Lack of Tyrosylprotein Sulfotransferase Activity in Hematopoietic Cells Drastically Attenuates Atherosclerosis in Ldlr<sup>−/−</sup> Mice

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Objective—Leukocyte recruitment is a major contributor in the development of atherosclerosis and requires a variety of proteins such as adhesion molecules, chemokines, and chemokine receptors. Several key molecular players implicated in this process are expressed on monocytes and require protein-tyrosine sulfation for optimal function in vitro, including human CCR2, CCR5, CX3CR1, and PSLG-1. We therefore hypothesized that protein-tyrosine sulfation in hematopoietic cells plays an important role in the development of atherosclerosis.

Methods and Results—Lethally-irradiated Ldlr<sup>−/−</sup> mice were rescued with hematopoietic progenitors lacking tyrosylprotein sulfotransferase (TPST) activity attributable to deletion of the Tpst1 and Tpst2 genes. TPST deficient progenitors efficiently reconstituted hematopoiesis in Ldlr<sup>−/−</sup> recipients and transplantation had no effect on plasma lipids on a standard or atherogenic diet. However, we observed a substantial reduction in the size of atherosclerotic lesions and the number of macrophages in lesions from hyperlipidemic Ldlr<sup>−/−</sup> recipients transplanted with TPST deficient progenitors compared to wild-type progenitors. We also document for the first time that murine Psgl-1 and Cx3cr1 are tyrosine-sulfated.

Conclusions—These data demonstrate that protein-tyrosine sulfation is an important contributor to monocytes/macrophage recruitment or retention in a mouse model of atherosclerosis.

Key Words: atherosclerosis ■ genetically altered mice ■ protein-tyrosine sulfation

Protein-tyrosine sulfation, a posttranslational modification of secretory and membrane proteins, is mediated by 2 Golgi isoenzymes, tyrosylprotein sulfotransferases 1 and 2 (TPST-1 and -2).<sup>1,2</sup> TPSTs are broadly expressed in multicellular organisms and catalyze the transfer of a sulfuryl group from 3′-phosphoadenosine 5′-phosphosulfate to the hydroxyl group of peptidyl-tyrosine.<sup>3</sup> Human and mouse TPST-1 are 67% identical to their TPST-1 counterparts.

Much of the data recognizing the importance of tyrosine sulfation in protein function has been obtained from in vitro studies.<sup>3–7</sup> Evidence for the importance of tyrosine sulfation in vivo is very limited. Thus, we developed Tpst<sup>1−/−</sup>, Tpst<sup>2−/−</sup> and Tpst<sup>−/−</sup>; Tpst<sup>2−/−</sup> double knockout (Tpst DKO) mice.<sup>8–10</sup> Our studies revealed that both Tpst<sup>1−/−</sup> and Tpst<sup>2−/−</sup> mice are viable and have distinct phenotypes. In part, this is because of differences in substrate specificity of the two isoenzymes.<sup>11</sup> In addition, Tpst DKO mice have early postnatal lethality attributable to pulmonary insufficiency of unknown cause.<sup>10</sup> Together, these studies demonstrate that TPST-1 and TPST-2 have distinct but overlapping function.

The role of tyrosine sulfation in protein function has been demonstrated for a number of proteins.<sup>3,12</sup> For example, sulfation of platelet gp1bα and factor VIII is required for efficient binding to thrombin<sup>13</sup> and von Willebrand factor<sup>14</sup> respectively, whereas sulfation of factor V and factor VIII is required for optimal proteolytic processing.<sup>15,16</sup> In the human system, sulfation of P-selectin glycoprotein ligand-1 (PSGL-1) is required for optimal binding to P- and L-selectin.<sup>17</sup> In addition, sulfation of the N-terminal extracellular domains of certain G protein–coupled receptors, including several chemokine receptors (ie, CCR2, CCR5, CX3CR1), glycoprotein hormone receptors (ie, thyroid stimulating hormone), and others is required for optimal ligand binding and ligand-induced responses in vitro.<sup>3,5–7,17</sup> Mouse orthologs of several of these proteins have been shown to play important roles in mouse models of atherosclerosis, including Psgl-1, Ccr2, Ccr5, and Cx3cr1. Significantly, attenuation of atherosclerosis is observed in ApoE<sup>−/−</sup> mice that are also deficient in Psgl-1, Ccr2, Ccr5, and Cx3cr1.<sup>18–22</sup> However, the importance of tyrosine sulfation in a complex chronic inflammatory disease such as atherosclerosis is unknown.
To explore this question we assessed the development of atherosclerosis in a model in which *Tpst* DKO hematopoietic progenitors were used to reconstitute hematopoiesis in hyperlipidemic *Ldlr<sup>−/−</sup>* mice. We observed that TPST deficient progenitors reconstituted hematopoiesis in *Ldlr<sup>−/−</sup>* recipients and that transplantation had no discernible effect on plasma lipids in *Ldlr<sup>−/−</sup>* recipients fed either a standard or atherogenic diet. However, we observed a substantial reduction in both lesion size and in the number of macrophages in lesions from *Ldlr<sup>−/−</sup>* recipients transplanted with *Tpst* DKO compared to wild-type hematopoietic progenitors.

**Methods**

**Atherosclerosis Model**

The generation and characterization of *Tpst1<sup>−/−</sup>*; *Tpst2<sup>−/−</sup>* double knockout mice in the 129S6 background was described previously. Therefore, fetal livers were used as the source of hematopoietic progenitors. *Tpst* DKO and wild-type fetuses were generated by timed matings of *Tpst1<sup>−/−</sup>*; *Tpst2<sup>−/−</sup>* and wild-type males and females, respectively. Fetal livers were harvested at E15.5 and single cell suspensions were prepared by passage through 40 μm nylon strainers. Cells were washed in Hank balanced salt solution (HBSS), 10 mmol/L HEPES, and 10% fetal bovine serum and stored at 4°C until transplanted. *Tpst* DKO fetuses were identified by flow cytometry using PS2, a monoclonal antibody (mAb) that recognizes sulfotyrosine residues in proteins (see supplemental Methods). Genotypes were confirmed by PCR for the ly10, ly23 and is discussed in detail in supplemental materials (available online at http://atvb.ahajournals.org).

**Lesion Analysis**

Lesion analysis was performed as previously described. Briefly, serial 10-μm sections through the entire aortic valve and sinus were collected on slides so that each slide contained every tenth section. Slides were stained with oil red O and counterstained with hematoxylin. For each section, lesion area (oil red O–reactive area) and the area within the internal elastic lamina were measured from digital images using a digitizing tablet (Model CTE-630BT, Wacom Technology) and ImageJ software. The ratio of lesion area to area within the internal elastic lamina was calculated and averaged over all sections spanning the lesion. The extent of lesion necrosis, defined as the ratio of acellular area to lesion area, was calculated and averaged over 3 midvalve sections.

Macrophages were quantitated using immunofluorescence microscopy in sections immediately adjacent to those stained with oil red O. Frozen sections were blocked in PBS/5% fish gelatin (Ted Pella Inc) for 1 hour. Macrophages were labeled using 25 μg/mL of the rat antimouse monocyte/macrophage mAb MOMA-2 (IgG2a, AbD Serotec) for 1 hour. After washing, sections were incubated in 10 μg/mL Alexa Fluor 488 streptavidin (Invitrogen) for 1 hour, washed, rinsed in H2O, and mounted in Vector hard mount containing DAPI. Fluo-3 indicator dye (250 mg/kg, i.p.) and exsanguinated. Blood was collected into Avertin (250 mg/kg, i.p.) and exsanguinated. Blood was collected into EDTA and blood counts were performed using a Hemovet HV950FS Hematology Analyzer (Drew Scientific).

**Results**

**Lesion Analysis**

*Ldlr<sup>−/−</sup>* recipients were transplanted with fetal liver hematopoietic progenitors from either *Tpst* DKO or wild-type fetuses
in the 129S6 background or wild-type C57BL/6J fetuses. Eighteen weeks after transplant, atherosclerotic lesions were evident in all transplant groups. In control B6-WT\(^3\)Ldlr and 129-WT\(^3\)Ldlr animals, the lesions encompassed 24.2\(\pm\)3.0\% (mean\(\pm\)SEM, n=6) and 19.2\(\pm\)2.7\% (n=10) of the area within the internal elastic lamina, respectively (Figure 2A and supplemental Figure I). In contrast, the lesions in 129-Tpst DKO\(^3\)Ldlr animals encompassed only 6.2\(\pm\)0.9\% (n=10) of the area within the internal elastic lamina, a \(\approx\)3-fold reduction in lesion size (Figure 2B). Lesion size in the 3 groups was significantly different (P<0.0001, 1-way ANOVA). Posthoc \(t\) testing showed that lesions in the 129-Tpst DKO\(^3\)Ldlr group were smaller compared to both the B6-WT\(^3\)Ldlr (P<0.0001) and 129-WT\(^3\)Ldlr (P=0.0003) groups. However, lesion size was not different between B6-WT\(^3\)Ldlr and 129-WT\(^3\)Ldlr mice (P=0.25).

We also quantitated the extent of necrosis within the atherosclerotic lesions. Necrosis was evident in lesions from all transplanted groups and the total necrotic area was reduced in 129-Tpst DKO\(^3\)Ldlr lesions compared to the control groups. However, there were no statistical differences in the ratio of necrotic area to lesion area between transplant groups (Figure 2C; P=0.32, 1-way ANOVA). The number of lesional macrophages was determined in 3 midvalve sections from 5 animals in each experimental group at 18 weeks posttransplant. Accumulation of macrophages was evident in all transplanted groups (Figure 2E). In control B6-WT\(^3\)Ldlr and 129-WT\(^3\)Ldlr animals, the number of macrophages in lesions was 4558\(\pm\)917 and 5447\(\pm\)758 cells/mm\(^2\), respectively (mean\(\pm\)SEM). In contrast, we observed only 2232\(\pm\)168 cells/mm\(^2\) in the lesions in 129-Tpst DKO\(^3\)Ldlr animals (Figure 2D). The number of macrophages in the 3
Fasting plasma total cholesterol, HDL cholesterol, and triglycerides were determined 2 weeks posttransplant while mice were on normal chow and 6 and 14 weeks posttransplant while on an atherogenic diet. Mean ± SEM (n = 10) of assays performed in triplicate. Statistical significances between the 3 experimental groups at each time point were determined by single factor ANOVA.

To assess whether the attenuation of lesion development was specific for the aortic root, en face oil red O staining of the abdominal aortas from 4 animals in each transplant group was performed focusing on the ostia of the intercostal arteries. We observed a similar degree of attenuation of lesion development in the 129-Tpst DKO→Ldlr group compared to the control groups as observed in the aortic root (supplemental Figure III).

Lipid Analysis
The impact of the transplants on lipid metabolism was assessed by measuring total cholesterol, HDL cholesterol, and triglyceride levels at 2 weeks posttransplant while mice were on a normal chow diet and then again at 6 and 14 weeks posttransplant while on an atherogenic diet (Table). No statistical differences were observed between the groups at 2 weeks posttransplant while on normal chow. As anticipated, total cholesterol, HDL cholesterol, and triglyceride levels increased in all 3 groups after 4 and 12 weeks on the atherogenic diet. However, there were still no statistical differences between groups.

Hematopoietic Reconstitution
Blood counts were performed 18 weeks posttransplant. Total leukocyte, lymphocyte, monocyte, and platelet counts were not statistically significant different between the transplant groups (supplemental Table 1). However, neutrophil and erythrocyte counts were statistically different as assessed by 1-way ANOVA. Posthoc t testing showed that neutrophil counts were higher in the 129-WT→Ldlr group compared to B6-WT→Ldlr groups (P = 0.022). In addition, erythrocyte counts were higher in the B6-WT→Ldlr compared to the 129-Tpst DKO→Ldlr (P = 0.003). Nevertheless, all parameters were within the normal range.

To assess the efficiency of reconstitution of Tpst DKO hematopoiesis in Ldlr−/− mice, the percentage of circulating PSG2-positive leukocytes was determined at 18 weeks posttransplant. Analysis of the leukocyte population from 129-WT→Ldlr mice showed that virtually all the circulating leukocytes were PSG2-positive (Figure 3, top). However, fluorescence (FL1) histograms of cells stained with isotype control mAb and PSG2 are not completely resolved. Thus, the valley between the 2 FL1 histograms was used to define the FL1 threshold for quantitating the percentage of PSG2-positive cells in 129-Tpst DKO→Ldlr mice (Figure 3, bottom). It should be noted that this definition might underestimate the degree of donor hematopoiesis in mice transplanted with Tpst DKO progenitors. Nevertheless, 82.4 ± 1.9% (mean ± SEM, n = 10) of the total leukocytes displayed a PSG2lo phenotype and thus were of donor origin. By the same criteria, 92.7 ± 1.1% (mean ± SEM, n = 10) of the total leukocytes in 129-WT→Ldlr mice were PSG2hi. Taken together, these data confirm efficient reconstitution of Tpst DKO hematopoiesis in the Ldlr−/− recipients at the time of lesion analysis.
Protein-Tyrosine Sulfation and Atherosclerosis

To address the question, a direct biochemical analysis of metabolically labeled, purified native mouse Psgl-1 and recombinant mouse Cx3cr1 expressed in mouse pre-B L1.2 cells was performed as described in supplemental Materials. This analysis unambiguously demonstrates that both molecules are tyrosine-sulfated (Figure 5).

**Discussion**

Atherosclerosis is a chronic inflammatory disease of the arterial wall initiated by injury of the vascular endothelium leading to endothelial dysfunction and subsequent intramural accumulation of oxidized LDL. This is followed by the elaboration of signaling molecules and induction of adhesion receptors that promote recruitment of monocytes into developing lesions, which subsequently mature into lipid-laden foam cells. A compelling body of evidence indicates that recruitment of monocytes is a dominant factor in the initiation and progression of atherosclerosis. Genetic studies in mice indicate that Ccr2, Ccr5, Cx3cr1, and the adhesion molecule Psgl-1, which are all expressed on monocytes, are proatherogenic and play important roles in monocyte recruitment in atherosclerosis. Furthermore, in vitro studies have shown that human CCR2B, CCR5, CX3CR1, and PSGL-1 contain sulfotyrosine residues that are required for optimal function in vitro. However, at present it is not known whether these proteins are sulfated in the mouse system or whether sulfation of these proteins is important for their optimal function in vivo.

Based on these data, we examined the effect of hematopoietic deficiency of TPST activity on the development of atherosclerosis in a model in which TPST DKO hematopoietic progenitor cells were used to reconstitute hematopoiesis in Ldlr<sup>−/−</sup> mice. We observed a 68% reduction in the size of aortic root lesions in recipients transplanted with TPST-deficient progenitors compared to 129S6 wild-type controls. The reduction in lesion size was accompanied by a 2- to 3-fold reduction in the number of lesional macrophages. As expected, attenuated lesions in 129-Tpst DKO→Ldlr animals contained smaller necrotic areas. However, no differences in the ratio of necrotic area to lesion size were apparent between the experimental and control groups. This suggests that although TPST deficiency in macrophages attenuates monocyte/macrophage recruitment or retention, it does not perturb phagocytic clearance of apoptotic/necrotic cells.

To address potential mechanisms(s) for the reduction in lesion size, factors known to be important in lesion development or progression were assessed. Lipid metabolism and especially LDL cholesterol levels are directly correlated with development of atherosclerosis. However, we observed no differences in cholesterol or triglyceride levels between the experimental groups. Thus, TPST deficiency in the hematopoietic compartment does not affect overall lipid homeostasis in the Ldlr<sup>−/−</sup> recipients, and the reduction in lesion size was not attributable to reduced exposure of the endothelium to plasma-derived lipids. Monocyte recruitment is the major cellular event to influence lesion progression and the level of circulating monocytes can directly impact lesion development. However, we observed no differences in monocyte counts between the experimental and control groups at 18 weeks posttransplant.

### Figures

**Figure 4.** Immunofluorescence staining of lesional macrophages. A representative lesion from a 129-Tpst DKO→Ldlr mouse at 18 weeks posttransplant stained with DAPI (A), the macrophage specific MOMA-2 mAb (B), and the anti-sulfotyrosine PSG2 (C). D. Merged pseudocolor image. DAPI (blue), MOMA-2 (red), and PSG2 (green). Red/green emission overlap=orange/yellow. E, Diagrammatic representation of the image shown in D. E indicates endothelium; NI, neointima; M, media; A, adventitia. Bar=200 μm.

To assess whether the macrophages in the lesions in 129-Tpst DKO→Ldlr mice were donor or recipient in origin, immunofluorescence staining was performed using MOMA-2 and PSG2 antibodies. We observed clear colocalization of MOMA-2 and PSG2 staining in 129-Tpst DKO→Ldlr lesions (Figure 4), indicating that some of the macrophages in these lesions were of recipient and not donor origin.

**Psgl-1 and Chemokine Receptor Expression**

To assess whether the absence of TPSTs in hematopoietic cells affects surface expression of Psgl-1 and a panel of chemokine receptors, we examined peripheral blood leukocytes in B6.SJL mice (CD45.1<sup>+</sup>) transplanted with 15.5-dpc fetal liver cells from Tpst DKO mice (CD45.2<sup>+</sup>) or wild-type 129S6 mice by flow cytometry. In 3 independent comparisons 20 to 24 weeks posttransplant, surface expression of Psgl-1, Ccr5, and Cx3cr1 was indistinguishable in the 129-Tpst DKO→B6.SJL and 129-WT→B6.SJL groups (supplemental Figure IIA). At the time of analysis, all recipients had normal blood counts, and 90% to 95% of circulating leukocytes were of donor (CD45.2<sup>+</sup>) origin (data not shown). We also examined thymocytes from a rare 2-week-old Tpst DKO survivor and a sex- and age-matched wild-type mouse. We observed no differences in the expression of Psgl-1, Ccr6, Ccr7, D6, Cxcr2, and Cxcr4 (supplemental Figure IIB).

**Sulfotyrosine Analysis**

It is widely assumed that mouse Psgl-1 and many chemokine receptors are tyrosine-sulfated like their human counterparts. However, this has never been experimentally verified. To...
weeks posttransplant. Reduced lesion size in 129-Tpst DKO→Ldlr mice may be attributable to reduced expression of chemokine receptors or adhesion molecules on TPST-deficient hematopoietic cells resulting in decreased recruitment of monocytes into developing lesions. However, we observed that expression of a panel of chemokine receptors and Psgl-1 on Tpst DKO hematopoietic cells was normal. This finding is consistent with previous observations that inhibition of tyrosine sulfation has no impact on the secretion or surface expression of a variety of tyrosine-sulfated proteins, including chemokine receptors.3,5

In our experimental group, Tpst DKO fetal liver cells from 129S6 donors were used to reconstitute hematopoiesis in Ldlr−/− recipients in the B6 background. However, no difference in any experimental end point was observed between the controls in which wild-type 129S6 or C57BL/6J mice were used as donors. Therefore, the reduction in lesion size in the 129-Tpst DKO→Ldlr group is not related to the difference in genetic background between donor and recipient.

In this study, we used the antisulfotyrosine mAb PSG2 to identify Tpst DKO donor fetuses and to assess the degree of chimerism in mice transplanted with Tpst DKO progenitors. In the 129-Tpst DKO→Ldlr mice at 18 weeks posttransplant, we found that on average ≈82% of the total leukocytes were PSG2+ and thus were of donor origin. This led us to ask whether the lesional macrophages in these mice were donor or recipient in origin. Immunofluorescence staining showed colocalization of PSG2 and MOMA-2 staining in 129-Tpst DKO lesions. This indicates that at least some of the lesion development observed in 129-Tpst DKO→Ldlr mice may be attributable to residual recipient hematopoiesis, suggesting that lesion development might have been further attenuated if a higher level of chimerism had been achieved. Alternatively, colocalization of PSG2 and MOMA-2 in lesions may be a reflection of close association of sulfated plasma proteins or membrane microparticles derived from the recipient. It is also possible, but very unlikely, that this colocalization reflects in-trans sulfation of donor macrophage proteins by TPSTs derived from recipient cells.

The 68% reduction in aortic root lesion area we observed is greater than that reported in ApoE−/−; Cx3cr1−/− (32%),22 ApoE−/−; Selplg−/− (≈40%),18 and ApoE−/−; Ccr5−/− double knockouts (≈50%),21 but comparable to that observed in ApoE−/−; Ccr2−/− double knockouts (≈60%).19,20 However, the relative impact that defective sulfation of these proteins has in lesion attenuation is unclear. Nevertheless, it is reasonable to conclude that the dramatic reduction in aortic
root lesion area observed is attributable to defective sulfation of multiple proteins involved in monocyte recruitment and/or retention, including Psgl-1, Cx3cr1 (Figure 5) as well as Ccr2, Ccr5, and other proteins yet to be identified.

Our findings suggest that strategies for treatment and prevention of atherosclerosis might be developed based either on drugs that inhibit the interaction between specific tyrosine-sulfated proteins and their cognate ligand(s) or ones that inhibit TPST activity. Targeting a specific receptor/ligand interaction might be favored if that interaction plays the dominant role in development of atherosclerosis. However, as discussed above, current data argue that the reduction in atherosclerosis we observe is attributable to defective sulfation of multiple proteins. In this circumstance, inhibition of TPST activity may be the preferred approach. However, potential off-target effects may limit the viability of this approach given the number of tyrosine-sulfated proteins both known and unknown.

In conclusion, posttranslational tyrosine sulfation of protein(s) in hematopoietic cells has a major impact on the development of atherosclerosis in hyperlipidemic LDLr−/− mice. Our findings represent the first direct evidence that protein-tyrosine sulfation plays a key role in the development of a common and clinically important inflammatory disorder.

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**Disclosures**

Kevin Moore is a cofounder of Siwa Biotech Corporation.

**References**

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