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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- β 1 and Correlation with Cardiac Dysfunction

Wei Wang, MD, Nathalie Burg, MD, Spandana Vootukuri, MS, and Collier S. Barry, MD
Allen and Frances Adler Laboratory of Blood and Vascular Biology, the Rockefeller University,
New York, NY

Abstract

Background—Transforming growth factor- β 1 (TGF- β 1) has been implicated in the pathogenesis of aortic valve stenosis (AS). There is, however, little direct evidence for a role of active TGF- β 1 in AS due to the sensitivity of current assays. We searched for evidence of plasma TGF- β 1 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- β 1 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- β 1 levels. Leukocyte p-Smad2/3 may be a valuable surrogate indicator of circulating active TGF- β 1.

Correspondence to Barry S. Collier, MD., The Allen and Francis Adler Laboratory of Blood and Vascular Biology, Rockefeller University, 1230 York Avenue, New York, NY 10065. collierb@rockefeller.edu. Tel: (212)-327-7490, Fax: (212)-327-7493.

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Contributions

W.W., N.B. and S. V. performed experiments. W.W. and N.B. designed the research and analyzed data along with B.S.C. W. W. wrote the manuscript. N.B. and B.S.C. edited the manuscript.

Disclosure of Conflicts of Interest

Dr. Barry Collier has royalty interests in abciximab (Centocor) and the VerifyNow assays (Accumetrics), and serves as a consultant to Platelet Biogenesis (pro bono) and Scholar Rock.

Keywords

aortic valve stenosis; blood platelets; heart failure; leukocytes; Tgfb1 protein; mouse

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

Material and methods

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 μ g, 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 previous reported [1].

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 μ M PGE₁ and 10 μ g/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 μ l) was also obtained from WT mice and treated with recombinant human TGF- β 1 (rhTGF- β 1, 240-B, R&D Systems) for 30 min at 37°C with or without an inhibitor of transforming growth factor- β receptor I (TGF β -RI) (SB-525334, 10 μ M; 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 (MWRReg30, 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).

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.

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.

Results and Discussion

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 (Figure 1A and 1B) [1]. Reversa mice on a WD had white blood cell counts that were similar to those of Reversa mice on a chow diet (Figure 1C), but they had somewhat higher platelet counts ($p=0.03$) (Figure 1D). Reversa mice in the WD group had a significantly high percentage of CD45(+) leukocytes with attached platelets ($26.1\pm3.1\%$ vs. $16.4\pm2.2\%$, $p=0.02$) (Figure 1E) and a higher total number of circulating PLAs ($1.01\pm0.13 \times 10^6$ vs. $0.71\pm0.1 \times 10^6/\text{ml}$, $p=0.03$) (Figure 1F).

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 (Figure 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) assay. 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 to assess p-Smad2/3 (data not shown) [5, 9, 11]. Thus, we sought an alternative method to detect circulating active TGF- β 1 in plasma and reasoned that active TGF- β 1 might induce phosphorylation of Smad2/3 in circulating PLAs and leukocytes. In fact, we found that the intensities of p-Smad2/3 staining of PLAs and leukocytes free of platelets were both higher in Reversa mice on a WD rather than a chow diet ($p<0.05$ and $p<0.01$, respectively) (Figure 2B and 2C). In addition, p-Smad2/3 staining was more intense in PLAs than in leukocytes free of platelets in both WD and chow diet groups (both $p<0.001$). There was a modest correlation between p-Smad2/3 staining intensity in PLAs and plasma total TGF- β 1 levels when combining data on mice from both diet groups ($r=0.38$, $p=0.05$) (Figure 2D). A borderline correlation also existed between p-Smad2/3 staining intensity in single leukocytes and plasma total TGF- β 1 levels ($r=0.37$, $p=0.06$) (Figure 2D). Using combined optical and fluorescence imaging and flow cytometry allowed us to confirm the presence of PLAs and assess the number of platelets per leukocyte in PLAs. It also allowed us to identify the location of p-Smad2/3 staining. We found most of the p-Smad2/3 staining was located in the leukocytes of PLAs, with occasional platelet staining (Figure 2E). However, the fluorescent area of p-Smad2/3 staining did not correlate with the intensity of platelet staining of PLAs ($r=-0.05$, $p=0.54$) (Figure 2F).

To assess the sensitivity and specificity of the assay, blood from WT mice was treated with various concentration of TGF- β 1 for 30 min with or without a specific inhibitor of TGF β -R1. There was a dose response between the p-Smad2/3 signal on flow cytometry in whole blood neutrophils and the concentration of rhTGF- β 1 between 5–500 pg/ml (Figure 3A, one-way ANOVA $p<0.001$). In contrast, the geometric mean value of total Smad3 did not change significantly as a result of TGF- β 1 stimulation (Figure 3B, one-way ANOVA $p>0.05$). The p-Smad2/3 signal in leukocytes treated with 500 pg/ml was completely blocked by an inhibitor of TGF-RI, supporting the specificity of the *in vitro* assay for TGF- β 1 (Figure 3C).

Correlation between p-Smad2/3 levels in PLAs and cardiac function

Reversa mice on a WD developed systolic cardiac dysfunction as judged by decreased ejection fraction (EF) (55 ± 3.0 vs. $67\pm2.2\%$, $p<0.01$) and fractional shortening (FS) (28 ± 2.0 vs. $36\pm1.7\%$, $p<0.01$) (Figure 4A and 4B). They also showed a trend toward cardiac hypertrophy as reflected by an increase in left ventricular mass (96.4 ± 5.1 vs. 84.6 ± 3.6 mg, $p=0.07$), an increase in left ventricular posterior wall thickness (0.90 ± 0.05 vs. 0.75 ± 0.02 mm, $p=0.01$), and an increase in end-diastolic inter-ventricular septum thickness (0.76 ± 0.04 vs. 0.64 ± 0.03 mm, $p=0.02$) (Figure 4C–4E). Of note, p-Smad2/3 intensity in PLAs correlated with cardiac dysfunction and cardiac hypertrophy as reflected by both ejection fraction ($r=-0.42$, $p=0.03$) and end diastolic inter-ventricular septum thickness ($r=0.50$, $p<0.01$) (Figure 4F and 4G). p-Smad2/3 intensity in single leukocyte also had a borderline correlation with ejection fraction ($r=-0.37$, $p=0.06$) and a significant correlation with end diastolic inter-ventricular septum thickness ($r=0.44$, $p=0.03$) (Figure 4F and 4G).

Conclusions

In summary, we found evidence of increased leukocyte p-Smad2/3 staining in both single leukocytes and PLAs in mice that developed AS, suggesting the presence of increased circulating active TGF- β 1. In addition, PLA p-Smad2/3 staining correlated with multiple parameters of cardiac dysfunction. Plasmas from these animals did not demonstrate active TGF- β 1 on ELISA nor did they induce detectable p-Smad2/3 signaling in MLECs. Since the ELISA has a sensitivity of approximately 31 pg/ml and the leukocyte p-Smad2/3 signal assay has a sensitivity between 5–50 pg/ml, they are relatively similar in sensitivity. As a result, the positive leukocyte p-Smad2/3 signal in the presence of undetectable active TGF- β 1 by ELISA may reflect the impact of exposure to low levels of TGF- β 1 over a longer period of time or potentially transient exposure to higher concentrations of TGF- β 1 when leukocytes occupy the marginal pool juxtaposed to the vasculature. Thus, leukocyte and PLA p-Smad2/3 staining may be a valuable surrogate indicator of in vivo circulating active TGF- β 1.

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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- β 1
TGFβ-RI	transforming growth factor- β receptor I
ELISA	enzyme-linked immunosorbent assay
PAI-1	plasminogen activator inhibitor

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Figure 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.

Figure 2. Reversa mice on WD develop elevated plasma total TGF- β 1 levels and increased p-Smad2/3 in single leukocytes and PLAs

(A) Plasma total TGF- β 1 levels, (B) p-Smad2/3 intensity in single leukocytes and PLAs with representative histograms from one set of experiments (C), (D) correlation between p-Smad2/3 staining intensity and plasma total TGF- β 1 levels, (E) representative fluorescent images of PLAs from Reversa mice on either a WD (upper 4 panels) or a chow diet (lower 3 panels), (F) correlation between p-Smad2/3 staining area and CD41 staining area in PLAs in both diet groups. * $p < 0.05$ between indicated groups; ** $p < 0.01$ leukocytes vs. PLAs in the same diet group. AU: arbitrary unit; WD: western type diet; PLA: platelet leukocyte aggregates.

Figure 3. p-Smad 2/3 signal is initiated by rhTGF-β1 in a dose dependent manner and blocked by a specific inhibitor of TGFb-R1 (SB-525334)

(A) p-Smad 2/3 geometric mean values and (B) total Smad3 geometric mean values in mouse neutrophils, (C) histogram of neutrophil p-Smad 2/3 in mouse neutrophils in response to 500 pg/ml TGF-β1 in the absence and presence of SB-525334 (n=3).

Figure 4. Reversa mice on WD develop cardiac hypertrophy and cardiac dysfunction that correlates with the intensity of p-Smad2/3 staining in PLAs

(A) Ejection fraction, (B) fractional shortening, (C) left ventricular mass, (D) left ventricular posterior wall thickness, and (E) end diastolic inter-ventricular septum thickness of Reversa mice on either a chow diet or WD. (F–G) correlation between p-Smad2/3 staining intensity in PLAs and single leukocyte with ejection fraction thickness (F) and end diastolic inter-ventricular septum (G). AU: arbitrary unit; WD: western type diet; PLA: platelet leukocyte aggregates.