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Evaluation of the bioactive and total transforming growth factor β1 levels in primary myelofibrosis

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ABSTRACT

TGFβ1 is secreted as latent protein that requires activation to become biologically active. It negatively regulates the progenitor cell growth, and favours the deposition of extra-cellular matrix in different tissues. We have studied TGFβ1 levels in Philadelphia-negative (Ph−) myeloproliferative diseases, evaluating patients with primary myelofibrosis (PMF) that is characterized by increased numbers of circulating progenitor cells and bone marrow (BM) fibrosis, and patients with polycythemia vera (PV) or essential thrombocythemia (ET) that do not present BM fibrosis.

We found that patients with PMF, PV or ET have higher peripheral blood (PB) plasma levels of both bioactive and total TGFβ1 than healthy controls, with a balance bioactive/total TGFβ1 in favour of the latter. The balance between bioactive/total TGFβ1 in the BM plasma of patients mirrored that of PB, with most of TGFβ1 in the latent form; on the contrary, in the BM plasma of healthy controls most of the TGFβ1 was in the bioactive form.

In conclusion, increased plasma levels of TGFβ1 and an altered ratio bioactive/total TGFβ1 in BM are not peculiar of patients with PMF suggesting that, whether altered levels of TGFβ1 have a role in myelofibrosis, this may not be related to the induction of BM fibrosis.

1. Introduction

TGFβ is one of the most powerful regulators of haematopoiesis selectively acting on progenitor cell growth by negatively regulating the cycling status through an autocrine mechanism [1]. It exists as three isoforms in mammals (TGFβ1, TGFβ2, TGFβ3) with TGFβ1 being the most abundant isoform.

TGFβ is secreted as a latent protein that requires activation for becoming biologically active [2]. Although physicochemical factors, such as local acidification or exposure to active oxygen species may participate in the regulation of TGFβ activation, mechanisms involving proteolytic cleavage or conformational modification of the latent forms are more likely to operate *in vivo* [1]. Therefore, the evaluation of the total amount (latent + bioactive) of TGFβ does not reflect the actual biological activity of TGFβ.

It has been shown in humans that the attenuation of the TGFβ1 signalling through its receptors is frequently associated with the clonal expansion that characterizes haematological malignancies [2]. In addition, its role as regulator of haematopoiesis has been related to the SDF1α–CXCR4 axis; in fact, TGFβ1 down-regulates SDF-1α expression influencing bone marrow (BM) cell cycle status, adhesion and migration [3–5]. TGFβ1 has also been shown to be involved in the induction of fibrosis in lung, liver, kidney and skin [6], whereas its possible role in BM fibrosis is still to be clarified [7].

Based on these findings, an altered TGFβ1 function may exert a role in primary myelofibrosis (PMF), a Philadelphia-negative (Ph−) myeloproliferative disorder of unknown aetiology, characterized by the presence of a $JAK2^{V617F}$ mutation in the myeloid cells of 50% of the patients [8], abnormal stem/progenitor cell trafficking [9], splenomegaly, marrow fibrosis leading to extensive extramedullary haematopoiesis [10], and alterations of megakaryocyte differentiation leading to thrombocytopenia [11]. It has been reported that at least in two animal models (GATA-1^{low} or TPO^{high}) the development of myelofibrosis was associated with high TGFβ1 content in extracellular fluids of marrow and spleen [11–12]. In humans, it has been previously shown that in CD34 + cells of

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patients with PMF, while the TGFβ1 expression is not altered, both the mRNA and the protein levels of TGFβRII are decreased with respect to controls, suggesting a possible resistance of these cells to TGFβ1 [13–14]. In favour of a role of TGFβ1 in the pathogenesis of the disease, also speaks the observation that patients with PMF have an increased TGFβ1 mRNA expression in peripheral blood mononuclear cells (PBMC) and increased levels of protein secreted in conditioned media from PBMC [15]. Increased quantity of TGFβ1 has also been localized in circulating megakaryocytes by immunostaining [15] or by mRNA expression [16]; in addition, TGFβ1 levels were found increased in culture supernatants of megakaryocytes [17] and monocytes [18] or in platelet lysates [19–20]. Rameshwar et al. [21] compared patients with/without BM fibrosis reporting that, despite the aetiology, bioactive circulating TGFβ1 is increased in presence of fibrosis; moreover, in a very limited number of patients in situ hybridisation and immunoistochemistry indicated an increase in TGFβ1 mRNA and protein in myelomonocytic-like areas of BM biopsy sections.

Polycythemia vera (PV) and essential thrombocythemia (ET), Ph− myeloproliferative diseases that do not present either increased circulating progenitor cells or BM fibrosis and are characterized by the presence of a $JAK2^{V617F}$ mutation in the myeloid cells of 97% and 75% of the patients [22], respectively, have also been investigated in a limited number of studies. Patients with PV or ET showed contrasting data on TGFβ1 mRNA expression in megakaryocytes, that was found to be either comparable [16] or higher [23] than that of healthy subjects; in addition, TGFβ1 mRNA expression was found increased in total BM cells of patients with ET with respect to those from subjects with non-neoplastic haematopoiesis [23].

In this study, we evaluate the plasma levels of both bioactive and total TGFβ1 in the peripheral blood (PB) of healthy subjects (CTRLs), patients with PMF, and patients with PV or ET, evaluated as disease controls. BM plasma samples and, in patients with PMF who underwent splenectomy, conditioned medium of spleen tissue cell cultures were also studied to compare TGFβ1 levels in different compartments involved in the altered haematopoiesis that characterizes patients with PMF.

2. Patients and methods

2.1. Patients

Patients with a diagnosis of PMF referred to the Hospital Fondazione Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS) Policlinico San Matteo (Pavia, Italy) for a period of three years were included in this study. Our institution is a teaching and tertiary-care hospital, and is a major referral site for patients with PMF because it coordinates the Italian Registry for Myelofibrosis, a nationwide prospective database. The diagnosis of PMF was established according to the Italian Consensus Conference Criteria [24], by which the diagnosis held if diffuse BM fibrosis was present and Ph chromosome or rearrangement in PB cells was absent (necessary criteria) and if an algorithm of optional criteria was satisfied. At the time that blood was drawn for the measurement of TGFβ1 plasma levels, the patients had also a complete blood count and a PB smear examination, and their spleen and liver measurements were taken. Spleen size was measured by ultrasonography. Two parameters of spleen size were recorded: the distance from the splenic tip to the costal margin (spleen grade) ranging from grade 1 (0–5 cm) to grade 4 (>15 cm) and the spleen index, the product of the longitudinal by the transverse spleen axis, the latter defined as the maximal width of the organ [25]. Liver enlargement was measured as the distance from the right costal margin in centimeters. Patients were assigned a prognostic score on the basis of the findings of Dupriez et al. [26]. Patients with other Ph− MPDs were also studied for comparison. They were categorized as having PV or ET according to the Polycythemia Vera Study Group (PVSG) criteria [27–28] and the evaluation of the $JAK2^{V617F}$ mutation. All the patients with PV or ET had mild signs of myeloproliferation and mild or no disease progression.

Healthy individuals were also studied. The informed consent was obtained from all the subjects included in the study.

2.2. Samples

Plasma samples. PB was collected without the use of a tourniquet in EDTA (ethylenediaminetetraaceticacid)-containing tubes and immediately placed on ice. After a centrifugation at 4000 rpm for 15 min at 4 °C, we obtained platelet-poor plasma samples from patients with PMF ($n = 35$), PV ($n = 10$), or ET ($n = 4$), and healthy subjects comparable for age and sex (CTRLs) ($n = 22$). The samples were stored at −80 °C until used. Platelet-poor BM plasma samples were obtained from bone marrow aspiration both from patients with PMF ($n = 10$), PV ($n = 2$) or ET ($n = 5$) at the time of diagnosis and from healthy BM donors ($n = 7$). BM aspirates were centrifuged at 200 g and the platelet-poor plasma collected following the same procedure as for PB.

The day of the experiment, to exclude interference from heterophilic antibodies, all the plasma samples were treated with polyethylene glycol 6000 (Merck, NJ, USA) (6% final concentration) for 2 h. Immunoglobulins were removed by centrifugation at 1000*g* for 45 min. Both incubation and centrifugation were performed at 4 °C.

Platelet lysate samples. Platelet-rich plasma obtained from patients and CTRLs was prepared by two steps of centrifugation at 200*g* for 10 min, washed twice in physiological saline containing 3.8% of sodium citrate. Platelets were counted and resuspended at a final concentration of 2.5×10^9/ml in Eagles Minimum Essential Medium modified (EMEM, LGC Promochem, Middlesex, UK). After five cycles of freezing and thawing, samples were centrifuged at 1000*g* for 30 min and immediately stored at −80 °C.

2.3. In vitro cultures

PBMC subsets PBMC were obtained by density gradient centrifugation (1077 g/ml) over Lymphoprep (Sentinel Diagnostic, Milano, Italy). After washing and 30 min incubation with anti-CD34 coated MicroBeads at 4 °C, CD34+ cells were separated with immunomagnetic MiniMacs columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according with the manufacturer's instruction. The CD34+ cells were plated at $1x10^6$/ml in Iscove's Modified Dulbecco's Medium (IMDM; Lonza, Verviers, Belgium) at 37 °C with 5% CO_2 for 24 h. The negative fraction obtained after the first separation step was used to isolate CD14+ cells by incubation with anti-CD14 coated MicroBeads for 15 min at 4 °C and separation with immunomagnetic Midi Macs columns (Miltenyi Biotec, Bergisch Gladbach, Germany). The CD14+ cells were plated at 1×10^6/ml with RPMI 1640 (EuroClone, Milano, Italy) supplemented with 2% human serum albumin (HAS; Alpha Therapeutic, Milano, Italy) for 24 h. PBMCs depleted of CD34+ and CD14+ cells were then plated at 2×10^6/ml for 48 h in RPMI 1640 supplemented with 2% FCS. All the supernatants were stored at −80 °C.

Megakaryocytes. Circulating CD34 + cells were separated as previously described from seven control apheresis and PB of seven patients with PMF. The cells were then cultured for 13 days in StemSpan medium (Stem-Cell Technologies, Vancouver, Canada) supplemented with 10 ng/ml TPO, IL-6, IL-11 (PeproTech EC Ltd. London, UK) at 37 °C in a 5% CO_2 fully-humidified atmosphere [29]. Supernatants were collected every 3 days and stored at −80 °C. The total amount of TGFβ1, evaluated by CCL64 cell line,