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Increased Smad2/3 phosphorylation in circulating leukocytes and platelet-leukocyte aggregates in a mouse model of aortic valve stenosis: Evidence of systemic activation of platelet-derived TGF-β1 and correlation with cardiac dysfunction☆

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ABSTRACT

Background: Transforming growth factor-β1 (TGF-β1) has been implicated in the pathogenesis of aortic valve stenosis (AS). There is, however, little direct evidence for a role of active TGF-β1 in AS due to the sensitivity of current assays. We searched for evidence of plasma TGF-β1 activation by assaying Smad2/3 phosphorylation in circulating leukocytes and platelet-leukocyte aggregates (PLAs) in a mouse model of AS (Reversa).
Methods: Echocardiography was used to measure AS and cardiac function. Intracellular phospho-flow cytometry in combination with optical fluorescence microscopy was used to detect PLAs and p-Smad2/3 levels.
Results: Reversa mice on a western diet developed AS, had significantly increased numbers of PLAs and more intense staining for p-Smad2/3 in both PLAs and single leukocytes (all $p < 0.05$). p-Smad2/3 staining was more intense in PLAs than in single leukocytes in both diet groups ($p < 0.05$) and correlated with plasma total TGF-β1 levels ($r = 0.38$, $p = 0.05$ for PLAs and $r = 0.37$, $p = 0.06$ for single leukocytes) and reductions in ejection fraction ($r = -0.42$, $p = 0.03$ for PLAs and $r = -0.37$, $p = 0.06$ for single leukocytes).
Conclusions: p-Smad2/3 staining is more intense in leukocytes of hypercholesterolemic mice that developed AS, suggesting increased circulating active TGF-β1 levels. Leukocyte p-Smad2/3 may be a valuable surrogate indicator of circulating active TGF-β1.

1. Introduction

Aortic valve stenosis (AS) is characterized by progressive fibrosis and calcification, leading to aortic valve (AV) narrowing and increased shear stress (SS) across the valve [1]. SS can activate platelets, leading to exposure of surface P-selectin and development of platelet-leukocyte aggregates (PLAs) [2,3]. Increased numbers of PLAs has been reported in the circulation of patients with AS and AV replacement decreases but does not normalize them [4].

Activated platelets release their granule contents, which includes transforming growth factor-β1 (TGF-β1). Our lab has reported that SS can activate latent TGF-β1 released from platelets in vitro and has provided presumptive evidence of TGF-β1 activation in in vivo mouse models of thrombosis and AS [1,5,6]. However, it is difficult to detect activated TGF-β1 in plasma because plasma levels of total TGF-β1 are low, active TGF-β1 has a short half-life ($t_{1/2}$ = 2–3 min) [7,8], and current assays lack high sensitivity [1,6,9,10]. Therefore, we searched for evidence of TGF-β1 activation in a mouse model of AS by assessing the level of phosphorylated Smad2/3, a downstream mediator of the classical TGF-β1 signaling pathway, in both circulating single leukocytes and PLAs.

2. Material and methods

2.1. Mice and diet

We studied 10–12 weeks old $Ldlr^{-/-}Apob^{100/100}/Mttp^{fl/fl}/Mx1Cre^{+/+}$ mice ("Reversa"), which spontaneously develop AS when fed a western style diet (WD) (n = 13) [1]. As a control, we studied the same mice fed a chow diet (n = 13) who received 4 injections of polyinosinic–polycytidylic acid (pI–pC, 225 μg, i.p.) at the age of 6 weeks to induce the Cre and thus lower their cholesterol levels. After 3 months, echocardiography and flow cytometry were performed as previously reported [1].

Abbreviations: AS, aortic valve stenosis; SS, shear stress; AV, aortic valve; PLA, platelet-leukocyte aggregate; WD, western type diet; PSGL-1, P-selectin glycoprotein ligand-1; PGE1, prostaglandin E1; MLEC, mink lung epithelial cells; EF, ejection fraction; FS, fractional shortening; rhTGF-β1, recombinant human TGF-β1; TGFb-RI, transforming growth factor-β receptor I; ELISA, enzyme-linked immunosorbent assay; PAI-1, plasminogen activator inhibitor.

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2.2. Flow cytometry and optical fluorescence microscopy to detect platelet leukocyte aggregates

Whole blood was obtained from the left ventricle of Reversa mice under ultrasound guidance as previously described [6] and anticoagulated with 3.8% sodium citrate containing 1 μM PGE_1 and 10 μg/ml purified anti P-selectin glycoprotein ligand-1 (anti-PSGL-1) antibody (4RA10, BD 557787) to prevent platelet activation and in vitro formation of PLAs. In some experiments, retroorbital blood (500 μl) was also obtained from WT mice and treated with recombinant human TGF-β1 (rhTGF-β1, 240-B, R&D Systems) for 30 min at 37 °C with or without an inhibitor of transforming growth factor-β receptor I (TGFb-RI) (SB-525334,10 μM; Selleck Chemicals).

Erythrocytes were then lysed and cells were fixed by adding BD phosflow™ lyse/fix buffer (BD 558049), permeabilized on ice with BD phosflow™ perm buffer III (BD 558050) for 30 min, and stained with antibodies to phospho(p)-Smad2 (pS465/pS467) and p-Smad3(pS423/pS425) (072–670, BD 562697, final concentration 10 μg/ml), integrin αIIb (MWReg30, Biolegend, final concentration 5 μg/ml), and CD45 (30-F11, Biolegend, final concentration 5 μg/ml) and total Smad3 (042021, USBiological, final concentration 10 μg/ml). Leukocytes and PLAs were analyzed by both flow cytometry (LSR-II, BD) and a combination of digital optical and fluorescence microscopy and flow cytometry (ImageStream-X; Amnis Corporation).

2.3. Flow cytometry of plasma treated mink lung epithelial cells (MLECs) to quantify p-Smad2/3

MLECs (10^5 in 150 μl of DMEM), a gift from Dr. Daniel B. Rifkin of New York University School of Medicine, were treated with rhTGF-β1, with or without SB-525334 or plasmas (50 μl) from Reversa mice before or 3 months after the initiation of WD diet. After a 60 min incubation at 37 °C, cells were fixed in BD phosflow™ lyse/fix buffer for 10 min at 37 °C, washed, and then permeabilized on ice with BD phosflow™ permeabilization buffer III (BD 558050) for 30 min. Cells were then stained with antibodies to p-Smad2/3 and total Smad3.

2.4. Statistics

All continuous data are reported as mean ± SEM. Differences in means between 2 independent groups were analyzed using a 2-sample Welch t test, whereas paired Student t test was used with paired samples. One-way ANOVA was used to analyze the differences between the means of three or more independent groups. A 2-tailed p value < 0.05 was considered significant. Flow cytometry data were analyzed using Flow Jo and the combined flow cytometry and microscopy data were analyzed by Ideas 6.0.

3. Results and discussion

3.1. AV stenosis, blood counts and induction of PLAs in Reversa mice on WD

Consistent with our previous report, feeding Reversa mice a WD rather than a chow diet led to a reduction in AV cusp separation distance and an increase in SS across the AVs (Fig. 1A and B) [1]. Reversa mice on a WD had white blood cell counts that were similar to those of Reversa mice on a chow diet (Fig. 1C), but they had somewhat higher platelet counts ($p = 0.03$) (Fig. 1D). Reversa mice in the WD group had a significantly high percentage of CD45(+) leukocytes with attached platelets (26.1 ± 3.1% vs. 16.4 ± 2.2%,$p = 0.02$) (Fig. 1E) and a higher total number of circulating PLAs ($1.01 \pm 0.13 \times 10^6$ vs. $0.71 \pm 0.1 \times 10^6$/ml, $p = 0.03$) (Fig. 1F).

3.2. p-Smad2/3 staining in PLAs and single leukocytes of Reversa mice

Similar to what we previously reported, Reversa mice on a WD had significantly increased plasma total TGF-β1 levels compared with those on a chow diet (Fig. 2A) [1]. We were not able to detect active TGF-β1 in the plasma of Reversa mice on either a chow diet or a WD diet using the enzyme-linked immunosorbent assay (ELISA). We also were unable to identify active TGF-β1 in plasmas collected from Reversa mice before and 3 months after of WD diet using a bioassay employing mink lung epithelial cells (MLEC) containing a plasminogen activator inhibitor-1 (PAI-1) promoter/luciferase reporter and flow cytometry

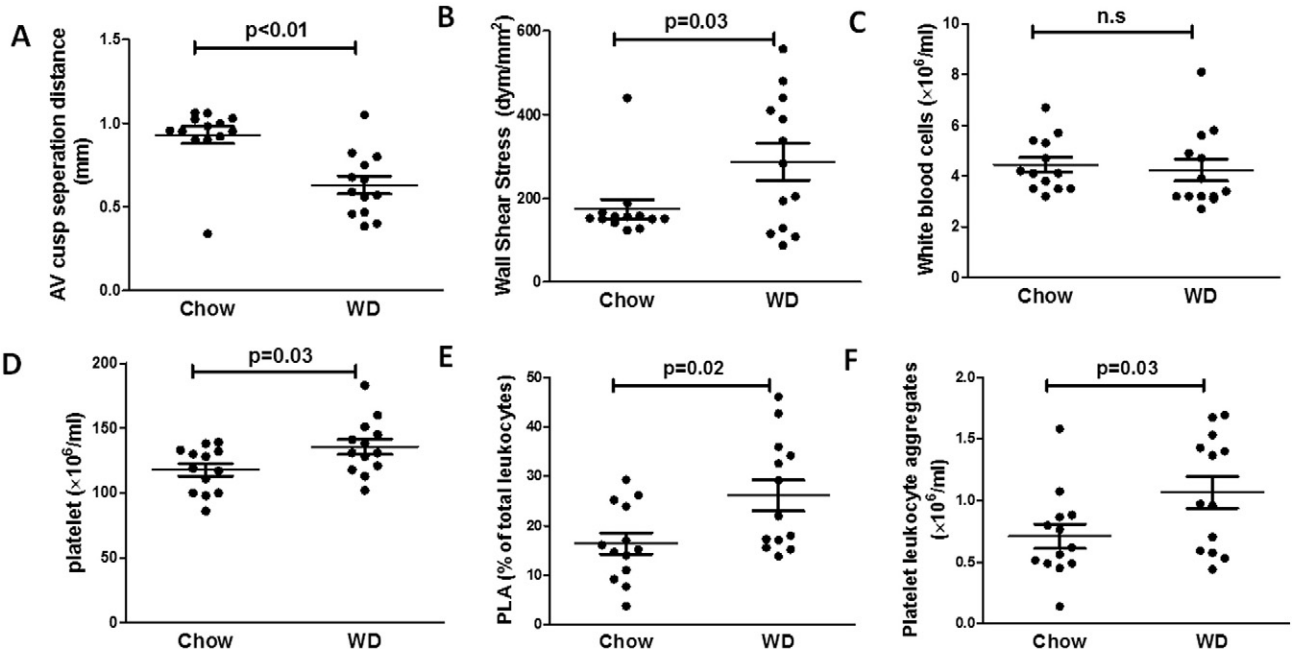

Fig. 1. Reversa mice on WD develop AS and increased PLAs. (A) AV cusp separation distance, (B) wall shear stress, (C) whole blood white blood cell counts, (D) whole blood platelet counts, (E) PLA percentage of CD45(+) leukocytes, and (F) total PLA counts. n.s: not significant; WD: western type diet; AV: aortic valve.