Involvement of TGFβ1 in autocrine regulation of proplatelet formation in healthy subjects and patients with primary myelofibrosis

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ABSTRACT

Megakaryocytes release platelets into the bloodstream by elongating proplatelets. In this study, we showed that human megakaryocytes constitutively release Transforming Growth Factor β1 and express its receptors. Importantly, Transforming Growth Factor β1 downstream signaling, through SMAD2/3 phosphorylation, was shown to be active in megakaryocytes extending proplatelets, indicating a type of autocrine stimulation on megakaryocyte development. Furthermore, inactivation of Transforming Growth Factor β1 signaling, by the receptor inhibitors SB431542 and Stemolecule ALK5 inhibitor, determined a significant decrease in proplatelet formation. Recent studies indicated a crucial role of Transforming Growth Factor β1 in the pathogenesis of primary myelofibrosis. We demonstrated that primary myelofibrosis-derived megakaryocytes expressed increased levels of bioactive Transforming Growth Factor β1; however, higher levels of released Transforming Growth Factor β1 did not lead to enhanced activation of downstream pathways. Overall, these data propose Transforming Growth Factor β1 as a new element in the autocrine regulation of proplatelet formation *in vitro*. Despite the increase in Transforming Growth Factor β1 this mechanism seems to be preserved in primary myelofibrosis.

Introduction

Megakaryocyte maturation and platelet generation in bone marrow (BM) result from megakaryocyte migration from the osteoblastic to the vascular niche, where megakaryocytes extend proplatelets and release newly generated platelets into the bloodstream.[1] Megakaryocytes are 'filled' with different fibrogenic factors, among which transforming growth factor beta1 (TGFβ1) mostly contributes to BM fibrosis associated with BM disorders, such as hairy cell leukemia and myelofibrosis.[2,3] Besides its fibrogenic activity, TGFβ is a pleiotropic regulator of all stages of hematopoiesis the activity of which depends on the differentiation stage of the target cell, the local environment and the concentration.[4,5] However, the role of TGFβ1 on megakaryocyte proliferation, differentiation and proplatelet formation has been poorly investigated. Kuroda *et al*. reported that TGFβ1 is involved in negative feed-back regulation of megakaryopoiesis in healthy volunteers and that megakaryocyte colony-forming units of patients with the myeloproliferative neoplasm essential thrombocythemia are less sensitive to TGFβ1 than normal subjects.[6] Moreover, Sakamaki *et al*. showed that TGFβ1 determines an arrest of megakaryocyte colony forming unit maturation by enhancing the thrombopoietin-dependent expression of TGFβ1 receptors on megakaryoblasts.[7]

There is evidence that hematopoietic precursors secrete several regulatory molecules that control various stages of normal human megakaryopoiesis in an autocrine and/or paracrine manner.[8-10] Given this, in this study we extended the search for autocrine growth factors for megakaryopoiesis to TGFβ1. We analyzed the influence of TGFβ1 on late stages of megakaryocyte maturation in cells from healthy subjects and we extended the study to patients with primary myelofibrosis (PMF), a chronic myeloproliferative neoplasm characterized by variable degrees of BM fibrosis associated with hyperplasia and atypia of megakaryocytes.

Design and Methods

Megakaryocytes were differentiated from human umbilical cord and peripheral blood hematopoietic progenitor cells as previously described.[10,11] Human cord blood was collected following normal pregnancies and deliveries with informed consent of the parents, in accordance with the Ethical Committee of the IRCCS Policlinico San Matteo Foundation in Pavia, Italy, and the principles of the Declaration of Helsinki. For peripheral blood studies, blood samples were obtained from 11 patients with PMF. All patients were referred to the Center for the Study and the Cure of Myelofibrosis of the IRCCS Policlinico San Matteo Foundation. None of the patients was receiving any disease-modifying therapy at the time of their enrollment in the study. All patients met the 2008 WHO criteria for PMF.[12] A normal, age- and sex-matched control population consisted of 13 healthy volunteers treated with granulocyte-colony stimulating factor (G-CSF) as donors for hematopoietic stem cell (HSC) transplantation. Further details of the Design and Methods are available in the *Online Supplementary Appendix.*

Results and Discussion

TGFβ1 mRNA was detected by qRT-PCR at a very early

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stage of megakaryocyte differentiation (Day 7 of culture) and increased during megakaryocyte maturation with a peak at Day 10 of culture (Figure 1A). Gene expression was paralleled by the release of the total and bioactive TGFβ1 proteins in the culture medium (Figure 1B and 1C). TGFβ1 signals through a heterotetrameric receptor complex comprising type I and type II receptors, followed by a canonical SMAD-dependent signaling cascade.[13] Thus, in order to investigate whether the TGFβ1 released by megakaryocytes in culture could exert a type of autocrine regulation on megakaryocyte development, we first performed a time course analysis of TGFβ1 receptor (TβR) expression (Figure 1D) demonstrating that TβRI and TβRII proteins were almost equally expressed during the entire process of megakaryocyte maturation *in vitro* (P=not significant). Subsequent Western blot analysis demonstrated the phosphorylation of SMAD2/3 during the entire process of megakaryocyte maturation, with a significant increase starting from Day 10 ($P<0.05$) indicating that TGFβ1 binding to its receptors had occurred (Figure 1E). TGFβ1 can rapidly activate phosphatidyl-inositol-3-kinase (PI3K), as indicated by the phosphorylation of its downstream effector Akt.[14] Activation of PI3K leads to downregulation of the phosphatase and tensin homolog (PTEN), enhances hematopoietic stem cell proliferation, and promotes thrombopoietin signaling.[15] Interestingly, time course analysis demonstrated a significant ($P<0.05$) increase in phosphorylated Akt and decrease in PTEN expression in mature megakaryocytes (Figure 1F) suggesting a possible role of these proteins in regulating platelet production. To address this hypothesis, megakaryocytes were pre-incubated at Day 13 of differentiation, with 10 μM of TGFβ1 receptor kinase inhibitor, SB431542, a small synthetic molecule that interrupts the activation of signaling pathways downstream to the TβRI, or 10 μM of Akt inhibitor VIII, AKTI-1/2. Moreover, in order to confirm the effects of the TGFβ1 receptor inhibition, megakaryocytes were also treated with a specific TβRI inhibitor, Stemolecule ALK5 inhibitor (10 μM). As shown in Figure 1G, inhibition of all signaling resulted in a significant decrease in the number of proplatelets extended by human megakaryocytes when compared to controls. Specifically, SB431542 inhibited pro-

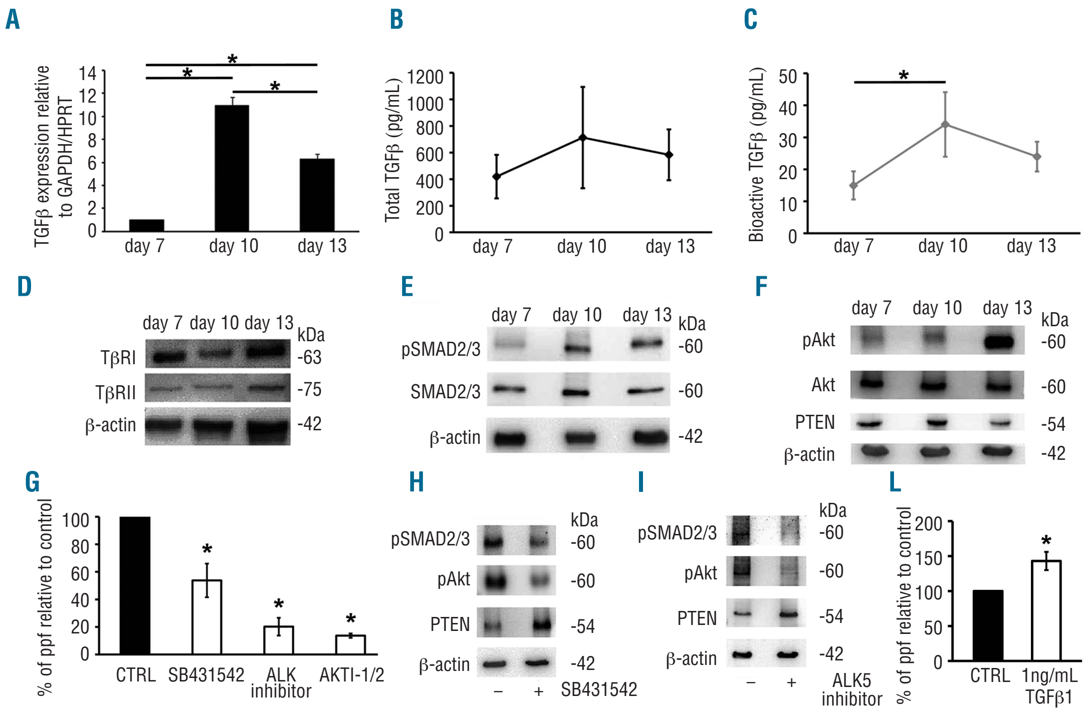

Figure 1. **Autocrine TGFβ1 impact on human megakaryocyte maturation and platelet production. Megakaryocytes were derived from human umbilical cord blood progenitor cells as described in the *Online Supplementary Appendix*. RNA was extracted from CD61+ megakaryocytes and qRT-PCR of the TGFβ1 expression was performed at day 7, 10 and 13 of megakaryocyte differentiation. mRNA levels were normalized to expression levels at Day 7 of culture (bars represent mean±SD, n=3 different experiments, *P<0.05, ANOVA and Bonferroni t-test as *post-hoc* test) (A). TGFβ1 was constitutively released into the conditioned medium during megakaryocyte differentiation in culture. Total (B) and bioactive TGFβ1 (C) levels in culture supernatants were determined by biological assay and data normalized to cell numbers (means±SD, n=3 separate experiments, *P<0.05, ANOVA and Bonferroni's t-test as *post-hoc* test). TGFβ1 receptors were expressed by human megakaryocytes in culture. Megakaryocytes were lysed and subjected to Western blot analysis. TβRI and TβRII receptors were detected in human megakaryocytes at day 7, 10 and 13 of culture. The membrane was reprobed with anti–β-actin to ensure equal loading (representative of 5 different experiments) (D). Western blot analysis of pSMAD (E), pAkt and PTEN (F) in human megakaryocytes revealed that the signaling involving pSMAD2/3, pAkt and PTEN was activated in mature megakaryocytes. Samples were also probed with anti-SMAD2/3, anti-Akt and anti–β-actin antibodies to ensure equal loading (representative of 5 different experiments). Mature megakaryocytes seeded in presence or absence of the TβRI inhibitors, SB431542 (10 μM) and specific ALK5 inhibitor (10 μM), or the Akt inhibitor, AKTI-1/2 (10 μM), showed a significant reduction of proplatelet formation (ppf) relative to not treated controls (bars represent means±SD, n=3 separate experiments, *P<0,05) (G). Western blot analysis of megakaryocytes treated (+) or not (–) with SB431542 (10 μM) (H) or ALK5 specific inhibitor (10 μM) (I) at Day 13 of culture. Decrease of SMAD2/3 phosphorylation determined by TβRI inhibition affected PTEN expression and subsequent Akt activation. The membranes were reprobed with anti–β-actin to ensure equal loading (representative of 5 different experiments). Mature megakaryocytes seeded in presence or absence of human recombinant TGFβ1 (1 ng/mL) showed a significant increase of proplatelet formation (ppf) relative to untreated controls (bars represent means±SD, n=5 separate experiments, *P<0.05) (L).**

platelet formation by approximately 50% (7±3%, mean±SD, n=3 separate experiments), Stemolecule ALK5 inhibitor by approximately 80% (2±0.5%, mean±SD, n=3 separate experiments), and AKTI-1/2 by approximately 90% (1.5±1%, mean±SD, n=3 separate experiments) relative to control samples (11±5%, mean±SD, n=9 separate experiments; $^{*}P<0.05$). No differences were observed in megakaryocyte ploidy (*Online Supplementary Figure S1A*). Interestingly, incubation with Stemolecule ALK5 inhibitor (10 μM) starting from Day 10 of differentiation totally inhibited proplatelet formation (*data not shown*). Importantly, the decreased capacity of extending proplatelets by human megakaryocytes (*Online Supplementary Figure S1B*) was supported by the findings that treatment with SB431542 or Stemolecule ALK5 inhibitor determined a decrease in SMAD2/3 and Akt pathway activation and an increase in PTEN expression (Figure 1H and I). Together, these results suggest that human megakaryocytes constitutively release TGFβ1 and, upon binding to its receptors, regulates proplatelet formation through SMAD2/3-PI3K-PTEN signaling. It is still to be discovered whether this process occurs also *in vivo*, but the high percentage of $CD41^{+}CD42b^{+}$ megakaryocytes in culture, together with high cell viability, demonstrated that increased TGFβ1 in the culture medium was consequent to active release from maturing megakaryocytes, rather than to megakaryocyte apoptosis or release by other contaminating cells in culture (*data not shown*).

The positive effect of TGFβ1 on proplatelet formation by human megakaryocytes was confirmed by adding to the culture media 1 ng/ml TGFβ1.[16] As shown in Figure 1L, proplatelet formation was increased (14±1.5%, mean±SD, n=5 separate experiments) relative to control samples (10±3%, mean±SD, n=5 separate experiments; $^{*}P<0.05$), indicating that TGFβ1 may be considered to be one of the modulators of the very late stage of megakaryocyte maturation.

Ciurea *et al.* demonstrated that $CD61^{+}$ megakaryocytes derived from PMF patient progenitor cells released more TGFβ1 in the culture supernatant when compared to controls, supporting the hypothesis that TGFβ1 has a crucial role in PMF pathogenesis.[3] Therefore, the authors claimed that, due to their increased ability to produce TGFβ1, megakaryocytes may promote the generation of the bone marrow fibrosis in these patients, through a TGFβ1-mediated mechanism. Nevertheless, how increased levels of TGFβ1 impact megakaryopoiesis in PMF has never been explored. As shown in Figure 2A, megakaryocytes, derived *in vitro* from peripheral blood $CD34^{+}$ cells of PMF patients, presented decreased capacity of extending proplatelets, compared to megakaryocytes obtained from healthy controls circulating $CD34^{+}$ cells. On this basis, we investigated whether the impairment in proplatelet formation by PMF-derived megakaryocytes could be related to altered TGFβ1 signaling. Thus, total and bioactive levels of TGFβ1 were measured in the supernatant of megakaryocyte cultures

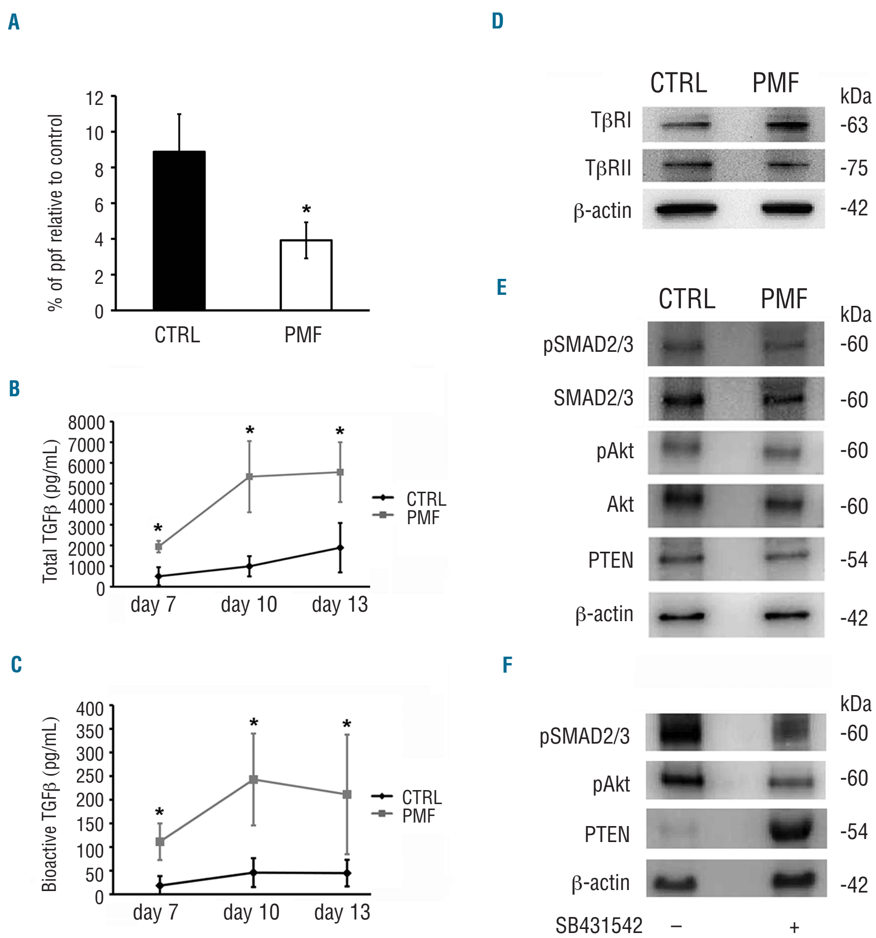

Figure 2. Autocrine TGFβ1 signaling in PMF derived megakaryocytes. Megakaryocytes were derived from peripheral blood progenitors of patients with primary myelofibrosis (PMF) and healthy donor controls (CTRL), as described in the *Online Supplementary Appendix*. Mature megakaryocytes from PMF, showed a significant reduction of proplatelet formation (ppf) relative to CTRL (bars represent means±SD, n=11 separate experiments, $^{*}P<0.05$) (A). Total (B) and bioactive TGFβ1 (C) levels in culture supernatants were determined by biological assay and data normalized to cell number (means±SD, n=11 separate experiments). PMF derived megakaryocytes showed a significant increase in both total and bioactive TGF secretion as compared to CTRL. Megakaryocytes were lysed and subjected to Western blot analysis. TGFβ1 receptors were equally expressed in CTRL and PMF mature megakaryocytes *in vitro*. The membrane was reprobed with anti–β-actin to ensure equal loading (representative of 3 different experiments) (D). Western blot analysis of pSMAD, pAkt and PTEN revealed a similar activation in CTRL and PMF derived megakaryocytes, despite the increased levels of TGFβ1 observed in PMF culture medium (representative of 3 different experiments) (E). PMF derived megakaryocytes seeded in presence (+) or absence (–) of the TβRI inhibitor, SB431542 (10 μM), showed a significant reduction of SMAD2/3 and Akt phosphorylation and conversely an increase in PTEN expression. The membranes were reprobed with anti–β-actin to ensure equal loading (representative of 3 different experiments) (F).

from PMF progenitor cells and compared to controls. As shown in Figure 2B and C, increased levels of both total and bioactive TGFβ1 were observed in culture supernatants of PMF-derived megakaryocytes when compared to controls, during the entire process of megakaryocyte maturation. Importantly, we observed no differences in TGFβ1 supernatant activity between patients with or without the *JAK2* V617F mutation (*data not shown*). As reported above, the high cell viability demonstrated that increased TGFβ1 in the culture medium was consequent to active release from maturing megakaryocytes rather than to damaged megakaryocyte in culture (*data not shown*). Thus, in order to investigate whether exposure to increased levels of TGFβ1 could enhance activation of TβR downstream signaling, we focused on mature megakaryocytes. First, we demonstrated similar expression of TβRI and TβRII in mature PMF-derived megakaryocytes when compared to controls (Figure 2D). Second, we showed no differences in TβR downstream signaling between PMF- and control-derived megakaryocytes as demonstrated by similar levels of SMAD2/3 and Akt phosphorylation and PTEN expression (Figure 2E). Overall, these data demonstrate that exposure to higher levels of released TGFβ1 did not lead to enhanced activation of downstream pathways. Finally, in order to verify whether pathway activation downstream TβRs was dependent on TGFβ1 binding in PMF-derived megakaryocytes as in controls, a Western blot analysis of SMAD2/3 and Akt phosphorylation and PTEN expression was performed in the presence or not of TGFβ1 receptor kinase inhibitor SB431542. The results demonstrated that PMF-derived megakaryocytes responded in the same way as controls to inhibition by SB431542, as shown by comparable decrease in SMAD2/3 and Akt phosphorylation, and consequent increase in PTEN expression (Figure 2F). Unfortunately, because of the low percentage of proplatelet formation by PMF-derived megakaryocytes, there was no significant evidence of inhibitory effect of SB431542 (*data not shown*).

Overall, this is the first demonstration that TGFβ1, released by megakaryocytes, regulates proplatelet formation. Autocrine components, together with environmental factors, seem to play an important role in the regulation of platelet production. We recently demonstrated that ADP, released by megakaryocytes, regulates proplatelet formation by interacting with $P2Y_{13}$.[10] The results we obtained in PMF provided confirmation that megakaryocytes produce an excess amount of TGFβ1.[3] However, increased levels of released bioactive TGFβ1 by PMF-derived megakaryocytes did not enhance TGFβ1 receptor downstream signaling in the latest stages of megakaryocyte differentiation. Based on this evidence, the aberrant megakaryopoiesis that occurs in the fibrotic bone marrow of these patients could be due to an excess of TGFβ1 that affects the first stages of cell differentiation rather than proplatelet formation. In conclusion, our results propose a new role for TGFβ1 in the regulation of platelet production and open new perspectives on the mechanisms that trigger proplatelet formation by mature megakaryocytes.

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Authorship and Disclosures

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