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Dysregulation of stromal derived factor 1/CXCR4 axis in the megakaryocytic lineage in essential thrombocythemia

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Summary

This study investigated the involvement of chemokines including stromal derived factor 1 (SDF-1), interleukin 8 (IL-8), growth-related oncogene alpha (GRO-α) and their receptors, CXCR4, CXCR2 and CXCR1 in essential thrombocythemia (ET), a chronic myeloproliferative disease characterized by megakaryocytic hyperplasia and high platelet count. Fifty-three ET patients were studied. Plasma levels of SDF-1, IL-8 and GRO-α, evaluated by enzyme-linked immunosorbent assay, and flow cytometric analysis of CXCR1 and CXCR2 on the platelet membrane, were found to be normal in ET patients. CXCR4 expression on platelet surface as well as platelet *CXCR4* mRNA detected by real-time reverse transcription polymerase chain reaction, were decreased. Platelet CXCR4 internalization rate was normal while SDF-1-induced platelet aggregation was delayed, decreased or absent. Immunohistochemical staining revealed that megakaryocytes were also affected. CXCR4 decrease was not observed either in peripheral white blood cells or in circulating $CD34^+$ precursors. These results show that CXCR4 is decreased in the megakaryocytic lineage in ET, mainly due to a reduced CXCR4 production, and an abnormal platelet response to SDF-1. This report is the first to describe platelet and megakaryocytic CXCR4 deficiency in a human disease and the presence of this abnormality in a megakaryocytic-related illness highlights the important role of SDF-1/CXCR4 axis in platelet development.

Keywords: CXCR4, stromal derived factor 1, platelets, essential thrombocythemia, chemokines.

Chemokines are small proteins that can be classified into two main subfamilies according to the presence or absence of an intervening amino acid between two conserved cystein residues, CXC or CC, respectively. These chemotactic cytokines direct cell migration and participate in a wide range of physiological and pathological processes (Rollins, 1997; Esche *et al*, 2005; Charo & Ransohoff, 2006). The involvement of chemokines in megakaryopoiesis and thrombopoiesis has been established in recent years. Stromal-derived factor 1 (SDF-1) has been shown to increase polyploidization and transendothelial migration of megakaryocytes favouring platelet production (Hamada *et al*, 1998; Guerriero *et al*, 2001). Furthermore, Avecilla *et al* (2004) have described the ability of SDF-1 and fibroblast growth factor 4 (FGF-4) to relocate megakaryocyte progenitors to the vascular niche, promoting survival, maturation and platelet release, thus allowing restoration of thrombopoiesis in thrombopoietin (TPO) or TPO receptor-deficient mice through a cytokine-independent mechanism. In addition to thrombopoietic chemokines, a number of members of the CXC family, such as platelet factor 4 (PF-4), interleukin 8 (IL-8) and growth-related oncogene alpha (GRO-α) as well as others from the CC family are described as negative regulators of megakaryocytopoiesis (Gewirtz *et al*, 1995). Chemokine receptors, named CXCR4 for SDF-1, CXCR1 and CXCR2 for IL-8 and GRO-α, are present on the surface of the megakaryocytic lineage (Wang *et al*, 1998; Kowalska *et al*, 1999; Riviere *et al*, 1999), and their functional activity has been established (Majka *et al*, 2000; Minamiguchi *et al*, 2001; Emadi *et al*, 2004). In addition, several reports have stated that chemokine receptors are also present on the platelet membrane (Wang *et al*, 1998; Kowalska *et al*, 1999). Indeed, some chemokines can potentiate platelet function triggered by specific agonists or even induce platelet activation by themselves (Kowalska *et al*, 1999; Abi-Younes *et al*, 2000; Gear *et al*, 2001).

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Essential thrombocythemia (ET) is a myeloproliferative disorder characterized by megakaryocytic hyperplasia and persistent thrombocytosis (Murphy *et al*, 1997; Harrison, 2005; Campbell & Green, 2006). Although a growing amount of data is available concerning its pathological features, the molecular abnormalities that give rise to this illness are not yet fully elucidated. The presence of either the activating somatic mutation of the regulatory domain of the Janus Kinase 2, *JAK2* V617F or the gain-of-function *MPL* mutation, *MPL*W515L/K, would only account for the phenotypic profile in about 50% of ET patients (Baxter *et al*, 2005; Kralovics *et al*, 2005; Levine *et al*, 2005; Pardanani *et al*, 2006).

The involvement of chemokines in other myeloproliferative diseases has already been stated. Increased IL-8 serum levels have been described in patients with primary myelofibrosis (Emadi *et al*, 2004) as well as in polycythemia vera (Hermouet *et al*, 2002). Recently, the decrease in CXCR4 membrane levels in circulating $CD34^+$ cells and granulocytes has been described in patients with primary myelofibrosis (Rosti *et al*, 2007).

The present study aimed to evaluate the implication of megakaryocyte-active CXC chemokines axis including SDF-1, IL-8 and GRO-α in ET.

Materials and methods

Patients and controls

Fifty-three consecutive patients with ET followed at a single institution were enrolled in the study. Diagnosis was based on clinical and laboratory features according to the Polycythemia Vera Study Group criteria (Murphy *et al*, 1997). Median age was 45 years, range 9–82 years; 39 were female. Samples from 53 healthy individuals matched by age and gender were studied as controls. The study was approved by the Institutional Review Board, and written informed consent was obtained from patients and controls, in accordance with the Helsinki declaration. The number of patient samples assayed in each experiment is described in results. Clinical and laboratory features were evaluated retrospectively by chart review and are summarized in Table I.

Table I. Clinical features at diagnosis and laboratory data in untreated ET patients and patients during anagrelide treatment.

	Untreated ET patients (n = 37)	ET patients on anagrelide treatment (n = 16)
Age, years, median (range)	43 (9–82)	50 (17–76)
Haemoglobin, g/l, median (range)	135 (101–162)	125 (92–138)
White blood cell count, $\times 10^9$/l, median (range)	9·3 (5·0–17·9)	8·2 (5·0–13·0)
Platelet count, $\times 10^9$/l, median (range)	1050 (470–1800)	460 (350–720)
JAK2 V617F-positive	44%	67%
Clinical features at diagnosis		
Thrombotic events or microvascular disturbances	45%	61%
Haemorrhagic manifestations	9%	8%

Data are expressed as median and range.

Samples

Platelet-poor-plasma (PPP) obtained from blood anticoagulated with 129 mmol/l trisodium citrate was separated by centrifugation at 2500 ***g*** for 20 min. An additional centrifugation at 13 800 ***g*** for 10 min ensured no platelet contamination. Samples were stored at −70°C until use. Platelet-rich plasma (PRP) was obtained by centrifugation at 200 ***g*** for 10 min. Anticoagulants used were: 0·342 mol/l EDTA for flow cytometry, 129 mmol/l trisodium citrate for platelet aggregation and internalization studies, and ACD-C (7 mmol/l citric acid, 93 mmol/l citrate, 139 mmol/l dextrose, pH 6·4) for reverse transcription polymerase chain reaction (RT-PCR). For flow cytometry and PCR studies, 42 mmol/l indomethacin (Sigma, St Louis, MO, USA) was added to prevent platelet activation. To reduce leucocyte and red cell contamination, PRP was subsequently centrifuged at 200 ***g*** for 5 min and then filtered through a high efficiency leucocyte reduction filter (Purecell PL, Pall Biomedical Products, East Hills, NY, USA). After this procedure the leucocyte:platelet ratio was less than $1:10^6$. For leucocyte analysis, peripheral blood mononuclear cells and granulocytes were separated from 77 mmol/l EDTA-anticoagulated blood.

Bone marrow biopsies were obtained by standard procedures, formalin-fixed and embedded in paraffin. This study was carried out retrospectively in samples from seven ET patients and, as a control group, samples from seven subjects who underwent bone marrow biopsy for non-haematological diseases.

Measurement of plasma chemokines

Plasma IL-8, GRO-α and SDF-1α levels were assayed using an enzyme-linked immunosorbent assay technique (R&D Systems, Minneapolis, MN, USA). Normal values obtained in our laboratory were similar to those given by the manufacturers. Samples were tested in duplicate. Minimum detectable levels were: IL-8, 2·8 pg/ml, GRO-α, 10 pg/ml, SDF-1α, 18 pg/ml. In order to avoid inter-assay variations all samples were tested in the same assay.

Flow cytometry for CXCR1, CXCR2 and CXCR4

Platelet-rich plasma was adjusted to 1×10^8 platelets/ml and labelled with saturating amounts of anti-CXCR1-fluorescein isothiocyanate (FITC), CXCR2-FITC or CXCR4-phycoeerythrin (PE) antibodies (Becton-Dickinson, San José, CA, USA). Irrelevant monoclonal antibodies from the same IgG subclass were used as negative isotypic controls. A FACScan flow cytometer (Becton-Dickinson) was used to identify platelets by their capacity to bind anti CD42b-PE or CD61-FITC

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(Becton-Dickinson). In order to evaluate CXCR4 expression on leucocyte and leucocyte progenitors membrane, subpopulations were individualized by the addition of specific monoclonal antibodies directed against CD45 labelled with peridinin chlorophyll (PerCP) and CD3 (lymphocytes), CD14 (monocytes) and CD34 (hematopoietic progenitors) labelled with FITC (Becton-Dickinson). Incubations were carried out at 4°C. At least 500 000 events were acquired for $CD34^+$ cells analysis. All samples were assayed in duplicate. Data were expressed as relative fluorescence intensity (RFI, ratio between the mean fluorescence intensity of each CXC receptor antibody and the corresponding isotypic control). The CXCR ratio was calculated as follows: Patient CXCR RFI/Control CXCR RFI.

Real-time RT-PCR analysis of platelet CXCR4 transcripts

CXCR4 gene expression was detected by real-time PCR. Total platelet RNA was isolated by Trizol reagent (Gibco-BRL, Grand Island, NY, USA) from 4×10^9 platelets according to Chomczynski and Sacchi (1987). cDNA was synthesized from 1 μg RNA using the Super Script™ preamplification system (Gibco-BRL) according to the manufacturer's instructions. Real-time PCR was performed using IQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in an iCycler iQ Real-Time PCR System (Bio-Rad Life Science). Quantification was carried out using *GAPDH* expression as an internal normalization control. Annealing temperatures were set as follows, 62°C for *CXCR4* and 68°C for *GAPDH*. Primer sequences for *CXCR4* were: forward 5′- AGGGGATCAGTATATACACTT-3′ and reverse 5′-TGCCCACAATGCCAGTTAAG-3′. Primer sequences for *GAPDH* were: forward 5′-CGACCACTTTGTCAAG-CTCA-3′ and reverse 5′-CCCTGTTGCTGTAGCCAAAT-3′. Quantitative normalization of cDNA in each sample was obtained by the Δ cycle threshold (ΔCt) method (*CXCR4* Ct – *GADPH* Ct) and fold change in mRNA expression in patients was expressed relative to normal controls using the ΔΔCt method and assuming 80% PCR efficiency. Melting curve analysis and electrophoresis in 2% agarose gel followed by ethidium bromide staining were performed to confirm identity of PCR products. Each sample was assayed in triplicate, and a negative control was included in each assay.

CXCR4 internalization studies

Platelets were adjusted to $1{\cdot}5 \times 10^4$/ml with Iscove's modified Dulbecco's medium (IMDM) medium (GIBCO BRL) and incubated with 500 nmol/l SDF-1 (PeproTech Inc.Rocky Hill, NJ, USA) for 10 min at 37°C. Platelets were washed and labelled as previously described with anti CXCR4-PE and CD61-FITC. Platelets incubated with IMDM instead of SDF-1 were taken as unstimulated controls. Samples were assayed in duplicate and analyzed by flow cytometry. The mean fluorescence intensity from stimulated (GmS) and resting platelets (GmR) were obtained. The internalization rate was calculated as follows: $100 \times [1-(GmS/GmR)]$.

Platelet aggregation studies

Platelet aggregation was evaluated by turbidimetric method as described by Born (1962) using a Lumi-aggregometer (Chrono-Log Corp., Havertown, PA, USA). PRP was adjusted to 3×10^8/ml and challenged with 10 to 100 nmol/l SDF-1 or 2·0 μmol/l ADP (Biopool Trinity Biotech plc, Bray, Co. Wicklow Ireland) under constant stirring.

Immunohistochemical CXCR4 assay

For immunohistological staining, sections 5 μm thick from patients and controls were cut and mounted on polylysine coated glass slides. In order to compare staining from patients and controls and to avoid methodological variations, each patient sample was mounted with a corresponding control sample on the same slide. Samples were deparaffinized through successive xylene baths and rehydrated through graded alcohols. In order to block endogenous peroxidase activity, slides were immersed for 20 min in methanol containing 0·3% hydrogen peroxidase. Non-specific binding was prevented by incubation with 2·5% blocking serum. Sections were incubated with anti-CXCR4 primary rabbit polyclonal antibody (Abcam, Cambridge, UK) overnight at 4°C at 1:500 dilution. Negative controls were performed by replacing the primary antibody with buffer. The LSAB detection kit (Dako, CA, USA) was used for visualization of antigen. Slides were counterstained with haematoxylin.

Estimation of CXCR4 staining of megakaryocytes: In an attempt to measure CXCR4 staining, images of all available mature megakaryocytes (between 12 and 60) in each specimen were obtained (ZoomBrowser EX, Canon USA Inc., Lake Success, NY). Using the VideoTesT - Master Morphology program Ltd (St. Petersburg, Russia), the average cytoplasmatic optical density (OD), excluding nucleus, was obtained for each cell. Afterwards, the median OD derived from all megakaryocytes of each specimen was calculated.

Statistical analysis

Data are presented as median values and ranges. The number of patients and control samples studied in each assay is detailed in the corresponding paragraph. The Mann–Whitney–Wilcoxon rank sum test was used to compare variables between patients and controls, and between patients grouped by different clinical or laboratory features. In order to avoid inter-assay variations in flow cytometry and immunohistochemical studies, each patient sample was tested at the same time with a normal control and analyzed as pairs using the Wilcoxon signed rank test. Kruskal–Wallis non-parametric analysis was applied for comparison among three groups. Spearman rank correlation was applied to search for the relationship between CXCR4 expression and clinical and laboratory data. Statistical significance was defined as $P < 0{\cdot}05$.

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Results

Plasma levels of the chemokines IL-8, GRO-α and SDF-1α

The first approach in the study of the chemokine/chemokine-receptor axis in ET was aimed at assessing plasma IL-8, GRO-α and SDF-1α levels in 19 untreated patients. Chemokine levels in patients were not different from those of controls (n = 19), and were respectively, IL-8, 2·5 pg/ml (0·8–28·2) (median and range) and 2·8 pg/ml (1·1–16·5); GRO-α, 30·0 pg/ml (7·4–463·1) and 23·9 pg/ml (9·6–148); SDF-1α 1895 pg/ml (1246–2719) and 1915 pg/ml (822–2424).

CXCR1, CXCR2 and CXCR4 on platelet surface

To further determine if chemokine receptors were normally expressed on platelet surface in ET, we measured IL-8, GRO-α and SDF-1α receptors on platelet membrane by means of flow cytometry. CXCR4 expression was decreased in 25 out of 29 ET patients (86%) evaluated, RFI 19·4 (1·3–39·4) compared to controls, 31·8 (2·4–73·5), P < 0·0001. The CXCR4 ratio (Patient/Control) was 0·65 (0·33–1·16) (Fig 1A). This group comprised 17 untreated patients and 12 patients on anagrelide treatment, an imidazo-quinazoline used to lower platelet counts. When analyzed separately, CXCR4 expression did not significantly differ between these groups, P = 0·19 (Mann–Witney–Wilcoxon rank sum test) (Fig 1B).

In contrast, platelet surface CXCR1 expression was normal in 10 ET patients, RFI 1·58 (1·33–1·78), compared to 10 controls, 1·57 (1·07–1·99), P = 0·99. Similar results were observed in the CXCR2 expression in 10 patients, 1·43 (0·94–1·74) compared to their respective controls, 1·28 (1·01–1·47), P = 0·47. The CXCR1 and CXCR2 ratios were 1·04 (0·85–1·42) and 1·04 (0·64–1·36), respectively (Fig 1A).

In order to investigate whether the decrease in CXCR4 on the platelet membrane was related to the platelet volume, CXCR4 RFI was analyzed in platelets classified according to their size into small, medium and large. There was a decrease in CXCR4 expression in every subset of platelets (Table II). A representative example is shown in Fig 1D. We then calculated the CXCR4 ratio among different-sized platelets. The degree of CXCR4 decrease was similar among these platelet subpopulations, small 0·68 (0·38–1·27), medium 0·60 (0·33–1·21) and large 0·65 (0·25–1·22), P = 0·37.

CXCR4 expression according to clinical and laboratory features

No differences were found in the CXCR4 ratio between patients displaying thrombotic events and/or microvascular disturbances, 0·65 (0·34–1·16) and those asymptomatic, 0·63 (0·33–0·85), P = 0·42. Similarly, there were no differences in CXCR4 ratio when patients were grouped by the presence 0·67 (0·34–1·15) or absence of *JAK2*V617F mutation 0·63 (0·33–1·06) (P = 0·52), which was assayed by allele-specific PCR (Heller *et al*, 2006). No correlations were found between platelet count (r = −0·07, P = 0·7) or white cell count (r = −0·02, P = 0·94) and CXCR4 ratio in untreated ET patients.

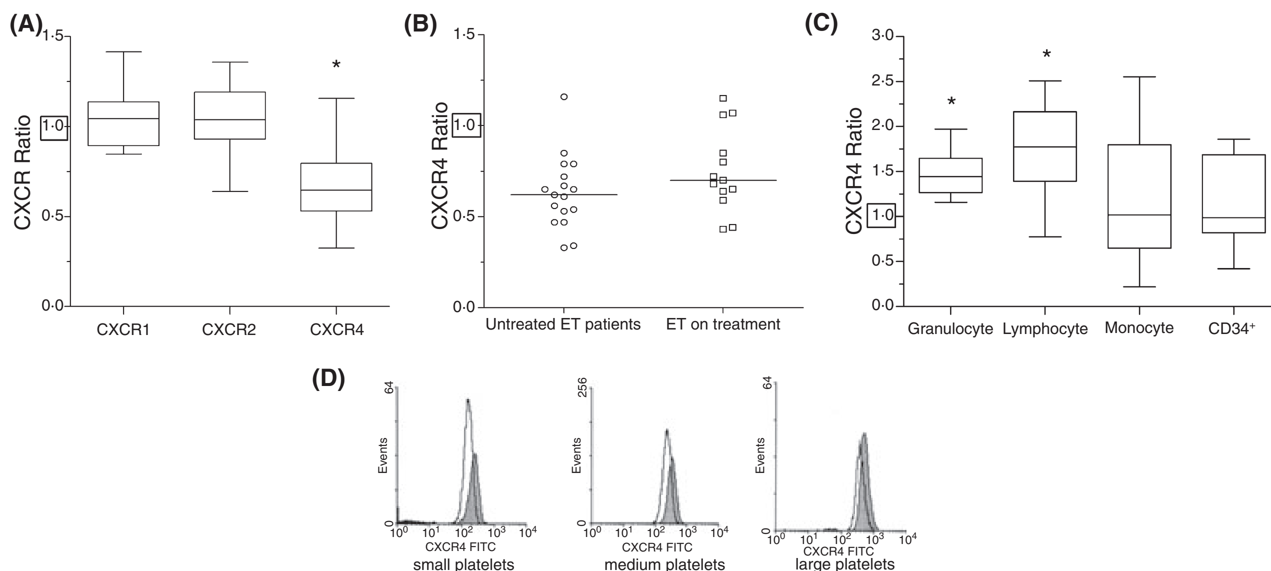

Fig 1. CXC receptors on cell membrane. (A) CXC receptor expression on platelet membrane in ET patients was assessed by flow cytometry and expressed as CXCR ratio (Patient RFI/Control RFI). Normal expression was taken as 1; (*) represents significantly decreased CXC receptor. (B) CXCR4 ratio in untreated patients and in patients on anagrelide treatment (Mann–Whitney–Wilcoxon rank sum test, P = NS). Median values are represented by horizontal lines. (C) CXCR4 ratio on cell surface in granulocytes, lymphocytes, monocytes and circulating $CD34^+$ progenitors; (*) represents populations with significantly increased CXCR4 expression. (D) Platelet populations were grouped according to their size and analyzed by flow cytometry for CXCR4 expression. Linear cell count (vertical axis) *versus* CXCR4 log fluorescence (horizontal axis) is shown. Traces refer to platelet fluorescence obtained in the patient (open histogram) and in a control (solid grey histogram) when small, medium and large subpopulations were examined. A representative example is shown.

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Table II. CXCR4 RFI according to platelet size.

CXCR4 RFI	Small	Medium	Large
Patients	14·6 (1·3–32·5)	18·0 (1·3–61·9)	26·1 (1·2–78·8)
Controls	20·6 (2·1–85·1)	31·6 (2·5–126·5)	45·8 (2·3–193·8)
P	0·0008	0·0004	0·0005

P values correspond to differences between patient and control CXCR4 RFI in each platelet subpopulation.

CXCR4 on leucocyte surface

With the aim of investigating whether the megakaryocytic lineage was the only one affected, we searched for CXCR4 receptors in peripheral blood leucocytes from 12 patients with low CXCR4 expression on platelet membrane and 12 normal controls. CXCR4 RFI in granulocytes from patients was found to be elevated, 7·1 (2·2–16·3) compared to controls, 5·0 (1·7–9·5), $P = 0{\cdot}006$. Similar results were found in CD3-positive lymphocytes; patients, 40·3 (6·8–67·9) and controls, 22·3 (5·2–30·0), $P = 0{\cdot}004$. On the other hand, CXCR4 on monocyte membrane was normal, RFI from patients, 16·2 (7·3–53·5), and controls, 20·1 (6·5–80·5), $P = 0{\cdot}64$. The CXCR4 ratio in each leucocyte subpopulation was: granulocytes, 1·44 (1·16–1·97), lymphocytes 1·77 (0·78–2·51), and monocytes 1·02 (0·22–2·55) (Fig 1C).

CXCR4 on circulating haematopoietic progenitors

In order to evaluate CXCR4 expression in haematopoietic progenitors, we studied this receptor on circulating $CD34^{+}$ cells from 10 ET patients and 10 normal controls. Similar results were found in both; patients CXCR4 RFI, 20·77 (13·6–43·19) and controls CXCR4 RFI, 20·4 (9·7–47·1), $P = 0{\cdot}92$. The CXCR4 ratio was 0·99 (0·42–1·86) (Fig 1C).

Platelet CXCR4 transcript levels

To determine the cause of the low expression of CXCR4 on platelet surface, platelet *CXCR4* mRNA levels from 13 ET patients were evaluated and compared to that of nine normal controls by real-time RT-PCR. The median ΔCt value (*CXCR4* Ct – *GAPDH* Ct) for ET platelets was significantly increased compared to controls, 6·21 (3·45–8·8) and 3·35 (2·55–3·82), respectively, $P < 0{\cdot}0001$, (Fig 2A). A median 5·37-fold decrease was found in the *CXCR4* mRNA expression in platelets from ET patients as calculated by the ΔΔCt method. A representative example is shown in Fig 2B. Six out of these 13 patients had been previously tested for CXCR4 membrane expression; all of them showed decreased CXCR4 on platelet membrane.

CXCR4 internalization

In order to test the functionality of CXCR4 in ET patients, we evaluated the receptor capacity for internalization upon the

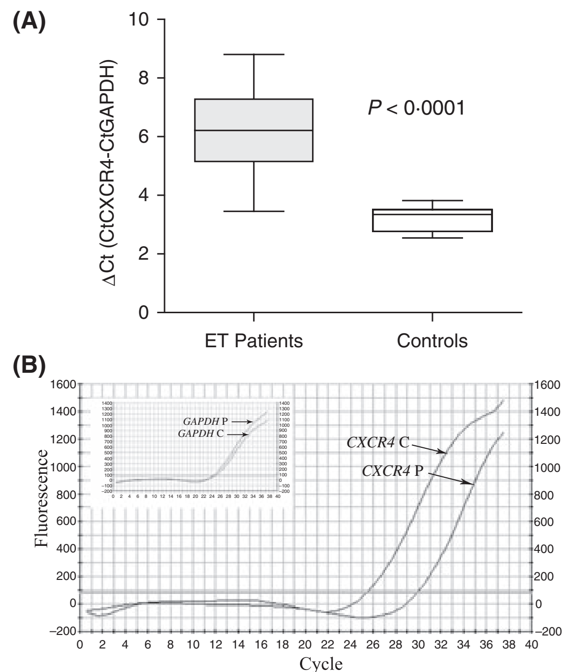

Fig 2. Real-time RT-PCR analysis of platelet *CXCR4* transcript levels. *CXCR4* expression was assessed by real-time RT-PCR using *GAPDH* as the housekeeping control gene. (A) Boxes represent *CXCR4-GAPDH* ΔCt in ET patients and controls, (Wilcoxon signed-rank test). (B) Results in an ET patient (P) showing higher Ct value for *CXCR4* compared to a control (C), reflecting reduced *CXCR4* mRNA levels; Ct values for *GAPDH* are similar (inset).

binding of SDF-1α. CXCR4 on platelet membrane was assessed by flow cytometry after incubation in the presence or absence of 500 nmol/l SDF-1. No significant differences were found in platelet CXCR4 internalization rate from nine untreated ET patients and their respective controls, 36% (24–61) and 14% (2–68), respectively, $P = 0{\cdot}358$. All these patients had decreased CXCR4 levels on platelet membrane.

Platelet aggregation triggered by SDF-1α

To further clarify the functional consequences of the CXCR4 decrease, we tested the effect of SDF-1α in platelet aggregation in 11 untreated ET patients. All but one of these patients had low CXCR4 platelet membrane levels. SDF-1α induced a biphasic, dose-dependent full platelet aggregation in PRP in normal controls ($n = 11$). On the contrary, abnormalities in SDF-1α response were observed in all patients, which consisted in a delay or absence of the second wave of aggregation or a lack of response. In order to evaluate if SDF-1α-induced aggregation is dependent on ADP response, ADP-induced platelet aggregation was tested in the same samples. Table III summarizes the results obtained in response to both agonists. A dissimilar platelet response to SDF-1α and ADP was seen in these patients. While some patients had an impaired response to ADP and SDF-1, others (cases I, II, VI and IX) displayed an altered SDF-1α response with a normal ADP-induced platelet

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Table III. Summary of platelet aggregation results induced by ADP and SDF-1 in eleven untreated patients studied.

Patient	ADP-induced platelet aggregation	SDF-1-induced platelet aggregation	CXCR4 ratio
I	Complete	2 waves, delayed*	0·54
II	Complete	2 waves, decreased	0·33
III	Absent	Absent	0·65
IV	First wave	Absent	0·62
V†	2 waves, decreased	2 waves, delayed	1·16
VI	Complete	First wave	0·85
VII	First wave	Absent	0·79
VIII	First wave	Absent	0·56
IX	Complete	Absent	0·72
X	Absent	Absent	0·79
XI	2 waves, delayed	Absent	0·47

*2 waves, delayed: means a delay in the second wave of aggregation.
†Patient who displayed normal membrane CXCR4 levels.

aggregation. No differences in CXCR4 ratio were found when patients who displayed no SDF-1α induced aggregation were compared to those who showed some kind of response, 0·65 (0·47–0·79) and 0·7 (0·33–1·16), respectively, $P = 0·9$. Representative examples are shown in Fig 3.

Immunohistochemical staining of CXCR4 in ET megakaryocytes from bone marrow biopsies

The decreased platelet CXCR4 expression in ET patients prompted us to search for the SDF-1 receptor on megakaryocyte membrane. With this aim, immunohistochemical CXCR4 staining in bone marrow biopsies was performed. Microscopic observation of the patient and control samples revealed a wide distribution of CXCR4 among the bone marrow cells. Concerning morphologically mature megakaryocytes, controls displayed stronger CXCR4 staining than ET patients.

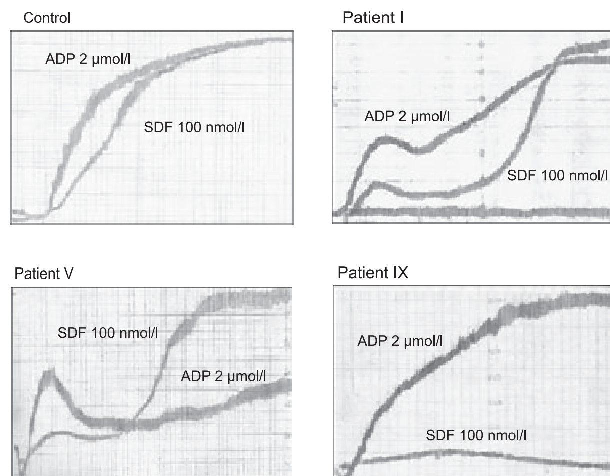

Fig 3. Platelet aggregation induced by SDF-1 and ADP. PRP was stimulated with either SDF-1 or ADP and platelet aggregation was recorded. Examples from patients I, V, IX and a control are shown.

Representative pictures are shown in Fig 4A. We found a decrease in CXCR4 staining as evaluated by cytoplasmatic OD in megakaryocytes from seven ET patients, 0·42 (0·33–0·51), compared to controls, 0·52 (0·39–0·57), $P = 0·015$, Fig 4B.

Discussion

In recent years an increasing amount of evidence has established the involvement of chemokines in megakaryopoiesis and thrombopoiesis. Of particular interest is the finding that SDF-1 enhances platelet production by favouring transendothelial megakaryocyte migration (Hamada *et al*, 1998; Avecilla *et al*, 2004). In this study we searched for abnormalities in chemokine/chemokine-receptor axis in ET, a disease characterized by megakaryocyte hyperplasia and high platelet count.

CXCR4 is a 7-transmembrane spanning G protein-coupled cell-surface glycoprotein receptor responsible for signal transduction in response to SDF-1 (Bleul *et al*, 1996). Here we found decreased CXCR4 levels on platelets in ET patients. Abnormalities in the mean platelet volume have been described in these patients (Bellucci *et al*, 1999). As we found a consistent decrease in CXCR4 among different-sized platelets, the involvement of platelet volume variations in CXCR4 decrease was ruled out.

This finding prompted us to search for CXCR4 in megakaryocytes. As shown in immunohistochemical studies, bone marrow megakaryocytes from ET patients display reduced CXCR4 staining, demonstrating that the CXCR4 decrease seen in ET platelets arises from their progenitors.

Protein CXCR4 reduction could be explained, at least in part, by decreased protein synthesis, as revealed by a low *CXCR4* mRNA expression. The latter might be a consequence of genetic and/or epigenetic modifications, as both mechanisms have been documented to affect *CXCR4* transcription (Mori *et al*, 2005; Sato *et al*, 2005; Busillo & Benovic, 2007). Indeed, Bogani *et al* (2008) recently described hypermethylation of the *CXCR4* promoter as the mechanism responsible for CXCR4 decrease in $CD34^+$ cells from patients with primary myelofibrosis. In addition, another possible cause of CXCR4 decrease could be an increased rate of internalization due to an increased stimulus. This hypothesis seems unlikely because we did not find high SDF-1 levels either in peripheral blood plasma in our cohort or in bone marrow blood samples from three ET patients studied (data not shown). The normal rate of CXCR4 internalization found in our study suggests that, although patients have a low number of receptors on platelet membrane, CXCR4 can be normally internalized after SDF-1 binding.

In order to evaluate the functional consequences of CXCR4 decrease, we sought to test SDF-induced platelet aggregation in ET. Patients showed an abnormal response characterized by a delay or absence of a secondary wave of aggregation or even a lack of response, suggesting that CXCR4 decrease in ET platelets is functionally relevant. However, the degree of the impairment in platelet aggregation was not related to the decrease in CXCR4 membrane expression. This led us to

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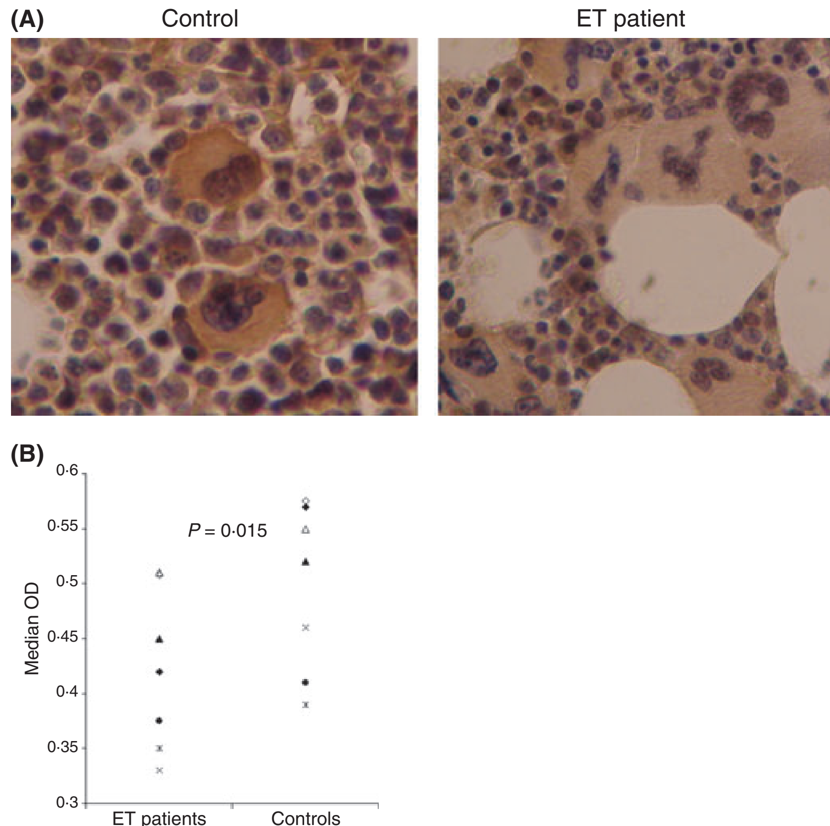

Fig 4. Immunohistochemical staining of CXCR4 in megakaryocytes. Bone marrow sections were stained with anti-CXCR4 primary rabbit polyclonal antibody and then counterstained with haematoxylin. (A) Representative sections of a patient and a control (magnification ×400) show decreased staining in the patient. (B) Megakaryocyte CXCR4 staining was evaluated by obtaining the cytoplasmatic OD using the VideoTesT - Master Morphology program. Median OD from megakaryocytes in seven ET patients and seven patients with non-haematological illnesses studied as controls are plotted (Wilcoxon signed-rank test). Each patient was plotted with the same symbol as their corresponding control.

consider that other abnormalities could contribute to this functional defect. In this regard, several reports have been published related to the involvement of ADP in SDF-1 induced platelet aggregation (Abi-Younes *et al*, 2000; Kowalska *et al*, 2000; Gear *et al*, 2001). Abnormal ADP-induced platelet aggregation is a well known defect in ET as well as in other chronic myeloproliferative disorders (Cortelazzo *et al*, 1981; Cesar *et al*, 2005). Indeed, 41% of ET patients studied in our laboratory since 1992 have displayed this abnormality (unpublished data). The altered platelet response to SDF-1 seen in these patients did not parallel that of ADP, because five out of seven patients who did not aggregate to SDF-1 displayed some response to 2 μmol/l ADP. These results demonstrated that, even though in some cases the involvement of a defective response to ADP in the SDF-1 impairment of platelet aggregation cannot be excluded, in others, where ADP-induced platelet aggregation is normal, the functional defect in SDF-1 response is clearly an independent phenomenon.

In addition to decreased CXCR4, a variety of receptors were found to be reduced in ET patients such as c-mpl, prostaglandin D2 receptors and glycoproteins Ib and IIb/IIIa complex (Cooper *et al*, 1978; Mazzucato *et al*, 1989; Horikawa *et al*, 1997). Therefore, it is possible that the molecular abnormalities involved in the development of ET might be related to the regulation of several gene products in the megakaryocytic lineage, including CXCR4. The fact that CXCR4 remains decreased during the normalization of the platelet count by anagrelide treatment further supports this hypothesis. It is interesting to note that CXCR1 and CXCR2, two CXC chemokine receptors, were normally expressed on the platelet surface, suggesting a specific chemokine receptor regulation for CXCR4.

Concerning clinical and laboratory features, the expression of CXCR4 on the platelet membrane did not differ between asymptomatic patients and those who experienced thrombosis and/or microvascular disturbances. Besides, similar to that described for c-Mpl, (Goerttler *et al*, 2005) platelet CXCR4 levels were independent of the *JAK-2* mutational status.

We show here decreased CXCR4 expression in ET megakaryocytes. The precise physiological role of CXCR4/SDF-1 axis in thrombopoiesis has not been yet fully clarified. On one hand, Berthebaud *et al* (2005) demonstrated that the increase in RGS16 down-regulated CXCR4 signalling during megakaryocyte maturation, postulating that CXCR4 desensitization leads to the release of mature megakaryocytes from bone marrow microenvironment to their interaction with the vascular

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endothelium for efficient platelet production. On the other hand, Avecilla *et al* (2004) identified SDF-1/CXCR4 as a key signalling pathway leading to thrombopoiesis. Thus, the effect of megakaryocyte CXCR4 reduction on platelet production is difficult to predict as, while according to Berthebaud *et al* (2005), CXCR4 reduction would result in enhanced platelet production, Avecilla *et al* (2004) suggested opposite effects. Overall, the functional consequences of CXCR4 decrease in ET megakaryocytes found in this study and its role in the pathogenesis of this entity remain to be established. Generation of megakaryocyte-specific *CXCR4*-null mice could be useful to further clarify the *in vivo* role of CXCR4 in thrombopoiesis.

Our results demonstrate that decreased CXCR4 is restricted to the megakaryocytic lineage as shown by the results of flow cytometric studies on leucocyte subpopulations. Unexpectedly, CXCR4 expression in lymphocytes and neutrophils in these patients was increased. Further studies are needed to shed light on this subject. Interestingly, granulocytes from primary myelofibrosis, contrarily to what we describe here in ET granulocytes, also displayed low CXCR4 levels (Rosti *et al*, 2007). Moreover, circulating $CD34^+$ haematopoietic progenitors from ET patients displayed normal CXCR4 membrane expression, indicating that CXCR4 abnormality occurs in cells that are already committed to the megakaryocytic lineage. Recently, CXCR4 decrease in circulating $CD34^+$ progenitors in primary myelofibrosis was described (Guglielmelli *et al*, 2007; Rosti *et al*, 2007). This differential feature between ET and myelofibrosis could be a contributing factor for the different degrees of $CD34^+$ mobilization observed in these two myeloproliferative disorders.

In conclusion, the results presented here show a dysregulation in the CXCR4/SDF-1 axis in megakaryocytes and platelets from ET patients. Indeed, the involvement of CXCR4/SDF-1 in megakaryopoiesis and thrombopoiesis makes this finding relevant to ET, a disease that predominantly affects the megakaryocytic lineage, and opens new perspectives in the elucidation of the mechanisms by which SDF-1 participates in platelet production.

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