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Letter to the Editors-in-Chief

Thrombolytic fucoidans inhibit the tPA-PAI1 complex, indicating activation of plasma tissue-type plasminogen activator is a mechanism of fucoidan-mediated thrombolysis in a mouse thrombosis model

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Thrombolysis by intravenous injection of recombinant tPA remains the most common non-interventional treatment to recanalize vessels occluded by acute thrombosis. Despite its recognized efficacy, its use is limited to a very short therapeutic window of 4.5 h after the first clinical signs [1–2] because beyond this term, the risk of hemorrhagic transformation [3] exceeds the benefit of thrombolysis. In addition, since rtPA is administered intravenously, it is also quickly neutralized and inhibited by circulating PA inhibitor (PAI1) [4]. Therefore, either blocking the inhibitory pathway or increasing the free tPA directly may lead to enhanced thrombolysis. Activation of rtPA by releasing it from PAI1 binding with a chaperon molecule, which can improve its bioavailability to thrombus, would thus be advantageous. (See Fig. 1.)

Fucoidans are well known to have antithrombotic effects and bind with either thrombin [5] or PAI-1 [4], but no direct experimental trial except our previous report [6–7] has assessed thrombolysis. Previously, we demonstrated fucoidan injection-mediated thrombolysis in an arterial thrombus model and dose-dependent enhancement of plasma tPA upon treatment of thrombus with a fucoidan fraction from Korean mariculture algae *U. pinnatifida* sporophylls (**KUpSpF**). Considering non-enzymatic nature of fucoidans as sulfated polysaccharides, we hypothesized that the molecular mechanism underlying fucoidan-mediated thrombolysis with enhanced plasma level of tPA may be *via* stimulated release of tPA from affected vascular endothelium or *via* enhanced active free tPA by inhibiting the plasma tPA-PAI-1 complex.

In this study, thrombolysis in a thrombosis model and direct interaction of the tPA-PAI1 complex were studied with various Russian fucoidan fractions including structurally modified analogs to elucidate the mechanisms underlying fucoidan-mediated thrombolysis.

1. Methods

Water-soluble fucoidan extracts were purified using a Macro-Prep DEAE column (Cl^- form, 2.5 × 9 cm, Bio-Rad, USA) equilibrated with 0.1 M HCl. Laminarin-containing fractions were eluted first with water, followed by eluting fucoidans with a linear gradient of NaCl (from 0.1 to 2 M) until the absence of a positive reaction for carbohydrates by the phenol-sulfuric acid method. The fucoidan (**F**) fractions were dialyzed against distilled water, and lyophilized. Monosaccharide composition was determined after polysaccharide hydrolysis by 2 M TFA (6 h, 100 °C) by HPLC using the column ISA-07/S2504 (0.4 × 25 cm, Shimadzu), the bicinchoninic assay, and the C-R2 AX integrating system (Shimadzu, Japan). Levels of sulfate groups were determined using the $BaCl_2$ gelatin method. Deacetylation of the fucoidans **FeF** (*F. evanescens*) and **RUpSpF** was performed in 12.5% aqueous NH_3 to obtain fractions of deacetylated (dA) fucoidans (**FeFdA** and **RUpSpFdA**). Fucoidan sulfated at C2 and acetylated at C3 (**FeF2S3Ac**) was prepared by the ultrasound extraction method combined with ion chromatography. Depolymerization of fucoidan was done by either enzymatic degradation using fucoidanase FFA2 (F28 from FeF) or hydrolysis (ScF4S from ScF: *Saccharina cichoorioides*). All the fucoidan fractions examined in this study were summarized with their structural features in Table 1.

The thrombus model was made in the ferric chloride-treated carotid artery of Balb/c mice while monitoring blood flow by a miniature Doppler flow probe (Model 0.5 VB, Transonic System, Ithaca, NY, USA) in accordance with the Institutional Animal Care and Use Committee of the Catholic University Hospital of Daegu (approval number, DCIAFCR-151007-7-Y). The time to occlusion was defined as the disappearance of the Doppler beat.

Thirty minutes after thrombotic occlusion, various fucoidan fractions were injected intravenously *via* the tail vein at a dose of 100 mg kg^{-1}. The time to reperfusion, defined as the appearance of the Doppler beat, was measured until a maximum of 3 h from the time of fucoidan injection. Each experimental thrombolytic or control group, containing 5–6 mice, was treated with various fucoidan fractions as listed in Table 1. The potent binding affinity of fucoidan with PAI-1 was tested on a preparation of tPA-PAI1 complex. Functionally active fucoidan binds to biotinylated human PAI1 that is covalently bound to a microtiter plate and induces the release of tPA from the complex, reducing the level of the remaining tPA-PAI1 complex. After treating the remaining tPA-PAI1 complex with a monoclonal antimurine tPA primary antibody, the tPA-PAI 1 complex bound with primary antibody was measured with a horseradish peroxidase-labeled secondary antibody in an ELISA kit (PA92 mouse tPA activity assay kit, Oxford Biomedical Research, Rochester Hills, Michigan, USA). Further color development with tetramethylbenzidine enabled monitoring the absorbance at 450 nm, rendered OD_{450} data on the tPA-PAI1 levels. OD_{450} data represent the relative level of remaining tPA-PAI1 complex after releasing tPA due

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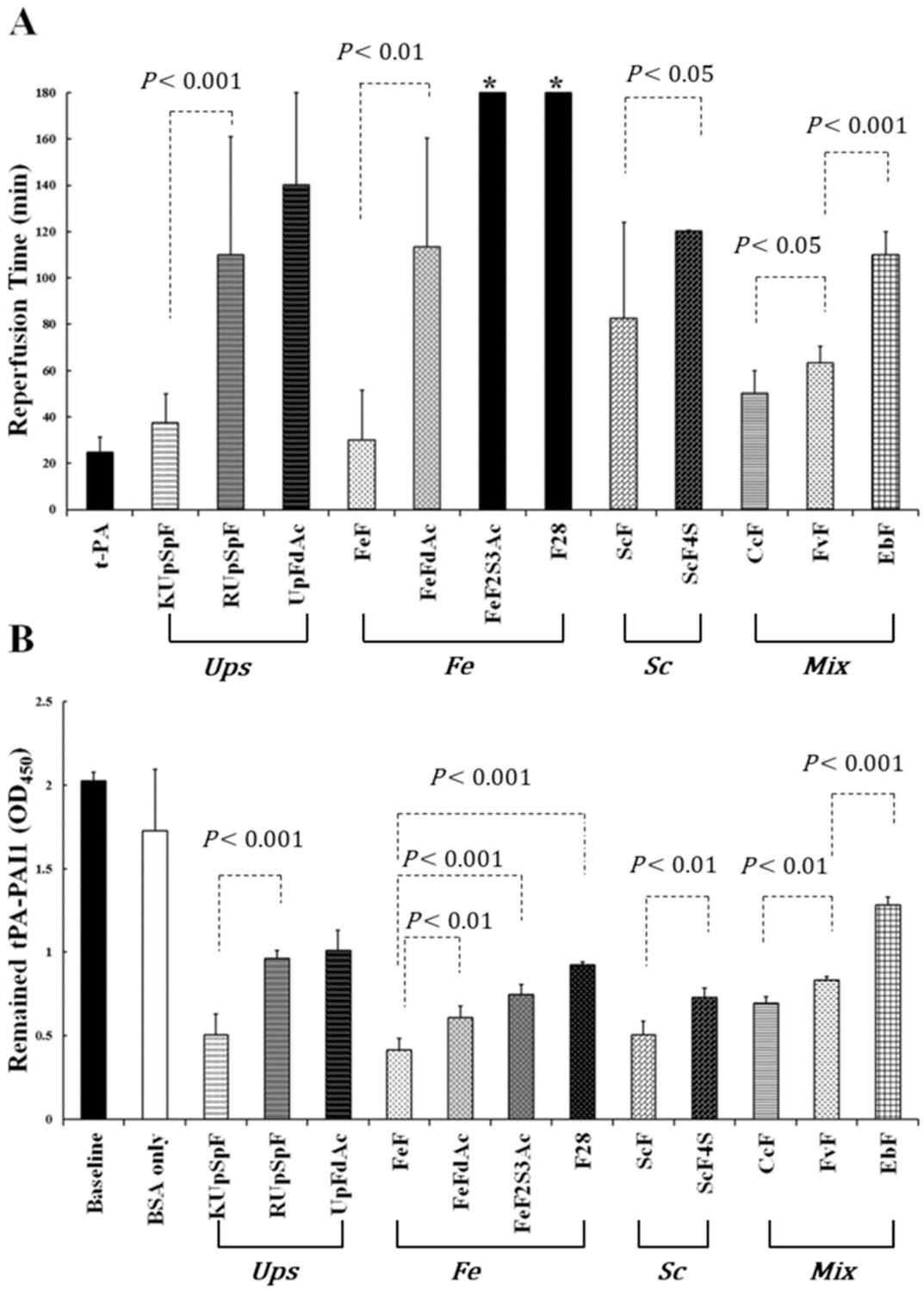

Fig. 1. A) Measurement of reperfusion time in mice that were treated with various Russian fucoidan fractions and a comparison with tPA and fucoidan from Korean *Undaria Pinnatifida Sporophyill* (KUpSpF). * indicates non-thrombolysis within the measurement time of 3 h. B). OD_{450} data represent the level of remaining tPA-PAI complex after treatment with fucoidan. Lower level with respect to baseline indicates the amount of free tPA release upon fucoidan binding with PAI-1. Fucoidan fractions are grouped depending on derived algal species; ***Ups*** (*Undaria Pinnatifida Sporophyill*), ***Fe*** (*Fucus evanescens*), ***Sc*** (*Saccharina cichorioides*), ***Mix*** (*Costaria costata*, *Fucus vesiculosus*, *Eisenia bicyclis*).

to competitive binding of fucoidan with PAI-1 [Fucoidan + tPA-PAI1 → tPA-PAI1 + (tPA + Fucoidan-PAI1)]. To test potential release of free tPA, relative amounts of tPA in the decanted solution after reacting three different doses (1, 5, 20 mg/ml) of **FeF**, **EbF**, **CcF**, and **ScF** fucoidans with tPA-PAI1 complex were measured in the biotinylated PAI-1 well using same antibody systems recognizing tPA-PAI1 complex. OD_{450} data on the tPA-PAI1 levels or reperfusion time are presented as the mean ± SD. Unpaired Student's *t*-test was used for comparisons between the fucoidan fractions either in each group of same algal species (***Ups***, ***Fe***, ***Sc***) or in ***Mix*** group as shown in Fig. 1. p values < 0.05 were defined as statistically significant.