



## Full length article

## Chondroitin sulfate protects vascular endothelial cells from toxicities of extracellular histones



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## ABSTRACT

Extracellular histones induce lethal thrombosis by promoting platelet aggregation, neutrophil migration, and cell injuries. Heparin, which has negative charges, can bind to extracellular histones; however, heparin strongly inhibits the activation of coagulation. Since chondroitin sulfate (CS) shows less effect on the coagulation system than heparin does, CS has the potential to become an effective drug for lethal thrombosis with high risk of bleeding. To elucidate the therapeutic mechanisms of CS in lethal thrombosis, we investigated the interaction between CS and extracellular histones. Mouse vascular endothelial cells were incubated with histones in the presence of heparin or CS, and the expression of caspase-3/7 was measured. The interactions between histones and heparin or CS were measured by surface plasmon resonance analysis. Vascular permeability, platelet counts, liver and renal functions, and coagulation times were evaluated in an in vivo assay. The apoptosis induced by histones was inhibited by treatment with heparin or CS. Heparin and CS showed strong binding to histones and inhibited vascular hyperpermeability. The platelet counts as well as liver and renal functions were not decreased by the treatment with heparin or CS. Moreover, CS showed less effect on the coagulation system than heparin did. These results suggested that CS can be a novel agent for lethal thrombosis with the risk for hemorrhage. Since vascular endothelial cell injuries occur at an early stage of lethal thrombosis, administration of CS might be a useful approach.

## 1. Introduction

Histones are basic proteins that form reversible complexes with DNA. In patients with disseminated intravascular coagulation (DIC), systemic inflammation promotes the release of histones from dying cells. Released histones promote platelet aggregation (Fuchs et al., 2010), neutrophil migration (Chaput and Zychlinsky, 2009; Xu et al., 2009), and thrombosis (Fuchs et al., 2010). Since histones are positively charged and can bind to the negatively charged cell plasma membrane (Freeman et al., 2013; Gillrie et al., 2012), circulating extracellular histones can function as tissue mediators (Abrams et al., 2013b; Nakahara et al., 2013) and induce cell injuries (Gillrie et al., 2012; Mena et al., 2016; Saffarzadeh et al., 2012). To reduce their toxicities, neutrophil extracellular traps, which include extracellular histones, are digested by DNase (Cooper et al., 2013) or undergo phagocytosis (Farrera and Fadeel, 2013; Nakazawa et al., 2016). However, excessive

release of histones promotes coagulation disorders (Ito, 2014; Nakahara et al., 2013; Xu et al., 2009) and liver injury (Kawai et al., 2016; Wen et al., 2016; Xu et al., 2011).

Initiation of coagulation is induced by tissue factor on endothelial cells (Levi and van der Poll, 2017); therefore, vascular injuries induce not only vascular hyperpermeability but also coagulation disorders. Severe hemorrhage exacerbates the prognosis of DIC patients, owing to vascular hyperpermeability and wasting of coagulation factors (Levi and van der Poll, 2017; Miller et al., 2002). To prevent severe hemorrhage, protection of the vascular system from histone toxicities could be a useful strategy for DIC patients.

Chondroitin sulfate (CS) forms side chains of proteoglycans, which are ubiquitously distributed on cell surfaces and in extracellular matrices in animals. Cartilage is rich in CS, and CS has been widely used as a nutraceutical and a drug for osteoarthritis (Toida et al., 2006; Uebelhart et al., 2006). Heparin is another linear polysaccharide, and

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its negative charge allows binding to extracellular histones (Kawai et al., 2016; Pemberton et al., 2010). However, heparin strongly inhibits the activation of coagulation (Pernerstorfer et al., 1999) and, therefore, is not recommended for the treatment of bleeding patients with lethal thrombosis (Levi et al., 2009). Since CS shows less effect on the coagulation system than heparin does (Bourin and Lindahl, 1993; Scully et al., 1986), CS has the potential to be an effective drug for the patients with high risk of bleeding. Previous reports have suggested that heparin protects cells from the toxicity of extracellular histones (Kawai et al., 2016; Wang et al., 2015; Wildhagen et al., 2014; Yoo et al., 2016); in addition anionic carbohydrates in CS have been shown to protect the toxicity of histones (Chaaban et al., 2015). However, there were no reports investigating whether the interaction between CS and extracellular histones affects coagulation system in lethal thrombosis induced by histones. Revealing the effects of CS might lead to the proposal of a new therapeutic strategy. To elucidate the therapeutic mechanisms of CS in lethal thrombosis, we investigated the interaction between CS and extracellular histones in lethal thrombosis.

## 2. Materials and methods

### 2.1. Reagents and antibodies

The following reagents were used: RPMI medium (Sigma–Aldrich, St Louis, MO, USA), fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), penicillin and streptomycin (Thermo Fisher Scientific), unfractionated histones from calf thymus (Sigma–Aldrich), the Cell Player™ Caspase-3/7 reagent (Essen Bioscience, Ann Arbor, MI, USA), heparin (Nacalai tesque, Kyoto, Japan), CS from shark cartilage (Wako, Osaka, Japan), ethylenediaminetetraacetic acid dipotassium salt (EDTA-2K; Dojindo Laboratories, Kumamoto, Japan), saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), a fluorescent-activated cell sorting (FACS) lysing solution (Becton Dickinson, San Jose, CA, USA), biotin-LC-hydrazide (Thermo Fisher Scientific, Pierce), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Thermo Fisher Scientific, Pierce), Amicon® Ultra-0.5 with a molecular mass cutoff of 3000 Da (Millipore, Milford, MA, USA), Evans blue dye (Wako), formamide (Wako), and sodium citrate (Sigma–Aldrich).

Antibody sources were as follows: allophycocyanin (APC)-conjugated rat IgG2b anti-mouse D45 (clone 30-F11; BioLegend, San Diego, CA, USA), phycoerythrin (PE)-conjugated rat IgG2b anti-mouse CD11b (clone M1/70; BioLegend), brilliant violet (BV) 421-conjugated rat IgG1 anti-mouse CD41 (clone MWRReg30; BioLegend), APC-conjugated rat IgG2b (BioLegend), PE-conjugated rat IgG2b (BioLegend), and BV421-conjugated rat IgG1 (BioLegend).

### 2.2. Cell apoptosis assays

Mouse vascular endothelial cells (RCB1994) were purchased from the RIKEN BioResource Center. Cells were cultured in RPMI medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin in humidified air with 5% CO<sub>2</sub> at 37 °C. Under sub-confluent conditions, the culture medium was replaced with serum-free medium containing calf thymus histones (0–0.2 mg/ml) and the Cell Player™ Caspase-3/7 reagent, and the cells were incubated for 24 h. After the incubation, the apoptosis rates of cells were measured according to the manufacturer's instructions and a previous report (Artymovich and Appledorn, 2015). Briefly, cells were monitored automatically via live cell imaging using an InCuCyte ZOOM™ video microscopy system (Essen Bioscience). Expression of caspase-3/7 was automatically calculated by the InCuCyte ZOOM™ software (Essen Bioscience). To elucidate the effects of heparin and CS on the cell injuries induced by histones, vascular endothelial cells were incubated with calf thymus histones (0.2 mg/ml) in the presence of heparin or CS (0.025–0.2 mg/ml each). Vehicle group was incubated with serum free medium. The expression of caspase-3/7 was calculated during 24 h

incubation.

### 2.3. Vascular permeability measurement

Vascular permeability was assessed by measuring the concentration of Evans blue dye according to previous reports (Daruwalla et al., 2009; Nishijima et al., 2013; Sugaya et al., 2016). To evaluate the vascular permeability in an acute phase of lethal thrombosis, the mice were injected with Evans blue dye 10 min after the injection of saline, calf thymus histones, or the mixture of heparin or CS with histones. Since equal amounts of CS and histones inhibited the toxicities of histones completely *in vitro*, the mice in the treatment groups received a single tail vein injection of calf thymus histones (45 µg/g weight) alone or mixed with CS or heparin (45 µg/g weight each). Lung, kidney, and liver samples were collected 1 h after the injection of Evans blue dye. The tissues were weighed and homogenized in formamide (100 mg/ml). The homogenates were incubated at 60 °C for 48 h to allow dye extraction. Following the incubation and centrifugation (12,000 × g for 20 min), the supernatant was analyzed using spectrophotometry (2300 EnSpire™ plate reader; Perkin Elmer, Waltham, MA, USA) at a wavelength of 620 nm.

### 2.4. Surface plasmon resonance analysis

Interactions between calf thymus histones and heparin or CS were measured using a BIAcore 2000 system (BIAcore AB, Uppsala, Sweden) as reported (Mizumoto et al., 2013; Nandini et al., 2004). Briefly, heparin was biotinylated on the carboxyl groups of glucuronic acid residues using biotin-LC-hydrazide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride in 100 mM 2-(*N*-morpholino) ethanesulfonic acid (MES)–NaOH, pH 5.5 (Mizumoto et al., 2013). CS was mixed with 3 mM biotin-LC-hydrazide and 0.35 M NaCNBH<sub>3</sub> in 0.1 M MES–NaOH (pH 5.5), yielding biotinylated CS at the reducing terminus (Mizumoto et al., 2013). The labeling reaction was carried out overnight at room temperature. Biotinylated heparin and CS were purified using Amicon® Ultra-0.5 with a molecular mass cutoff of 3000 Da. Biotinylated CS or heparin chains (~1.0 ng) were individually immobilized on the surface of a streptavidin-derivatized sensor chip. The binding reactions were carried out at 25 °C, and 93 µM histones, which were dissolved in running buffer, 10 mM HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% (w/v) Tween® 20, pH 7.4, were individually injected onto the sensor chips.

### 2.5. Treatment with heparin or CS against lethal thrombosis

All animal experiments were approved by the Experimental Animal Board of Meijo University (approval number 2017-PE-31) and were conducted in compliance with the Animal Experiment Guidelines of Meijo University. Animals were maintained under conventional laboratory conditions and were provided free access to food and water. A lethal thrombosis animal model was prepared according to a previous study (Mizuno et al., 2017) using male DBA/1JLmsSlc mice (9–12-week-old; Japan SLC, Shizuoka, Japan). To evaluate the effects of heparin and CS on lethal thrombosis, the mice were divided into four groups: vehicle, histone, CS-treated, and heparin-treated groups. To evaluate the effects of histone-heparin or histone-CS complexes *in vivo*, histones were pre-incubated with heparin or CS. The mice in the treatment groups received a single tail vein injection of calf thymus histones (45 µg/g weight) alone or mixed with CS or heparin (45 µg/g weight each). The mice in the vehicle group were injected with saline. One h after the injection, blood samples were collected from anesthetized mice.

### 2.6. Blood cell counts and liver and renal functions

Blood samples were collected from anesthetized mice 1 h after the

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