Effect of Chondroitinase on Dermatan Sulfate-Facilitated Arginine Amidase Released from Rabbit Ear Artery

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We have previously reported that trypsin (EC 3.4.21.4)-like enzymes, showing lysine and arginine p-nitroanilide-derivative hydrolyzing activities (arginine amidase activity, AA activity), which can be separated using aprotinin and lima bean inhibitor affinity columns, are secreted from rabbit arteries. 1,2 Moreover, we showed that AA released from isolated rabbit ear arteries was accelerated by the addition of mucopolysaccharide sulfate derivatives; heparin, chondroitin sulfate, and dermatan sulfate. 3–4 The molecular weight of natural DS is within the range of 12–45 kDa with an average molecular weight of 20–30 kDa. 3–9

Vascular endothelial cells are thought to produce and secrete tissue plasminogen activator (t-PA, EC 3.4.21.69). 5 We have already confirmed the presence of some fibrinolytic enzymes, plasminogen, plasmin (EC 3.4.21.7)-like activity and two types of plasminogen activators, urokinase (EC 3.4.21.73) and t-PA, in rabbit arteries; however, no thrombin (EC 3.4.21.5)-like enzymes were released from rabbit arteries by treatment with chondroitin sulfate. 1,3 Furthermore, the enhancement of AA activities produced by dermatan sulfate (chondroitin sulfate B; DS) depends on the endothelium, whereas spontaneous AA activities do not depend on the endothelium. 5,9

DS is a polysaccharide in the family of glycosaminoglycans. DS-containing proteoglycans widely exist on the surface of cells and in the extracellular matrix. DS has important anticoagulant and antithrombotic activities. 7 The structure of DS is largely accounted for by the repeating disaccharide sequence [IdoA-GalNAc4SO3], in which IdoA is α-L-iduronic acid and GalNAc4SO3 is N-acetyl-β-D-galactosamine 4-O-sulfate, 1.3- and 1.4-linked, respectively. 8 The molecular weight of natural DS is within the range of 12–45 kDa with an average molecular weight of 20–30 kDa. 9

The existence of enzymes that depolymerize chondroitin sulfate has been recognized in various biological systems. 10 Two specific glycosaminoglycan lyases were utilized: chondroitinase ABC (substrates are chondroitin sulfate A, B (DS), C, chondroitin and hyaluronic acid) 11 and chondroitinase ACII (substrates are chondroitin sulfate A, C, chondroitin and hyaluronic acid). 12 The substrates such as chondroitin sulfates were almost completely degraded to disaccharides by these two chondroitinases. 11,12

In the present study, we hypothesized that DS-induced AA release from the rabbit ear artery could depend on the molecular size of DS and we examined the effect of DS digested by enzymes using chondroitinases (chondroitinase ABC; ABCase and chondroitinase ACII; ACIIase) on DS-facilitated AA release in rabbit ear artery.

MATERIALS AND METHODS

Rabbit Ear Artery Preparation and Experimental Protocol The rabbits used in the present study were anesthetized and sacrificed by exsanguination as approved by the Institutional Ethics Committee for Animal Research at Meiji Pharmaceutical University, which was complied from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Sciences. Male Japanese white rabbits (weighing 2.5 kg) were anesthetized with pentobarbital sodium (50 mg/kg, intravenously (i.v.)) and exsanguinated. The ear artery was removed, cleaned of connective tissue and suspended in a water-jacketed organ chamber containing 2 ml of a modified Krebs solution continually aerated with 95% O2 and 5% CO2 at 37 °C. The composition of the Krebs solution was as follows (mM): NaCl 110; KCl 4.6; CaCl2 2.5; NaHCO3 24.8; KH2PO4 1.2; MgSO4 1.2; glucose 5.6. DS and other drugs were added to Krebs solution for 30 min. After a 30-min equilibration period, the extracted solution containing enzyme activity was used to assay Val-CHA-Arg-pNA amidolytic activity.

Measurement of Arginine Amidolytic Activity D-Valyl-cyclohexylalanyl-L-arginine-p-nitroanilide (Val-CHA-Arg-pNA, American Diagnostica Inc., U.S.A.) was used as the substrate for tissue kallikrein (EC 3.4.21.35) to measure arginine amidase activity released from the isolated artery. The amidolytic method described by Amundsen et al. 13 was employed with slight modifications to enable the use of microplates, and the reaction was performed in microplates at 37 °C in 0.05 mol/l Tris–HCl buffer (pH 8.5) containing

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0.15 mol/l NaCl. The final reaction volume and final substrate concentration were 0.25 ml and 0.5 mmol/l, respectively. Amidolytic activity was expressed in terms of pmol of substrate hydrolyzed per minute (pmol/min), after measuring 405 nm absorbance of p-nitroaniline (pNA, molar extinction coefficient=10000), a reaction product released by the amidolytic enzyme reaction from the above tripeptidyl substrate. A microplate reader (Multi Scan Bichromatic; Labosystem Japan Co., Tokyo) was used to measure the absorbance of pNA, and activity was calculated based on this measurement.

**Digestion of Dermatan Sulfate by Chondroitinases**

DS was treatment with chondroitinases before it experimented beforehand. The condition was shown as follows. DS was digested with ABCase (10 units in 20 mM Tris–HCl, pH 8.0, at 37 °C for 18 h) and ACIIase (5 units in 20 mM sodium acetate, pH 6.0, at 37 °C for 18 h). Heat-inactivated ABCase and ACIIase were prepared by heating the enzyme at 100 and 60 °C for 10 min, respectively. Each sample of DS was mixed with an equal volume of 2 sodium dodecyl sulfate (SDS) sample buffer and boiled. Each sample was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (15% acrylamide) at 200 V and stained with Alcian Blue, which stain acid mucopolysaccharides and glycosaminoglycans.

**Materials**

The following drugs were obtained from Seikagaku Corporation (Tokyo, Japan): chondroitin sulfate B sodium salt (avian crown) (DS), chondroitinase ABC (*Proteus vulgaris*), chondroitinase ACII arthro (*Arthrobacter aurescens*), heparan sulfate (HS) sodium salt (bovine kidney), hyaluronic acid (HA) sodium salt (Pig skin). All other chemicals were of reagent grade.

**Statistical Analysis**

All values are expressed as the mean±S.E.M. Student’s paired t-test and ANOVA before Fisher’s protected least significant difference post-hoc test were used for comparisons of means. p<0.05 was considered significant. These statistical analyses were performed using a computer program (Stat View 5; Abacus Concepts, Inc., CA, U.S.A.).

**RESULTS**

Figure 1a shows the effect of 0.1 U/ml ABCase on spontaneous and 0.02% DS-induced AA release from the rabbit ear artery. The secretion of AA activity from the ear artery was increased markedly after treatment with 0.02% DS. DS-induced AA release was significantly decreased by ABCase treatment. ABCase did not affect spontaneous AA release. Figure 1b shows the effect of heat-inactivated ABCase on spontaneous and DS-induced AA release from the rabbit ear artery. Heat-inactivated ABCase did not affect spontaneous and DS-induced AA release.

Figure 2a shows the effect of 0.1 U/ml ACIIase on spontaneous and 0.02% DS-induced AA release from the rabbit ear artery. ACIIase enhanced spontaneous and DS-induced AA release from the ear artery. Heat-inactivated ACIIase also did not affect spontaneous and DS-induced AA release.

Figure 3 shows the SDS-PAGE of DS digested by ABCase (a) and ACIIase (b). The band of DS was abolished by treatment with ABCase, but not ACIIase and heat-inactivated chondroitinases.

Figure 4 shows 0.02% hyaluronic acid (HA; a) and 0.02% heparan sulfate (HS; b)-induced AA release from the rabbit ear artery. Both HA and HS significantly increased AA release from the rabbit ear artery.
DISCUSSION

DS is a sulfated glycosaminoglycan consisting of repeating disaccharide units, i.e., \( \text{d-N-acetyl-galactosamine and uronic acid (mainly L-iduronic acid and some L-glucuronic acid), which are heterogeneously O-sulfated. } \)

DS has been reported to exhibit antithrombin activity by interacting with heparin cofactor II (HCII).

Furthermore, DS was suggested to enhance fibrinolytic activity by the release of t-PA from endothelial cells.

In the present study, we examined the effects of chondroitinases (ABCCase and ACIIase) on DS-facilitated AA release from the rabbit ear artery. Substrates of ABCCase are chondroitin sulfate A, B (DS), C, chondroitin and HA, and substrates of ACIIase are chondroitin sulfate A, C, chondroitin and HA. DS-induced AA release was significantly decreased by ABCCase in the rabbit ear artery without the change in spontaneous AA release. On the other hand, ACIIase enhanced spontaneous and DS-induced AA release in the rabbit ear artery; therefore, we examined the effects of other glycosaminoglycans (HS and HA) on the release of AA to test the possibility that the enhancement of spontaneous AA results from endogenous glycosaminoglycans release by ACIIase (Fig. 4). HS and HA induced AA release from the rabbit ear artery. Furthermore, heat-inactivated ABCase and ACIIase did not affect spontaneous and DS-induced AA release from the rabbit ear artery (Figs. 1b, 2b). These results suggest that the enhancement of spontaneous AA induced by ACIIase, but not ABCase, may be due to endogenous glycosaminoglycan release by ACIIase. Moreover, we confirmed that the band of DS was abolished by treatment with ABCase, but not ACIIase, and cleavage products of DS by ABCase do not operate to enhance AA release. In addition, it was previously reported that DS was almost completely degraded to disaccharides by ABCase for 18 h.

DS has been widely used as an antithrombotic drug. Evidence of the anticoagulant activity of low molecular weight DS is accumulating, however, the relationship between the structure of DS and their anti-thrombotic mechanisms is still unclarified. Oursulfated sequences, especially those containing 4,6-disulfated GalNAc residues or IdoA-2-\( \text{OSO}_3^- \rightarrow \)GalNAc-4-\( \text{OSO}_3^- \)disulfated disaccharide sequences, have been reported to enhance their antithrombotic activities. Du et al. demonstrated the relationship between the structure of DS-derived oligosaccharides by chemical depolymerization and their anti-thrombotic activities. Furthermore, Halldosdottir et al. showed that oligosaccharides (containing 2—12 monosaccharide units) from partial enzymatic digests of DS bind to HCII. Thus, the molecular weight and sulfated degree of DS might be of great importance in the development of new anti-thrombotic DS-derived drugs. Low molecular weight DS with a mean molecular weight of 5.6 kDa, desmin, has been obtained by the limited depolymerization of natural DS. Desmin shows antithrombotic activity and thrombosis prevention in various animal models. Recently, Alberto et al. reported that a very low molecular mass DS (ca. 5 kDa) obtained by peroxy-radical depolymerization was as effective as DS to prevent thrombus formation.

These investigations indicate that low molecular DS shows antithrombotic activity and the prevention of thrombus formation; however, antithrombotic activity does not depend on the molecular size of DS. Furthermore, fibrinolytic activity of DS has rarely been reported. The antithrombotic activity of DS acts directly on HCII; however, the fibrinolytic activity of DS appears by indirectly release of t-PA from the vascular endothelium. Details of the AA activity release from endothelium by DS remain unclear. In addition, it seems that spontaneously AA release prevent thrombus at any time.

These results indicate that the facilitatory effect of DS on AA release from the rabbit ear artery is affected by the molecular size of DS; thus, very low molecular weight DS may show fibrinolytic activity. However, more precise studies will be required to clarify the signal transduction pathway in which DS and its derivatives play their roles.

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