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Protease activated receptor-1, but not -2, -3 and -4, is the player in the pathogenesis of atrial fibrosis; The experiment by neonatal rat atrial fibroblasts

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We have recently found the co-localization of protease activated receptor (PAR)-1 with α-smooth muscle actin (α-SMA) in the subendocardial space from autopsied hearts of human [1]. α-SMA is activated by factor Xa (FXa) via PAR-1 in the human and murine fibroblasts [2]. However, there are four types of PARs; PAR-1, -2, -3 and -4 [3]; and little is known about whether the PAR-2, -3 and -4 are associated with promoting fibrosis. In this study, we examined which types of PARs accelerate the pro-fibrotic activity of cultured neonatal rat atrial fibroblasts.

FXa from human plasma was purchased from Haematologic Tehnologies (River Road, VT). Rivaroxaban was provided from Bayer Pharma AG (Leverkusen, Germany). We purchased SFLLR-NH_2 (Sigma–Aldrich, St. Louis, MO), SLIGRL-NH_2 (Sigma–Aldrich, St. Louis, MO), SFNGGP-NH_2 (Ana Spec, Fremont, CA), AYPGKF (Ana Spec, Fremont, CA), FR171113 (TOCRIS bioscience, Bristol, United Kingdom).

All animals received humane care and study protocols comply with the guidelines of the Animal Research Committee at Jikei University School of Medicine. Primary atrial fibroblast cultures were prepared from four-day-old Sprague–Dawley rat heart atria using the Neonatal Cardiomyocyte Isolation System with a differential plating procedure. The fibroblasts were cultured in DMEM/10% fetal bovine serum and harvested on days 3–4 of the primary culture.

Western blot analyses were performed using anti-β-actin (Sigma–Aldrich, St. Louis, MO), anti-α-SMA (AnaSpec, Fremont, CA), anti-PAR-1 (Santa Cruz, Delaware Avenue, CA), anti-PAR-2 (Santa Cruz, Delaware Avenue, CA), anti-PAR-3 (Santa Cruz, Delaware Avenue, CA) and anti-PAR-4 (Santa Cruz, Delaware Avenue, CA) as the primary antibodies.

Atrial fibroblasts were cultured to form a monolayer on 24-well tissue culture plates (FALCON; Becton Dickinson Labware). The cells were mechanically "wounded" and treated with FXa with or without FR171113 (3 μM). After 24 h of incubation, the fibroblasts were washed with PBS. Photographs were taken at 40× magnification. The migration rate was calculated by counting the cells that crossed into the "scratch" (wounded) area, and the values were presented as a percentage of the total cells on identical "non-scratched" areas (100%).

Continuous variables are expressed as the means ± standard error of the mean (SEM). All statistical analyses were performed using the SPSS software program (version 11.5 J, SPSS Japan Inc., Tokyo, Japan), and differences were considered to be statistically significant for values of $p < 0.05$. All data were analyzed using Welch statistics with post-hoc testing.

We examined the existence of PAR-1, 2, 3 and 4 in the fibroblasts by Western blot analysis (Fig. 1a). All PARs have been demonstrated by Western blot analysis.

We examined the α-SMA expression in atrial fibroblasts (Fig. 1b), and found that FXa (50 nM) significantly increased the α-SMA expression levels, which was significantly inhibited by treatment with the direct FXa inhibitor rivaroxaban (10 μM) ($p < 0.01$). The PAR-1 agonist peptide, SFLLR-NH_2 (100 μM) also increased the expression of α-SMA (Fig. 1b). However, SLIGRL (100 μM), AYPGKF (100 μM) and AYPGKF (100 μM) + SFNGGP (100 μM) did not increase the expression of α-SMA (Fig. 1c). We have next used PAR-1 antagonist peptide, FR171113 and stimulated fibroblast with FXa (Fig. 2a). The increase of the expression of α-SMA by FXa was inhibited by FR171113.

We observed that the fibroblasts stimulated with FXa (50nM) migrated more extensively than the control fibroblasts. This migration was significantly inhibited by treatment with FR171113 (Fig. 2b).

We have found that FXa activates proliferation of neonatal rat atrial fibroblasts. PAR-2 agonist peptide, SLIGRL-NH_2 could not activate PAR-2.

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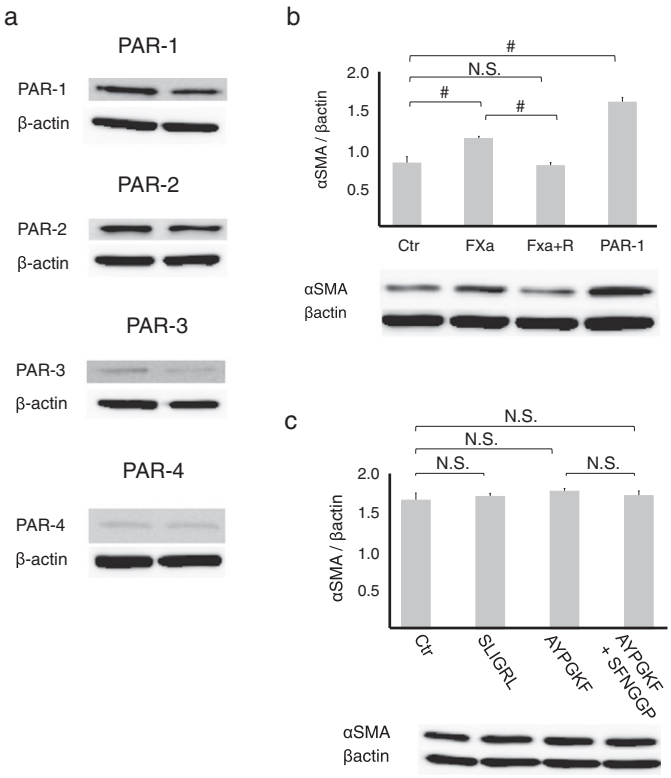

Fig. 1. a. The results of Western blot analysis show PAR-1, -2, -3 and -4 expressions. **b**. The results of the Western blot analysis showing the αSMA expression in neonatal rat atrial fibroblasts stimulated with FXa ($n = 4$). #: $p < 0.01$, N.S.: not significant, PAR-1: SFLLR-NH_2 (100 μM), R: rivaroxaban. **c**. The results of the Western blot analysis show the αSMA expression stimulated with SLIGRL-NH_2 (100 μM), AYPGKF-NH_2 (100 μM), AYPGKF-NH_2 (100 μM) + SFNGGP-NH_2 (100 μM) ($n = 4$). N.S.: not significant.

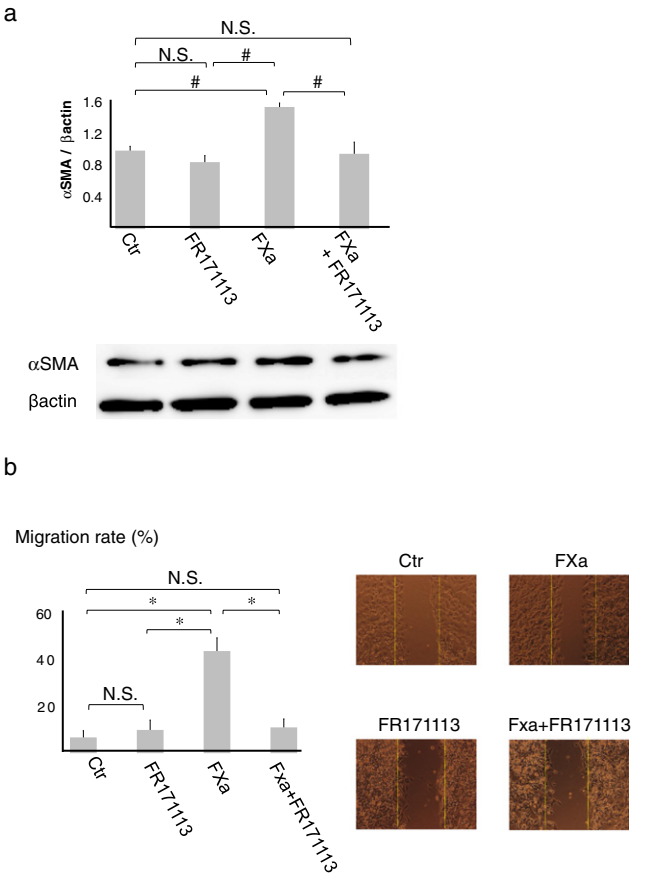

Fig. 2. a. The results of the Western blot analysis show the αSMA expression in neonatal rat atrial fibroblasts ($n = 5$). #: $p < 0.01$, N.S.: not significant. **b**. The results of the cell migration wound healing "scratch" assay ($n = 4$). ∗: $p < 0.05$, N.S.: not significant.

PAR-3 is known to augment the function of PAR-4 [4], but in this study, PAR-3 agonist peptide SFNGGP and PAR-4 agonist peptide AYPGKF did not show the pro-fibrotic effects in the fibroblasts.

PAR-1 may be related to the pathogenesis of atrial fibrosis [1]. But other PARs (PAR-2, -3 and -4) are little known in the view point of atrial fibrosis. The present study highlights that the rat atrial fibrosis is mainly played by the PAR-1 but not PAR-2, -3 and -4.

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