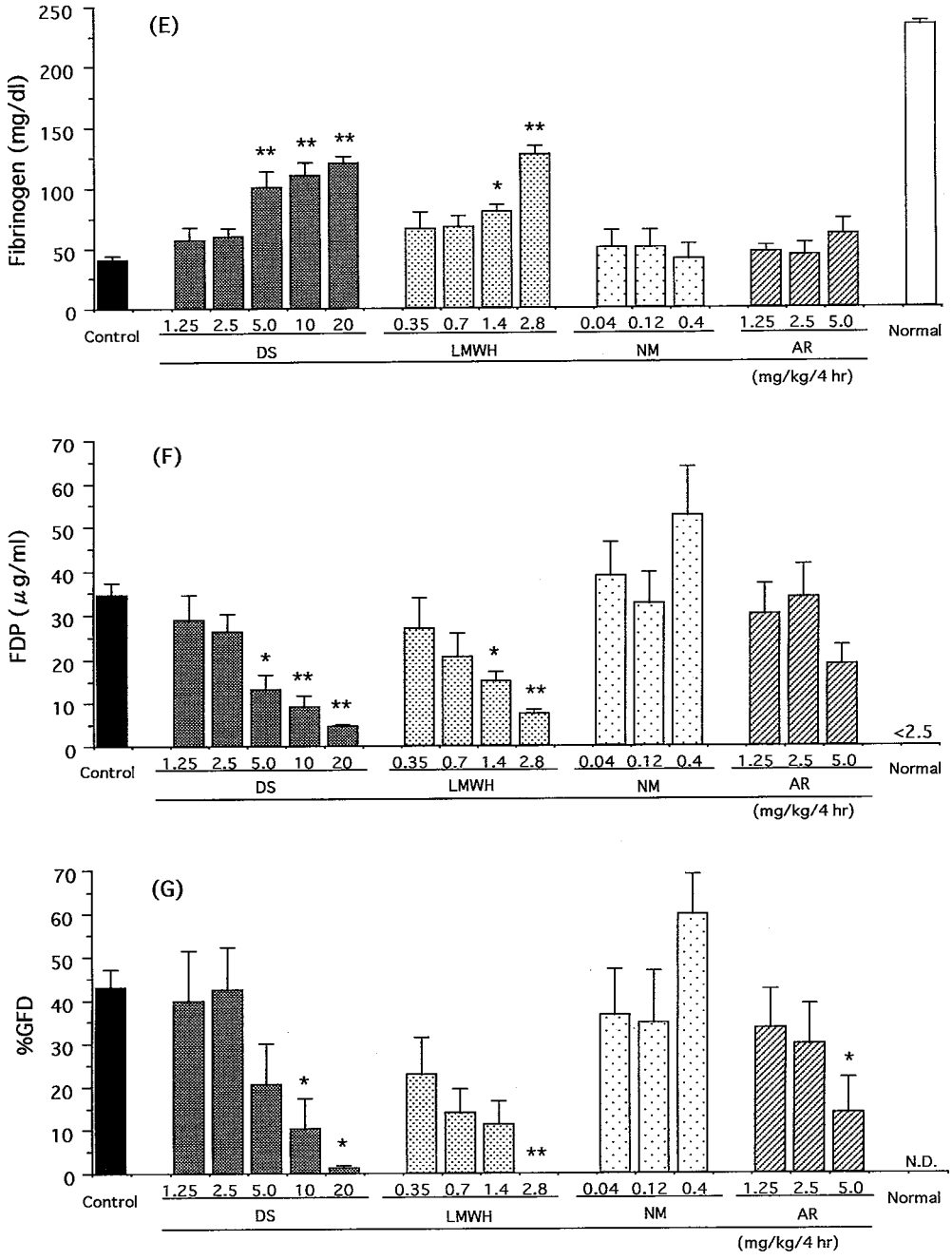

Fig. 2. Effects of DS and the other anti-DIC agents on the endotoxin-induced changes in fibrinogen (E), FDP (F) and %GFD (G). Each value represents the mean±S.E.M. of 7–38 rats. *P<0.05, **P<0.01, compared to the corresponding control group. Each test substance was infused simultaneously with the endotoxin for 4 hr. DS: dematansulfate, DIC: disseminated intravascular coagulation, FDP: fibrin-fibrinogen degradation products, %GFD: percentage of glomerular fibrin deposits, LMWH: low-molecular weight heparin, NM: nafamostat mesilate, AR: argathroban.

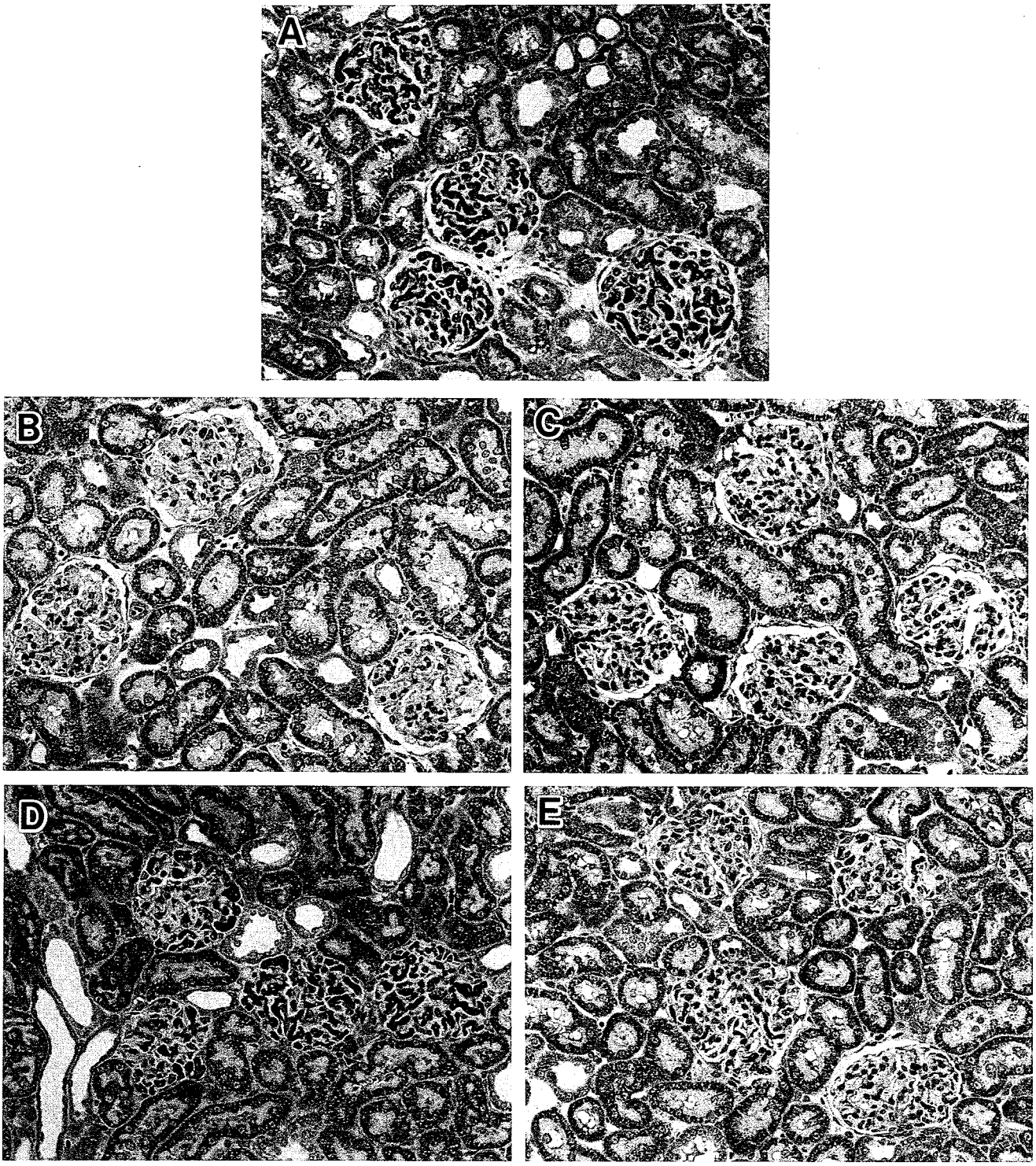

Fig. 3. Histological study of kidneys in the endotoxin-treated (A) rats that were given 20 mg/kg/4 hr of dermatan sulfate (B), 2.8 mg/kg/4 hr of low-molecular weight heparin (C), 0.4 mg/kg/4 hr of nafamostat mesilate (D) or 5 mg/kg/4 hr of argathroban (E). Each section was stained with phosphotungstic acid hematoxylin. Original magnification ×200.

tumor necrosis factor-α and tissue factor), destroying the balance in the blood coagulation system (12). As a result, thrombi form in microvessels, inducing organ failure.

Human plasma contains two heparin-dependent inhibitors of thrombin, namely, heparin cofactor II (HC II) and anti-thrombin III (AT III). While HC II inhibits only

thrombin, AT III inhibits various coagulation factors, especially thrombin and factor Xa. DS accelerates the antithrombotic activity by interacting with HC II. On the other hand, LMWH mainly exhibits anti-factor Xa activity by interacting with AT III, but has little effect on thrombin (18). NM is a synthetic serine protease inhibitor that has inhibitory effects against various proteases including thrombin (19), while AR is a synthetic selective thrombin inhibitor that possess a specific binding site for thrombin (20). Both NM and AR exhibit their antithrombotic effect independent of AT III. Thus, DS is a different type of anti-coagulant agent that acts by a different mechanism from the reference agents described above. When DS or one of the reference agents was continuously infused into normal rats for 4 hr, the prolongation of APTT was observed in the DS, LMWH and AR groups. At the dose necessary to prolong the APTT by about 1.5- to 2-fold, DS (5, 10 mg/kg/4 hr) had no effect on any other parameter. It is unclear why DS did not prolong the TCT, as it possess anti-thrombotic activity. In contrast, LMWH (2.8 mg/kg/4 hr) and AR (5 mg/kg /4 hr) significantly prolonged not only the APTT but also PT and TCT. These results may indicate that the anti-coagulant activity of DS is milder than that of LMWH or AR.

In this study, all the test substances except NM showed an ameliorative effect in the DIC model. Especially at the highest doses, these substances significantly improved the %GFD, one of the most important parameters for assessing drug efficacy on DIC. These results demonstrate that DS, LMWH and AR all prevent the production or deposition of fibrin in the glomeruli. In experiments performed by continuous administration of the test substances to normal rats, neither DS nor LMWH improved the pathological changes induced by endotoxin at the doses exhibiting no anti-coagulant activity. Thus, it was suggested that the anti-coagulant activity of the drugs was important for exertion of their effects. Meanwhile, DS did not improve thrombocytopenia observed from the initial stage of endotoxin administration. Thrombocytopenia is caused by aggregation of activated platelets due to the stimulation of platelet-activating factors produced by endotoxin or various cells (vascular endothelial cells, platelets) (12). Since DS did not inhibit aggregation of platelets by collagen and ADP (21, 22), it was speculated that DS did not affect platelets in this model. NM showed few effects in this model. The effects of NM on an endotoxin-induced rat DIC model have varied between researchers (15, 16), and its detailed actions remain to be clarified. Since changes in parameters of the coagulation system were rarely observed when NM was continuously administered to normal rats, it was thought that the effects of NM did not appear in this model.

The most noticeable effect of DS was that on the TCT. Namely, at the dose necessary to suppress the increase of %GFD, DS alone decreased the prolongation of TCT, while LMWH and AR further increased the prolongation of TCT, probably because of their strong anticoagulant activities as shown in the results of experiments using normal rats. The most serious side effect of agents with anti-coagulant activities is hemorrhage. It has been reported that prolongation of the TCT is one of the risk factors that is closely related to the bleeding frequency observed clinically (23). Although it is well-known that the bleeding action of DS is weak (4–6, 24), until the present study, there has been no proof of this fact in a DIC model. Our observations suggest that there is little possibility that DS induces a bleeding tendency in DIC rats that accelerates the prolongation of TCT. These results suggest that the efficacy of DS on DIC rats is comparable to that of LMWH. We expect that DS can be used as a safe drug for the treatment of DIC.

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