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Increased levels of plasma interleukin-6 soluble receptor in patients with essential thrombocythemia

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A B S T R A C T

Background and Objectives. The pathogenesis of essential thrombocythemia (ET), a disease characterized by megakaryocyte hyperplasia and persistent thrombocytosis, is not completely clarified. Interleukin-6 (IL-6), one of the cytokines related to megakaryocytic development, exerts its effect through binding to a cell surface receptor, IL-6Ra, and a signal transducing unit, gp130. Interestingly, the soluble form of the IL-6Ra, IL-6sR, is an agonist for IL-6 activity. In order to evaluate the possible participation of IL-6sR in ET we measured its levels in plasma, platelets and in the supernatant of a mononuclear cell culture. We also evaluated IL-6R on leukocyte membrane and IL-6R/IL-6sR mRNA expression in mononuclear cells.

Design and Methods. Fifty-five patients with ET were evaluated. IL-6sR and IL-6 were measured by an ELISA technique. Mononuclear cells were cultured for 48 hr and IL-6sR released into the supernatant was measured. IL-6R on leukocyte surfaces was evaluated by flow cytometry. IL-6R and IL-6sR mRNA levels were assessed by semi-quantitative reverse transcription polymerase chain reaction.

Results. Plasma IL-6sR levels were increased while intraplatelet levels were low in untreated ET patients. Plasma levels decreased during treatment. Non-stimulated mononuclear cells from ET patients released greater amounts of IL-6sR than did cells from normal controls in 48-hour culture. No abnormality was found in IL-6R or IL-6sR mRNA expression by mononuclear ET cells. IL-6R on leukocyte surfaces was normal.

Interpretations and Conclusions. Increased plasma IL-6sR levels might have a role in the abnormal megakaryocytic proliferation seen in ET patients, while platelets and mononuclear cells could be the source of the above-mentioned high levels of plasma IL-6sR.

Key words: essential thrombocythemia, IL-6 soluble receptor, myeloproliferative diseases.

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Essential thrombocythemia (ET) is a myeloproliferative disorder characterized by megakaryocytic bone marrow hyperplasia and persistent thrombocytosis.[1] Abnormal megakaryocytic proliferation is also often present in the other myeloproliferative diseases, polycythemia vera, agnogenic myeloid metaplasia and chronic myelogenous leukemia.[2,3]

It has been postulated that a deregulation of growth factors involved in megakaryocytopoiesis could be responsible for the development of ET. In this regard, hypersensitivity of megakaryocytic progenitors to interleukin-3[4] and thrombopoietin[5] has been described. Furthermore, there is an increasing number of reports on spontaneous megakaryocyte growth in the absence of exogenous cytokines[6-8] or even in the absence of accessory cells capable of producing these cytokines.[9] However, these studies are not conclusive and the mechanism responsible for the increased expansion of the megakaryocytic lineage still remains unclear.

Interleukin 6 (IL-6) is a multifunctional cytokine that plays an important role in megakaryocytic development. IL-6 serum levels have been proposed to be useful for differentiating between primary and reactive thrombocytosis[10] because of their known association with acute-phase response.[11] This cytokine exerts its effect by acting through a specific receptor protein, IL-6Rα, and a signal transducing unit, gp130. The latter is shared by other cytokines such as oncostatin M, leukemia inhibitor factor and interleukin 11.[12] A 55 kD soluble IL-6R (IL-6sR) has also been described which retains the ability to bind to IL-6 and associate with gp130 acting agonistically on cells that do not express IL-6R.[13] IL-6sR can be generated by alterna-

tive splicing or by shedding from the anchored receptor through receptor by a metalloprotease.[14,15]

Raised plasma IL-6sR levels have been found in other hematologic disorders such as multiple myeloma.[16] However, to our knowledge, the possible relationship between IL-6sR and platelet production in myeloproliferative states remains to be explored. Here we describe increased plasma IL-6sR levels in ET patients and search for the source of this abnormal production.

Design and Methods

Patients

Fifty-five consecutive patients with ET were enrolled in the study. The diagnosis was based on clinical and laboratory features according to the Polycythaemia Vera Study Group criteria.[1] Forty were female. The age at the time of the study before treatment was 48.5 years (19-83) (median and range). Twenty-four patients (44%) had symptoms, including 6 with thrombotic events, 13 microvascular disturbances, 4 minor hemorrhagic events and one had coexistence of thrombotic and hemorrhagic events. Spontaneous platelet aggregation was found in 23 patients (42%). Samples taken during treatment with anagrelide were collected after the second month of starting therapy, when patients were in hematologic remission. None of the patients had taken aspirin for at least 8 days before blood withdrawal. In a first approach, we evaluated IL-6sR levels in plasma from a group of 46 consecutive ET patients. The number of individuals included in further studies, as indicated in the appropriate paragraphs, depended on availability of the patients`samples and were not selected by their plasma IL-6sR levels. Blood samples from 55 healthy individuals selected for having a similar range of age (45.3 years, 22-79) and gender distribution (40 were female) were studied as controls. This project was approved by our institutional Ethics Committee (*IDIM, A. Lanari*). Informed consent, according to the declaration of Helsinki, was obtained from patients and healthy donors.

Plasma and platelet preparation

Blood samples were taken before and during treatment, when hematologic remission had been reached. Platelet-poor plasma was separated from blood anticoagulated with 129 mmol/L trisodium citrate by centrifugation at 2,500 g for 20 min. An additional centrifugation at 13,800 g for 10 min ensured no platelet contamination. Blood samples for the platelet preparations were collected on citric acid-citrate dextrose (7 mmol/L, 93 mmol/L and 139 mmol/L, respectively, pH 6.4), centrifuged at 200 g for 10 min and then the upper half of the platelet-rich plasma (PRP) was used. Indomethacin (41.9 μmol/L) was added to prevent platelet activation. Contaminating leukocytes and red blood cells were removed by additional centrifugation at 200 g for 5 min and a later filtration of PRP samples through a high efficiency leukocyte reduction filter (Purecell PL, PALL Biomedical Products Company, NY, USA). With this procedure the leukocyte:platelet ratio was reduced to less than $1:10^6$. Platelets were pelleted, washed twice with Hanks buffer (KCl 2.7 mmol/L, NaCl 137 mmol/L, Na_2CO_3 12 mmol/L, NaH_2PO_4 0.36 mmol/L, $CaCl_2$ 2 mmol/L, $MgCl_2$ 1 mmol/L, glucose 55 mmol/L, pH 7.4) and supplemented with 1 mmol/L adenosine, 2 mmol/L theophyl-line, 129 mmol/L trisodium citrate and 1.54 mmol/L EDTA. Platelet suspensions were adjusted to 10^6/μL and stored at -70°C until tested.

Plasma IL-6sR and IL-6 measurement

Plasma IL-6sR and IL-6 levels were assayed by an ELISA technique (R&D Systems, Minneapolis, USA). Normal values (mean±2SD) obtained in our laboratory in 39 controls were 27.43±10.59 ng/mL for IL-6sR and 1.60±2.88 pg/mL for IL-6, similar to those given by the manufacturer. Lower detection limits given by the manufacturer were 6.5 pg/mL for IL-6sR and 0.7 pg/ml for IL-6.

Intraplatelet IL-6sR measurement

Platelet suspensions were lysed by five cycles of 30 sec sonication (Fisher, NY, USA) and centrifuged at 60,000 g for 30 min. Intraplatelet IL-6sR levels were assayed in the supernatant of undiluted samples by the ELISA technique (R&D Systems). Results are expressed as pg/10^9 platelets.

Mononuclear cell culture

Mononuclear cells were separated from heparinized whole blood by Ficoll Hypaque gradient (density 1,077 g/cm^3) (SIGMA Laboratory, St. Louis, USA). Cells were washed three times with Iscove's modified Dulbecco's medium (IMDM) at 200 g for 15 min to minimize platelet contamination. A total of 1×10^6 cells were incubated in 1 mL IMDM containing 10% fetal calf serum, 15 μg/mL penicillin, 15 μg/mL streptomycin and 2×10^{-5}M 2-mercaptoethanol (Gibco BRL, Gaithersburg, MD, USA) at 37°C in a 5% CO_2 atmosphere. Platelets, at a concentration similar to that found contaminating mononuclear cells, were seeded in a separate control well. Assays were performed in triplicate and the viability of cultured cells was over 92%. After 48 hr the supernatants were collected and stored at –70°C.

Flow cytometry for IL-6R on leukocytes

Fresh blood drawn into 0.342 mol/L EDTA, was labeled with saturating amounts of anti-CD126-PE that recognizes IL-6R (Immunotech, Miami, Florida, USA). White cell subpopulations were detected by the addition of specific monoclonal antibodies (MoAbs) directed against CD45 and CD3 labeled with PerCP and CD19 and CD14 labeled with FITC (Becton-Dickinson, San José, CA, USA). Irrelevant MoAbs of the same Ig subclass were used as negative controls. Samples were analyzed by immunofluorescence using a FACScan flow cytometer (Becton-Dickinson). List mode data were collected for 60,000 ungated events to determine receptor expression on the different subsets of cells. Results were analyzed as relative fluorescence intensity (RFI) (ratio between mean fluorescence intensity obtained with CD126 antibody and the isotype control) and as percentage of the CD126-PE positive population in T and B-lymphocytes, granulocytes and monocytes.

Semi-quantitative polymerase chain reaction after reverse transcription of IL-6R and IL-6sR

For RNA extraction, mononuclear cells were prepared from whole blood by density gradient centrifugation (Ficoll Hypaque). Total RNA extraction was carried out according to Chomczynski & Sacchi,[17] using the TRIZOL reagent (Gibco BRL). Single-stranded cDNA was synthesized utilizing the SuperScript Preamplification System (Gibco BRL). Briefly, 1 μg RNA and 50 ng random hexamers were incubated at 70°C for 10 min and then placed on ice for 1 min. Afterwards, 10×PCR buffer (200 mmol/L Tris-HCl pH 8.4, 500 mmol/L KCl, 25 mmol/L $MgCl_2$, 10 mmol/L of each dNTP and 0.1 mol/L DTT were added and the mixture was incubated at 25°C for 5 min. Two hundred units of SuperScript II reverse transcriptase (Gibco BRL) were added and incubated at 25°C for 10 min, then at 42°C for 50 min and finally at 70°C for 15 min. The mixture was then placed on ice for 1 min and 2 units of *E. coli* RNase H were added (Gibco BRL). After 20 min incubation at 37°C, samples were stored at –20°C until use. For IL-6R and IL-6sR cDNA amplification, the polymerase chain reaction was performed by a standard technique using Taq Polymerase and 36 cycles of a 60-second denaturation step at 94°C, a 60-second annealing step at 58°C, and a 60-second extension step at 72°C. The oligonucleotide primers used were as follows, upper: 5′-ACGCCTTGGACAGAATCCAG-3′ and lower: 5′-TGGCTCGAGGTATTGTCAGA-3′ as previously described by Horiuchi.[18] For semi-quantification of IL-6R gene expression on mononuclear cDNA, we performed co-amplification of a GADPH internal control sequence using oligonucleotide primers as follows, upper: 5′-TGCACCACCAACTGCTT-3′ and lower: 5′-TACTCCTTGGAGGCCAT-3′, rendering a 554 bp fragment.[19] The amplified PCR products were analyzed on 2% agarose gel stained with ethidium bromide. Semi-quantification of the results was carried out by densitometric analysis using an imaging densitometer (Bio-Rad Laboratories, Hercules, CA, USA). Samples were amplified at different cDNA dilutions, 1:10, 1:20, 1:40 and 1:80, to ensure that band intensity corresponded to a working linear region of the PCR reaction curve. Restriction analysis using RSA I and Sph I enzymes (Promega Corporation, WI, USA) confirmed the identity of the PCR products.

Statistical analysis

Data are presented as median values and ranges. The rank sum test (Mann-Whitney-Wilcoxon) was applied to compare data from patients and normal controls. Wilcoxon's signed rank test was applied to compare data before and during treatment. Statistical significance was defined as $p<0.05$.

Results

Plasma IL-6sR levels in ET patients before and during treatment

Plasma IL-6sR levels were higher in untreated patients (n=46), 37.2 ng/mL (19.2-64.8) than in normal controls (n=39), 26.4 ng/mL (19.1-37.2), $p<0.00001$ (Figure 1A). Twenty-two out of 46 patients had values above the upper normal limit, 38.0 ng/mL (mean±2SD).

Platelet and monocyte blood counts in this group of patients were 992×10^9/L (564-3,742) and 650×10^3/L (300-900), respectively. There was no statistical relationship between plasma IL-6sR levels and either platelet or monocyte counts. No differences in plasma IL-6sR levels were found between patients with or without thrombotic events or spontaneous platelet aggregation in PRP. Twenty-one cases were evaluated before and during anagrelide treatment. Plasma IL-6sR levels decreased significantly during treatment, (median and range of the difference between levels before and during treatment, Δ) 5.45 ng/mL (-7.1-13.8) $p=0.01$, but did not reach the normal range (p between levels in patients during treatment and in normal controls, 0.019), (Figure 1B). There was no correlation between Δ of IL-6sR levels and Δ of platelet count before and during treatment (701×10^9 p/L, 220-3478×10^9).

Plasma IL-6 levels

Plasma IL-6 levels were normal in all 22 untreated patients evaluated: 1.68 pg/mL (0.85-10.2).

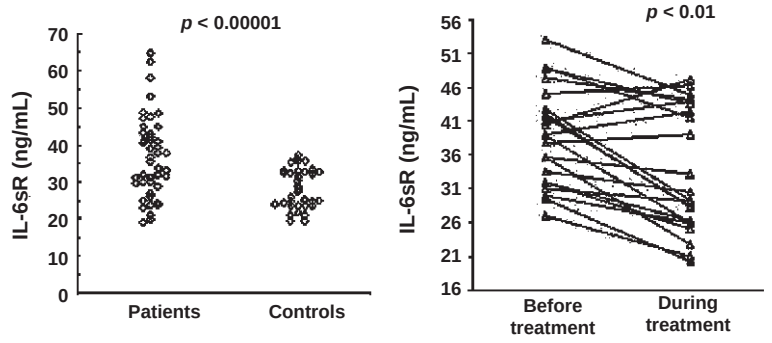

Figure 1. Plasma IL-6sR levels in ET patients. a) Using Wilcoxon's rank sum test a significant difference was found between levels in 46 untreated ET patients and in 39 normal controls (p<0.00001). b) Plasma IL-6sR levels before and during treatment in 21 ET patients. Wilcoxon's signed rank test showed a significant difference (p=0.01).

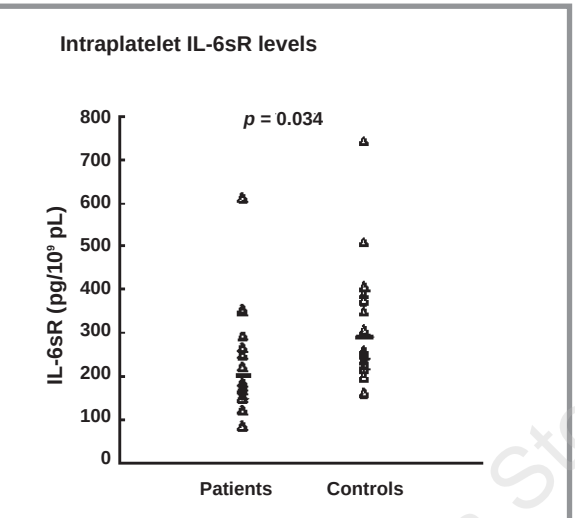

Figure 2. Intraplatelet IL-6sR levels. IL-6sR levels were expressed as pg/10^9 platelets. Fifteen untreated ET patients and fifteen normal controls were studied (p= 0.034, Wilcoxon's rank sum test).

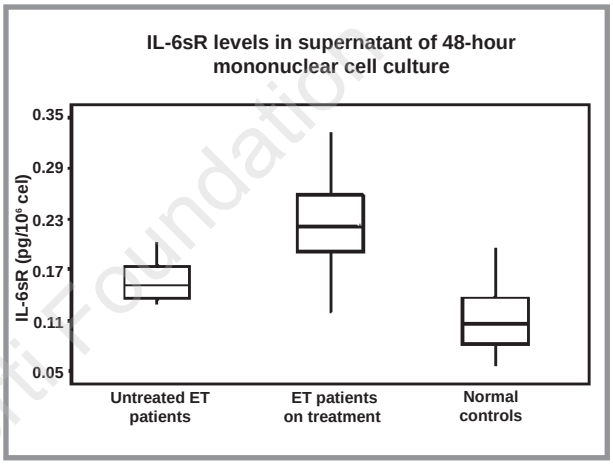

Figure 3. IL-6sR levels in the supernatant of 48-hr mononuclear cell cultures. IL-6sR levels are expressed as pg/10^6 cells in 8 untreated ET patients, 12 patients on treatment and 16 normal controls. Significant differences were seen between untreated patients and normal controls (p=0.009), between patients during treatment and normal controls (p=0.0001) and between untreated and patients on treatment (p=0.007).

Intraplatelet IL-6sR levels

We measured IL-6sR platelet content in order to evaluate a possible contribution of platelet release to plasma IL-6sR levels. Intraplatelet IL-6sR levels were lower in untreated ET patients (n=15) than in normal controls (n=15): 202.0 pg/10^9 platelets (86.3-612.8) and 288.9 pg/10^9 platelets (161.9-743.4) respectively, p=0.034 (Figure 2). There was no correlation between intraplatelet and plasma IL-6sR levels.

IL-6sR in the supernatant of mononuclear cell cultures

IL-6sR levels in the supernatant of mononuclear cell cultures from 8 untreated ET patients were higher than those from 16 normal controls (153 pg/10^6 cells [130-202]) and 105 pg/10^6 cells [58-195], respectively; p=0.009). Besides, ET patients under treatment (n=12) also had increased IL-6sR levels in the supernatant of cell culture, 221 pg/10^6 cells (120-332), p=0.0001 (Figure 3). The amount of IL-6sR released by mononuclear cells from patients under treatment was significantly higher than that obtained from untreated patients (p=0.007). Clinical as well as pretreatment platelet counts and spontaneous platelet aggregation were not different between groups. Wells seeded with platelets in a concentration similar to that found contaminating mononuclear cells (1×10^3 to 1×10^5 platelets per well) had undetectable IL-6sR levels. There was no relationship between IL-6sR levels released from mononuclear cells and plasma IL-6sR levels or monocyte counts from mononuclear cell cultures.

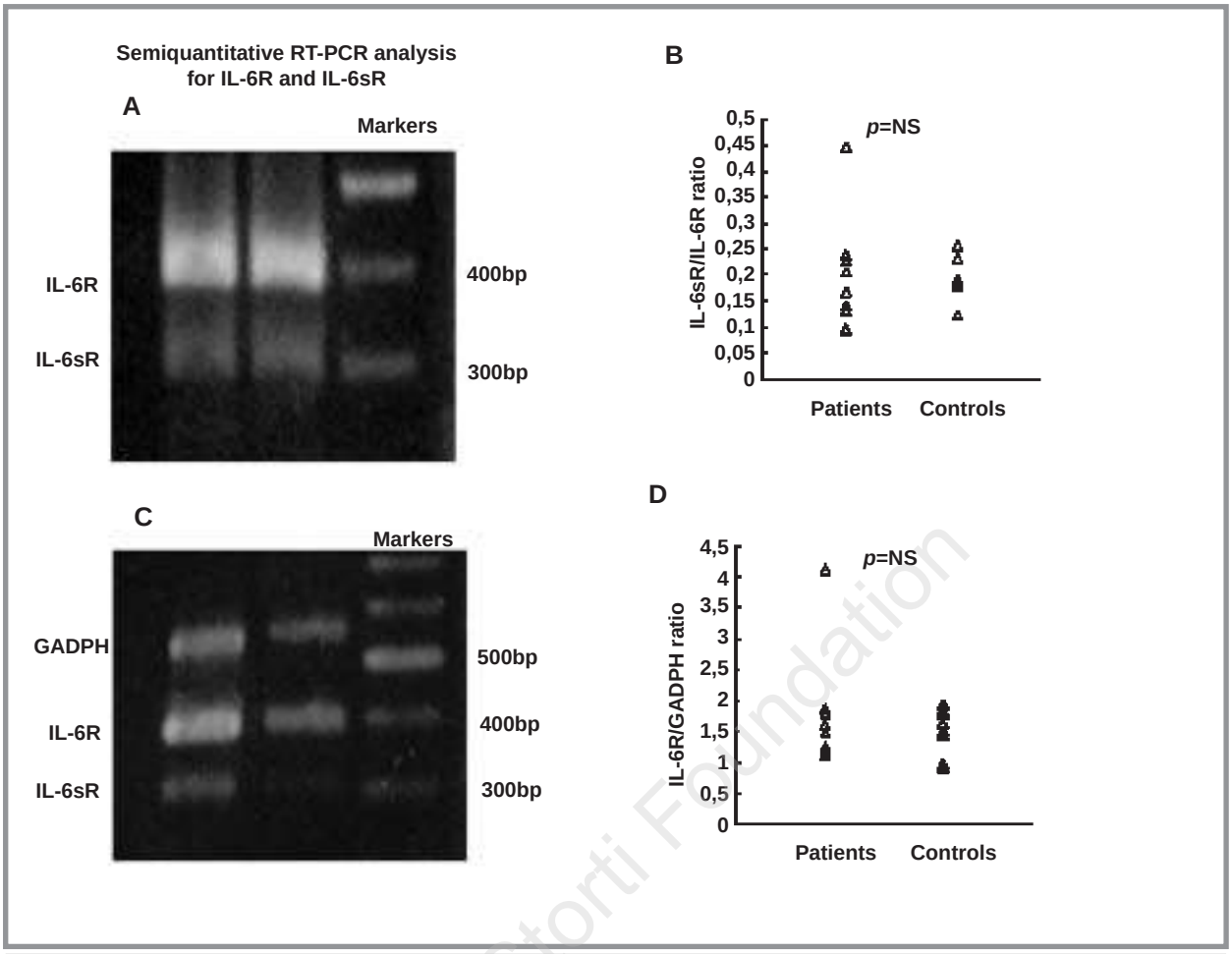

Figure 4. Semi-quantitative RT-PCR analysis for IL-6R and IL-6sR. a) Representative example of IL-6R and IL-6sR mRNA expression in mononuclear cells. RT-PCR was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. b) Ratio of IL-6sR/IL-6R bands in 10 ET patients during treatment and in 6 normal controls. IL-6sR amplification was not detected in one patient and four normal controls (*p*=NS). c) Representative example of IL-6R and GADPH expression in mononuclear cells by RT-PCR. d) Ratio of IL-6R/GADPH scanned bands in 11 ET patients on treatment and in 10 normal controls (*p*=NS).

IL-6R on leukocyte membranes

As IL-6sR can be generated by shedding, an increase in the activity of the metalloprotease responsible for this cleavage would lead to a decrease in IL-6R on the cell surface. In an attempt to evaluate this possibility in ET patients we studied IL-6 receptor expression on leukocyte surfaces using anti-CD126-PE and flow cytometric analysis. Leukocytes examined included monocytes ($CD14^+$ cells), B and T lymphocytes ($CD19^+$ and $CD3^+$ cells, respectively) and granulocytes (selected by forward, side scatter and $CD45^+$ fluorescence). Neither RFI nor percentage of $CD126^+$ events was different from that in normal controls (n=17) in any of the cell populations analyzed in untreated ET patients (n=10) ($p>0.1$ in each case). Samples from ET patients under treatment (n=9) showed similar results. Normal medians and ranges were as follows: monocytes-RFI, 10.8 (6.3-28.2), B lymphocytes-RFI, 1.1 (0.93-1.4), T lymphocytes-RFI, 6.6 (4.8-10.9), and granulocytes, 17.5 (14.6-25.9). Normal values for percentage of $CD126^+$ events were: monocytes, 98.8 (82.1-99.9), B lymphocytes, 5.3 (3.1-9.1), T lymphocytes, 71.8 (62.6-80.3), and granulocytes, 99.8 (97.8-100).

RT-PCR semi-quantification of IL-6R and IL-6sR in mononuclear cells from ET patients

In order to evaluate the transcription rate of IL-6 receptors, RT-PCR was used to detect IL-6R and IL-6sR expression sensitively at the mRNA level. All patients studied were receiving anagrelide treatment. The selected oligonucleotide primers flank the transmembrane domain of the receptor, generating a 398-bp fragment from the anchored IL-6R mRNA. A 304-bp molecule, lacking the 94-bp fragment, is also generated from the IL-6sR mRNA. A representative example is shown in Figure 4A. The ratio between the intensity of the scanned bands corresponding to the soluble and the anchored fragments in 11 patients during

treatment and 10 normal controls were not significantly different (Figure 4B). One patient and four normal controls did not show IL-6sR amplification. Semi-quantification of the IL-6R gene expression was evaluated by concomitant amplification of the housekeeping gene GADPH as an internal control. A representative agarose gel is shown in Figure 4C. No significant difference was found when the ratio between the band corresponding to the anchored receptor and the housekeeping gene were compared in the 11 ET patients and 10 normal controls (Figure 4D).

Discussion

IL-6, one of the cytokines involved in megakaryocytopoiesis, exerts its activity through either the membrane bound IL-6Rα or through the soluble agonistic form, IL-6sR, both mediated by gp130. Here we describe elevated IL-6sR plasma levels in patients with essential thrombocythemia, an illness characterized by abnormal megakaryocytic expansion. Although plasma levels of IL-6sR were significantly higher among patients, an overlap was found between levels in patients and control samples. This finding could be related to the heterogeneity of ET patients as shown by clonality studies and variable disease progression.[20-21]

Plasma IL-6sR levels have been found to be increased in other hematologic and non-hematologic disorders such as multiple myeloma[16] and hemorrhagic fevers.[22] In the latter, an inflammatory state is responsible for the increase of IL-6sR. In our patients with ET, inflammation was ruled out as IL-6 levels were found to be within the normal range in all cases. Besides, patients with a plasma IL-6sR higher than the upper normal limit had undetectable C-reactive protein values (*data not shown*). Oleksowicz *et al.*[23] found that IL-6 enhances *in vitro* platelet aggregation induced by various agonists. We, therefore, searched for a possible relationship between the occurrence of thrombosis and/or spontaneous platelet aggregation and elevated IL-6sR levels. However, we did not find any difference in plasma IL-6sR levels between ET patients with or without thrombotic events or spontaneous platelet aggregation.

Plasma IL-6sR levels decreased during anagrelide treatment, when the platelet count returned to normal values, although they were still higher than those of controls. In a previous work, we have described that normal platelets possess IL-6sR.[24] In the present study, we showed that intraplatelet IL-6sR levels were lower in untreated ET patients than in normal controls. Taken together, these results may suggest that IL-6sR is released from platelets in ET patients. This is in agreement with what has been described for other platelet proteins in ET patients, such as PDGF, which shows increased plasma levels while intraplatelet levels are low, suggesting that platelet release contributes to the plasma increase.[25] However, given the amount of intraplatelet IL-6sR, we believe that this source of IL-6sR made only a minor contribution to the plasma increment. Therefore, we searched for an additional source of IL-6sR.

Following this line, we found increased IL-6sR levels in the supernatant of unstimulated mononuclear cell cultures, both in untreated and anagrelide-treated ET patients. It is therefore tempting to speculate that these cells might be another possible contributor to the plasma IL-6sR increase in our ET patients. Moreover, IL-6sR levels found in the supernatant of mononuclear cell cultures from patients on treatment were statistically higher than those found in untreated patients. The difference between these groups is unlikely to be due to clinical or laboratory features since both groups had similar characteristics. However, the possibility of a direct or indirect effect of anagrelide on mononuclear cells function must be taken into account.

Among the mononuclear cells, monocytes are strong candidates for being the source of this abnormal plasma IL-6sR value. Monocyte activation in ET patients cannot be disregarded considering that in another myeloproliferative disorder, agnogenic myeloid metaplasia, this activation has been confirmed to exist.[26-27]

The generation of IL-6sR has been shown to occur via translation from alternatively spliced mRNA.[28] In an attempt to elucidate whether this mechanism contributes to the elevated IL-6sR generation in ET patients, we evaluated the expression of IL-6R and IL-6sR mRNA in mononuclear cells by semi-quantitative RT-PCR. The expression levels of both types of receptors were similar to those in normal controls. These findings would suggest that the abnormally high IL-6sR levels in our group of patients with ET is not due to an increased rate of gene transcription. On the other hand, IL-6sR can be generated by proteolytic cleavage of membrane IL-6R by a transmembrane metalloprotease.[14,29] If this mechanism was enhanced in ET patients, IL-6R on the cell surface would be decreased. In this regard, we could not find any abnormality in the expression of IL-6R on the plasma membrane of leukocyte subpopulations. However, we must take into account that levels of IL-6R on plasma membrane result from both protein synthesis and proteolytic cleavage. It cannot be ruled out that abnormal mRNA translation makes a contribution since this aspect was not evaluated. Thus, further studies are needed to clarify the mechanism

of abnormal IL-6sR production by mononuclear cells. The increased plasma IL-6sR levels in ET we describe here could contribute to the abnormal megakaryocytic proliferation seen in these patients since the IL-6/IL-6sR complex participates in hematopoietic and megakaryocytopoietic development.[30-31] Studies are in progress to elucidate the mechanism of IL-6sR generation and the involvement of monocytes.

RM and NG main authors, RM conception and design, RM, NG, PL and AG developed the assays, PH, PV, LK and FM were involved in collection of clinical data including follow-up examination of patients. RM and NG drafted the article. CP helped in analyzing the data and revised the paper. FM participated in the design of the study, interpretation of the results, revised the manuscript and is the principal clinician involved. The authors reported no potential conflicts of interest.

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