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Full Length Article

A complementary role for tetraspanin superfamily member TSSC6 and ADP purinergic $P2Y_{12}$ receptor in platelets

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ABSTRACT

Tumor-suppressing subchromosomal transferable fragment cDNA 6 (TSSC6) expression is restricted to hematopoietic organs and tissues where it plays a role in hematopoietic-cell function. The ADP purinergic receptor $P2Y_{12}$ is mainly expressed by platelets with important clinical significance as a target for several clinically approved antithrombotic agents. We have previously shown a physical association between $P2Y_{12}$ and TSSC6 in platelets. Hence our aim was to investigate whether this physical association is translated to functional effects. To investigate this possibility, we used wild-type or TSSC6 knockout (KO) mice treated with either PBS or 50 mg/kg clopidogrel. TSSC6 KO mice treated with clopidogrel exhibited synergy in delayed kinetics of clot retraction, reduced collagen-mediated platelet aggregation, and platelet spreading on fibrinogen. Platelets derived from TSSC6 mice with $P2Y_{12}$ blockade form smaller thrombi when perfused over a collagen matrix under arterial flow. Clopidogrel treated TSSC6 KO arterioles showed smaller and less stable thrombi with increased tendency to embolise *in vivo*. These studies demonstrate a complementary role between TSSC6 and $P2Y_{12}$ receptor in platelets in regulating 'outside in' integrin $\alpha_{IIb}\beta_3$ signalling thrombus growth and stability.

1. Introduction

Platelets play a pivotal role in haemostasis and thrombosis, by limiting blood loss following vascular injury or in the context of pathological conditions such as atherosclerosis [1]. There are various receptors and signalling molecules in platelets responsible for signalling pathways involved in platelet thrombus formation [2]. Among them, the tetraspanin superfamily, TSSC6, also termed as Tspan32 or Pan hematopoietic expression (Phemx), was confirmed as a member of this family by Robb et al. [3]. In their study, they identified the presence of the four transmembrane domains and the highly conserved cysteines in the large extracellular loop which is a characteristic of all other tetraspanin members [3]. However, the expression of TSSC6 appears to be restricted to the hematopoietic cells and organs [3] unlike other tetraspanin superfamily members where their expression is more broadly distributed.

In mice, TSSC6 was mapped to chromosome 7 in a region syntenic with human chromosome 11p15 [4,5] and found to be present on the surface and intracellular pools in both human and mouse platelets [6]. Similar to other tetraspanins, TSSC6 contains two extracellular loops and another two intracellular domains which consist of N- and C-terminal domains. The latter is relatively large compared to other tetraspanin superfamily members.

There are at least 10 tetraspanins present in platelets and among them CD151, CD9, and CD63 have been well-studied. In 2006, TSSC6 protein was found to be present on the surface of murine platelets as well as in intracellular pools [7]. The study demonstrated a constitutive physical relationship of TSSC6 with integrin $\alpha_{IIb}\beta_3$ in resting wild-type mouse platelets [7]. The same study showed that 'outside-in' signalling of integrin $\alpha_{IIb}\beta_3$ in TSSC6 KO platelets was impaired causing a delay in kinetics of clot retraction, a defect in platelet aggregation and reduced ability of platelets to spread on fibrinogen. These data suggested a functional relationship between integrin $\alpha_{IIb}\beta_3$ and tetraspanin superfamily member, TSSC6 [7]. Unstable haemostasis in TSSC6 KO mice was also observed as indicated by a prolonged bleeding time as well as an increased tendency for rebleeds. Additionally, thrombus formation and stability *in vivo* in TSSC6 KO mice was clearly affected indicating that this tetraspanin superfamily member is important for regulation of

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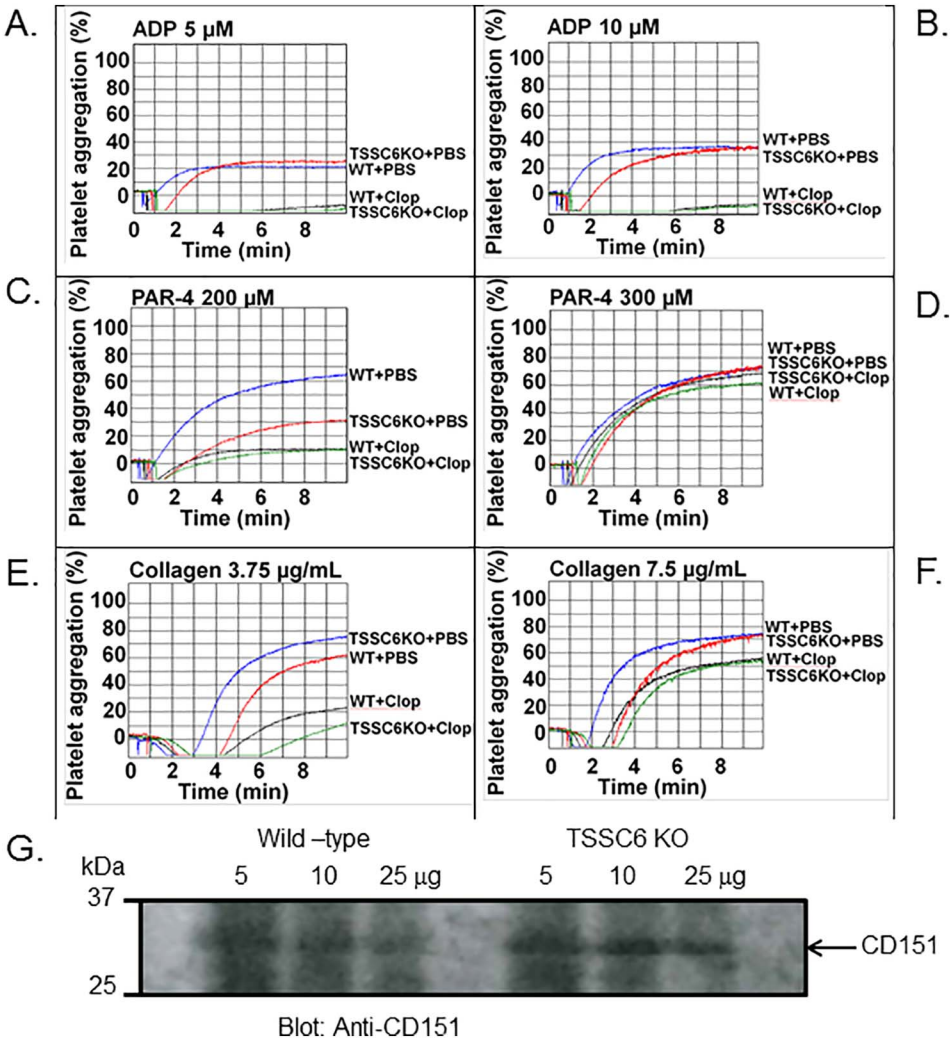

Fig. 1. TSSC6-deficient mice treated with Clopidogrel demonstrate better synergy in the inhibition of collagen–mediated platelet aggregation than TSSC6 KO or $P2Y_{12}$ blockade alone. (A–F) Aggregation responses of normalised PRP from wild-type, $P2Y_{12}$ blockade, TSSC6 KO, and TSSC6 KO with $P2Y_{12}$ blockade platelets were determined after activation with different concentrations of various agonists; including ADP agonist (5 and 10 µM), PAR-4 agonist peptide (200 and 300 µM), and collagen agonist (3.75 and 7.5 µg/mL), respectively using light platelet aggregometry (n = 4 replicate experiments). G. CD151 western blot. 5, 10 and 25 µg of either wild-type or TSSC6 KO platelet lysates were electrophoresed on a non-reduced 12.5% SDS-PAGE followed by western blot. Immunostaining was done using polyclonal anti-CD151 H-80 antibody 1:1000 followed by anti-rabbit HRP conjugated 1:5000 and ECL development.

platelet thrombus formation [7].

The ADP purinergic receptor $P2Y_{12}$ has attracted a lot of attention due to its limited distribution on tissues and selective expression in platelets [8]. $P2Y_{12}$ receptor is a member of the G protein-coupled receptor family that is linked to the α_{i2} subunit of G protein. In contrast, to the ADP purinergic receptor $P2Y_1$, $P2Y_{12}$ distribution appears to be restricted to the brain and platelets [8,9]. Recent studies reported that $P2Y_{12}$ receptor is additionally expressed by glial cells [10] and smooth muscle cells [11]. $P2Y_{12}$ receptor is expressed on platelets at a moderate level (600 copies/platelet) when compared to ADP purinergic receptor $P2Y_1$ which is expressed only at 150 copies/platelet [12]. However, the expression of both purinergic receptors is relatively low when compared to other platelet glycoproteins [12]. $P2Y_{12}$ receptor plays a pivotal role in amplifying platelet aggregation through the major platelet integrin, $\alpha_{IIb}\beta_3$ [13]. In addition, $P2Y_{12}$ receptor contributes to all signalling pathways in platelets. This is clear by its critical role in amplification of platelet aggregation *via* all known platelet agonists that activate different signalling pathways [14,15]. Humans [16] or mice [8,17] deficient in ADP purinergic receptor, $P2Y_{12}$, display a significant increase in bleeding time. This increase was even more obvious in Clopidogrel treated mice [18]. $P2Y_{12}$ receptor is involved in mechanisms including granule secretion [19] and stabilisation of thrombus formation [18]. Application of $FeCl_3$ induced injury in mesenteric arterioles in $P2Y_{12}$ receptor deficient mice revealed delayed thrombus formation, smaller blood clots, clot instability and embolisation [19]. Deficiency of $P2Y_{12}$ receptor in humans has also been reported and resulted in platelet dysfunction and bleeding diathesis similar to platelet profiles after administration of thienopyridines (*e.g.* Clopidogrel) to humans [20,21]. All these characteristics make $P2Y_{12}$ receptor a good target for anti-thrombotic therapy.

We have recently reported the first detected physical interaction between tetraspanin superfamily and G-protein-coupled receptor family in platelets [22]. More specifically, the physical interaction showed that CD151 forms a lateral association with $P2Y_{12}$ oligomers and monomers on the platelet membrane while TSSC6 forms a lateral association with $P2Y_{12}$ oligomers, monomers as well as dimers. We also demonstrated that tetraspanin superfamily member, CD151 forms a functional contribution with the purinergic ADP receptors, $P2Y_{12}$ receptor in the regulation of 'outside-in' integrin $\alpha_{IIb}\beta_3$–mediated platelet aggregation, clot retraction and cytoskeletal reorganisation [22]. In addition, the complementary role was found to be required in the regulation of the *in vitro* (through examining thrombus growth under physiological arterial flow conditions) or *in vivo* (through evaluating thrombus and stability using $FeCl_3$–induced vascular injury of mesenteric arterioles). Our findings excluded the role of this co-operative interaction in the regulation of granule secretion or 'inside-out' integrin $\alpha_{IIb}\beta_3$–mediated signalling properties [22]. In the current study, we aimed to examine whether the complementary role for tetraspanin superfamily member TSSC6 and ADP purinergic $P2Y_{12}$ receptor in platelets does exist since we have previously shown that they are physically associated [22].