Progenitor cell trafficking in physiologic conditions and in myeloproliferative diseases: quantification of CD34+ cells by polymerase chain reaction

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Background and Objectives. Previous studies using flow cytometry have shown that CD34+ cell trafficking is increased in patients with chronic idiopathic myelofibrosis. Few data exist on physiologic CD34+ cell trafficking and the quantification of very low cell ranges requires reliable and sensitive measurement techniques. The aim of this study was to establish a quantitative polymerase chain reaction (PCR) technique for studying CD34+ cell trafficking in physiologic conditions, and in patients with myeloproliferative diseases.

Design and Methods. CD34+ cell trafficking was measured in 56 controls [(healthy controls (n=21), patients with ischemic cardiopathy (n=21), patients with secondary thrombocytosis or erythrocytosis (n=14)], and in 37 untreated patients with myeloproliferative diseases diagnosed according to the WHO-criteria [(essential thrombocythemia (n=10), polycythemia vera (n=14) and chronic idiopathic myelofibrosis (n=13)]. Quantitative PCR was used to determine CD34 mRNA expression in peripheral blood samples.

Results. Physiologic CD34 mRNA expression ranges were determined in the healthy control group. Mean CD34 mRNA expression was within the physiologic range in patients with ischemic cardiopathy, secondary thrombocytosis or erythrocytosis, essential thrombocythemia and polycythemia vera (p=0.146), but was significantly increased in patients with chronic idiopathic myelofibrosis (p<0.001). When analyzed individually, 12/13 patients with chronic idiopathic myelofibrosis and 3/14 patients with polycythemia vera showed CD34 mRNA expression above the physiologic range.

Interpretation and Conclusions. This is a first report about CD34+ cell trafficking measured by quantitative PCR. Quantitative PCR is a reliable method suitable for the quantification of very low cell populations. Our study confirms the significant increase of CD34+ cell trafficking in patients with chronic idiopathic myelofibrosis, and in a subset of patients with polycythemia vera. Prospective studies are underway to characterize these circulating CD34+ cells and to investigate their role in the pathophysiology of myeloproliferative diseases.

Key words: myeloproliferative diseases, trafficking, CD34, PCR, ischemic cardiopathy.

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Trafficking of hematopoietic precursor cells, i.e. that hematopoietic precursor cells are not only confined to the bone marrow, but also circulate in the peripheral blood, has been described recently.[1] The role of precursor cell trafficking is not completely understood yet, but it has been postulated that circulating progenitor cells are involved in organ homeostasis. The first reports suggest that impaired trafficking is associated with ineffective organ repair in patients with cardiovascular diseases.[2-6] In contrast, increased trafficking has been described in patients with myeloproliferative diseases, and it has been shown to be associated with disease progression and poor outcome.[7-9]

All these studies used flow cytometric analysis to quantify the circulating CD34+ cells. This is a very useful technique for the characterization of circulating cells as it allows identification of marker co-expression on defined cell populations. However, this technique is hampered by the fact that quantification is imprecise for small cell populations, and that it is difficult to standardize for external quality controls.[10] Given the increasing interest of several research groups in quantifying CD34+ cells, a reliable technique that is easy to perform, allows precise quantification of rare cell populations, and can be adapted to the special requirements of clinical settings, is required. Quantitative polymerase chain reaction (PCR) techniques fulfill such requirements and are used in our diagnostic facility. We established a PCR method for CD34 quantification and report about CD34+ cell trafficking in healthy controls, patients with ischemic cardiopathy, secondary erythrocytosis and thrombocytosis, and myeloproliferative diseases.

Design and Methods

Study design

Blood samples from healthy controls and patients enrolled in prospective clinical studies in our Cardiology Department and Hematology Department were analyzed for CD34 mRNA expression. All patients signed informed consent and protocols were approved by our hospital's ethics committee.

In order to set baseline values for trafficking of $CD34^+$ cells a control group was analyzed. This group was composed of normal controls, cardiac controls and control subjects with secondary erythrocytosis. There were 21 normal healthy controls; 13 males, mean age 35 years (range 21-57) and 8 females, mean age 45 years (range 26-58). The cardiology control group was formed of 21 patients (20 males, 1 female; mean age 64 years (range 42-83) with ischemic cardiopathy enrolled in two different clinical studies investigating collateral coronary flow. Blood samples were taken at the time of enrolment in the protocols before treatment. The third control group comprised 14 patients investigated at our center for thrombocytosis (n=2) or erythrocytosis (n=12) and found to have secondary erythrocytosis or thrombocytosis; there were 7 males and 7 females with a mean age of 48 years (range 32-74). The study group was composed of patients with myeloproliferative diseases (10 with essential thrombocythemia, 14 with polycythemia vera and 13 with chronic idiopathic myelofibrosis) diagnosed according to the WHO-criteria. Patients were not under treatment at the time of analysis. The 10 patients with essential thrombocythemia (male/female=6/4, mean age 61 years [range 33-83]), had a mean leukocyte count of 8.6×10^9/L (range 7-12), and a mean spleen size of 12.3 cm (range 11-14); bone marrow fibrosis was present in one patient. The 14 patients with polycythemia vera (male/female=10/4, mean age 62 years [range 36-81]) had a mean leukocyte count of 13.9×10^9/L (range 5-31), and a mean spleen size of 14.1 cm (range 11-19); none had bone marrow fibrosis. The 13 patients with chronic idiopathic myelofibrosis (male/female=7/6, mean age 65 years [range 42-77] had a mean leukocyte count of 31.1×10^9/L (range 3-258) and a mean spleen size of 18.8 cm (range 12-33); bone marrow fibrosis was present in all 13 patients (Table 1). All bone marrow biopsies were reviewed by a trained hematologist.

Sample preparation

Total cellular RNA was extracted from 10^7 leukocytes using the QIAmp RNA Blood Mini Kit (Qiagen, Basel, Switzerland). cDNA was synthesized from 2 μg total RNA applying the Superscript II system (Invitrogen, Basel, Switzerland) and random hexamer primers in a total volume of 40 μL.

Table 1. Control and study groups.

Control and study groups	N	m/f ratio	Age (mean)	Leukocyte count (mean×10^9/L)	Spleen size (cm) (mean)	Bone marrow fibrosis
Normal controls	21	13/8	40 (21-58)	6.8 (4-11)	nd	nd
Controls with ischemic cardiopathy	21	20/1	64 (42-83)	7.3 (5-12)	nd	nd
Controls with secondary thrombocytosis or erythrocytosis	14	7/7	48 (32-74)	8.4 (4-11)	nd	nd
Essential thrombocythemia	10	6/4	61 (33-83)	8.6 (7-12)	12 (11-14)	1/10
Polycythemia vera	14	10/4	62 (36-81)	14.1 (11-19)	14 (11-19)	0/14
Chronic idiopathic myelofibrosis	13	7/6	65 (42-77)	31.1 (3-258)	19 (12-33)	13/13

m/f: male/female; nd: not done.

TaqMan real-time quantitative RT-PCR for CD34

CD34 mRNA expression was determined by quantitative real-time PCR based on a specific primers and probe set (TaqManGene Expression Assays, Hs00156373_m1, *http://myscience.appliedbiosystems.com*) supplied by Applied Biosystems (Rotkreuz, Switzerland). The assay is designed to span the exon boundary of exons 4 and 5 of the CD34 gene, the probe being located at the exact exon-exon boundary at position 689 (Reference Sequence NM_001773). A similar set of specific primers and probe (TaqManGene Expression Assays, Hs00609297_m1) was used for determination of the internal control gene, porphobilinogen deaminase (PBGD), spanning the exon boundary of exons 1 and 2 of the PBGD gene. All PCR reactions were performed in duplicate. PCR reactions contained 125 ng cDNA, 12.5 μL of 2x universal master mix (Applied Biosystems) and 1.25 μL of 20x Primer-probe mix in a final reaction volume of 25 μL. Amplification was carried out at 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. All reactions were performed in Micro-Amp optical 96-well plates using an ABI Prism 7700 sequence detection system (Applied Biosystems). A mean cycle threshold (Ct) was calculated for each duplicate. Values above Ct 32 for the housekeeping gene, PBGD, were not accepted and these patients were excluded. Standard curves were obtained using a serial dilution of total RNA extracted from KG-1 cells. Since very similar slopes (Y) of the

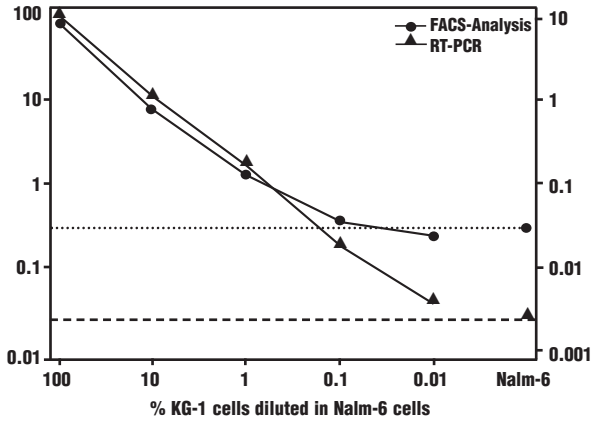

Figure 1. Correlation of PCR quantification with flow cytometry. A serial dilution of KG-1 cells in CD34- Nalm6 cells was used to compare PCR quantification of CD34 expression with flow cytometric measurements. The linear range for quantitative CD34 measurement by flow cytometry was limited to 2 logs, while the linear range for quantification by PCR remained linear for two additional logs. Residual intrinsic CD34 expression of the Nalm6 cell line is indicated for flow cytometric (dotted line) and RT-PCR measurements (dashed line). Left: percentage of CD34 expression by FACS; right: CD34mRNA expression level.

Table 2. CD34 mRNA expression in patients and controls.

Control and study groups	*Number of controls/ patients*	*Median CD34 mRNA expression*	*Range*
Normal controls	21	0.020	0.0004-0.130
Controls with ischemic cardiopathy	21	0.007	<0.0001-0.074
Controls with secondary thrombocytosis or erythrocytosis	14	0.010	<0.0001-0.054
Essential thrombocythemia	10	0.046	<0.0001-0.120
Polycythemia vera	14	0.027	<0.0001-0.300
Chronic idiopathic myelofibrosis	13	0.790	0.1300-6.500

A total of 56 controls and 37 patients with myeloproliferative disease were analyzed. The mean value of CD34 mRNA expression and ranges are given for each group: normal controls, controls with ischemic cardiopathy, patients with secondary thrombocytosis or erythrocytosis, essential thrombocythemia, polycythemia vera and chronic idiopathic myelofibrosis.

standard curves were obtained (Y_{CD34}=3.5, Y_{PBGD}=3.5), standard curves were omitted from further TaqMan reactions and the ΔCT method was used. Thus, relative CD34 expression was determined as PBGD-normalized CD34 levels and was calculated using the equation: relative expression level = $2^{-\Delta CtNORM}$, where $\Delta Ct_{NORM}=Ct_{CD34}\,Ct_{PBGD}$. The sensitivity of the CD34 PCR quantification was determined by serial dilutions of the cell line KG-1. KG-1 RNA was reproducibly detected down to a dilution of $1:10^5$, but a negative result was consistently obtained at a dilution of $1:10^6$. Thirteen samples from healthy controls were extracted and analyzed twice in two independent experiments in order to assess the reproducibility of results. The overall variation between results was less than 3%.

Flow cytometry

For flow cytometric analysis 2.5×10^5 cells were washed with cellWASH (BD Biosciences, Basel, Switzerland). The cells were centrifuged at 450×g and stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD34 antibody or FITC-IgG1 isotype control (both from BD Biosciences). Cells were analyzed on FACScalibur using CellQuest Pro software.

Clonogenic stem cell assays

Clonogenic stem cell assays for the determination of CFU-E (colony forming unit-erythroid) were performed as described elsewhere.[11]

Statistical analysis

Statistical analysis of the data was performed with the SigmaStat® software from Jandell Corporation (San Rafael, CA, USA). Groups were compared using multiple comparison procedures (Kruskal-Wallis one way analysis of variance on ranks) and pairwise multiple comparisons (Dunn's method). Linear regression analysis was used for the determination of correlations.

Results

Correlation of CD34 real-time PCR and flow cytometry

A serial dilution of KG-1 cells in CD34-negative Nalm6 cells was used to compare PCR quantification of CD34 expression with flow cytometric measurements (Figure 1). A correlation was obtained down to a dilution of 1:100. The linear range for quantitative CD34 measurement by flow cytometry was limited to 2 logs, while the linear range for quantification by RT-PCR was extended to 4 logs.

Definition of normal levels of CD34 mRNA expression

First, CD34 mRNA expression levels were assessed in blood samples of the 21 healthy controls. All values were above the detection limit (the detection limit of our PCR assay system being 0.0001) and ranged from 0.0004-0.13, with a median value of 0.02 (Table 2).

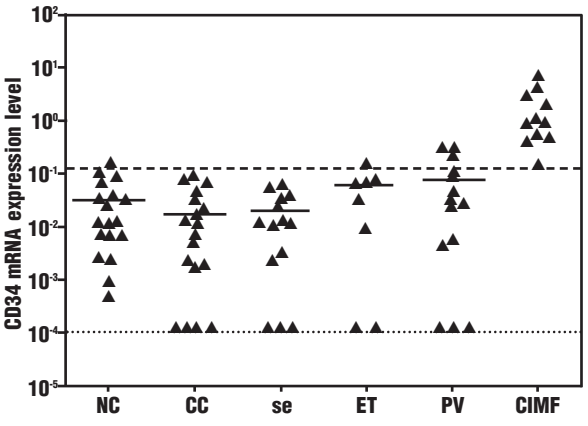

Figure 2. Individual CD34 mRNA expression for each patient and control CD34 mRNA expression in normal controls (NC), controls with ischemic cardiopathy (CC), patients with secondary thrombocytosis or erythrocytosis (se), essential thrombocythemia (ET), polycythemia vera (PV) and chronic idiopathic myelofibrosis (CIMF). The dashed line indicates the *cut-off* value for normal CD34 mRNA expression. The dotted line indicates the defined detection limit of 0.0001 CD34/PBGD.

The highest value of 0.13 measured within this group was chosen as the *cut-off* value for physiologic CD34 mRNA expression (Figure 2). Sixteen of 21 patients with ischemic cardiopathy showed detectable CD34 mRNA expression, as did 11 of 14 patients with secondary thrombocytosis or erythrocytosis, the median CD34 levels in these two control groups being slightly lower than in the healthy controls. None of the patients in these two control groups showed increased CD34 mRNA expression above the physiologic cut-off level (Figure 2). Statistical analysis showed no significant difference (p=0.146) in CD34 mRNA expression between the different control groups (i.e. healthy controls, patients with ischemic cardiopathy and patients with secondary thrombocytosis or erythrocytosis).

CD34 mRNA expression in patients with myeloproliferative diseases

Thirty-seven peripheral blood samples from patients with myeloproliferative diseases were analyzed for CD34 mRNA expression (Table 2). The group was composed of 10 patients with essential thrombocythemia, 14 patients with polycythemia vera and 13 patients with chronic idiopathic myelofibrosis.

Statistical analysis revealed a significantly higher level of CD34 mRNA expression in patients with chronic idiopathic myelofibrosis when compared to the CD34 mRNA expression of the 3 control groups (healthy controls, patients with ischemic cardiopathy and patients with secondary thrombocythosis or erythrocytosis) (p<0.001). Patients with essential thrombocythemia and polycythemia vera did not differ significantly from the control group (p>0.05). In patients with myeloproliferative diseases, CD34 mRNA expression was associated with the presence of bone marrow fibrosis, and was partly correlated to the presence of splenomegaly (R=0.59) but not to elevated leukocyte count (R=0.32).

The individual CD34 mRNA expression of each control subject or patient is shown in Figure 2. All healthy controls had detectable CD34 mRNA expression, the lowest value being 0.0004 and the highest 0.13. CD34 mRNA expression was below the detection limit of our assay in 5/21 patients with ischemic cardiopathy, in 3/14 patients with secondary thrombocytosis or erythrocytosis, in 2/10 patients with essential thrombocythemia, and in 3/14 patients with polycythemia vera. CD34 mRNA expression was detectable in all 13 patients with chronic idiopathic myelofibrosis. None of the patients with essential thrombocythemia had CD34 levels above the cut-off value, but 3/14 patients with polycythemia vera and 12/13 patients with chronic idiopathic myelofibrosis showed increased CD34 mRNA levels in the pathologic range. When analyzed for its value as a diagnostic test for chronic idiopathic myelofibrosis, CD34 quantification by PCR was shown to have a specificity of 98% and a sensitivity of 92%.

Clonogenic progenitor cell assays were performed for all patients with secondary thrombocytosis and erythrocytosis, essential thrombocythemia, polycythemia vera and chronic idiopathic myelofibrosis. The number of CFU-E (colony forming unit-erythroid) counted in each group is shown in Figure 3, and was significantly higher in patients with chronic idiopathic myelofibrosis than in subjects of other groups (p<0.05). A correlation was found between the number of CFU-E and CD34 mRNA expression (R=0.54).

Discussion

Correlation of CD34 mRNA expression with flow cytometry and sensitivity of the PCR method

A very good correlation of CD34 protein expression measured by flow cytometry and CD34 mRNA expression was found down to a dilution of 1:100 in a dilution experiment of the $CD34^+$ KG-1 cell line diluted in the $CD34^-$ cell line, Nalm6 (Figure 1). However, the linear range of CD34 quantification by flow cytometry is limited. In order to overcome this technical limitation we chose the real-time PCR method which displays a linear range of an additional two logs, hence adding a considerable gain of sensitivity. The PCR approach enabled us to determine the exact level of CD34 mRNA not only in pathologic but also in physiologic conditions, and thus to define a cut-off value for pathologic expression of CD34 mRNA. Very little is known about physiologic

CD34 mRNA expression, its stability over time, the relation of gene copies to CD34 molecules and the correlation between CD34 molecules and mRNA copies. The latter are particularly important when looking at diseases with pathologic levels of CD34+ cells. With this quantitative PCR technique we have now established a reliable method for quantifying CD34 mRNA expression in very low ranges and prospective studies are underway at our center which specifically address the other issues. Flow cytometry procedures are considered the gold standard in the setting of hematopoietic stem cell transplantation. However, quantitative PCR represents a very interesting research tool for the study of the pathophysiology of hematopoiesis and progenitor cell trafficking as a considerable additional level of sensitivity can be achieved with this technique.

CD34+ cell trafficking in physiologic conditions

In previous studies, CD34+ cell trafficking was measured by flow cytometry.[2-6,7-9] Physiologic values of trafficking can be deduced from the different control groups described in these studies. However, CD34+ cell trafficking has not been studied systematically until now and very little is known about physiologic variations such as differences between males and females, young and elderly, eventual circadian fluctuations or other external influences. Although our group of normal controls was too small to analyze such parameters with enough statistic significance, no trend could be identified. To exclude a possible impact of high hematocrit values or high platelet counts on CD34 mRNA expression, 14 patients with secondary thrombocytosis or erythrocytosis were analyzed, and CD34+ cell trafficking was found to be within the range of normal controls (Figure 2).

CD34+ cell trafficking in patients with ischemic cardiopathy

Previous studies reported impaired CD34+ cell trafficking in patients with cardiovascular diseases.[2-6] As this pathology is frequent, and thus often present as a co-morbidity in patients with myeloproliferative diseases, we measured CD34 mRNA expression in 21 patients with ischemic cardiopathy to detect an eventual bias in our analysis. We found no significant difference in CD34 mRNA expression in those patients when compared to normal controls (Figure 2). However, 5 of 21 patients with ischemic cardiopathy (24%) showed no detectable CD34 mRNA expression. Whether these very low values for CD34+ cell trafficking are of clinical significance for those individual patients cannot be answered in the setting of this study, but as this question is worth closer attention, it will be further investigated in larger groups of patients.

CD34+ cell trafficking in myeloproliferative diseases

Increased CD34+ cell trafficking has been described in patients with myeloproliferative diseases.[7-9] Elevated numbers of circulating CD34+ cells are associated with a bad prognosis and predict evolution towards blastic transformation.[8,9] Our study using quantitative PCR confirms previous data obtained by flow cytometry, i.e. that CD34+ cells are increased in the blood of patients with chronic idiopathic myelofibrosis. A 10- to 30-fold overexpression of CD34 mRNA was found in 12 of the 13 patients with chronic idiopathic myelofibrosis. In contrast to previous reports[7-9] our study showed no increase in CD34 mRNA expression in patients with essential thrombocythemia or polycythemia vera (mean value of the group). When analyzed individually, however, we did find CD34 mRNA expression above the cut-off value in three patients with polycythemia vera (Figure 2). These three patients showed no particular clinical feature suggestive of advanced disease, their leukocyte count and spleen size were close to the median of their group, and they had no bone marrow fibrosis. This raises the question of the origin of increased trafficking. We postulate that this phenomenon is linked with a change in adhesion molecule expression, but, to our knowledge, this point has not been commented upon in the literature and needs to be confirmed. Given the bad prognosis associated with increased CD34 mRNA expression[8,9] and given the high positive predictive value of our test for chronic idiopathic myelofibrosis, the three patients with polycythemia vera and increased CD34 mRNA expression will be re-evaluated regularly for disease progression.

In the overall series of patients, CD34 mRNA expression was associated with bone marrow fibrosis and splenomegaly but not with the leukocyte count.

In patients with chronic idiopathic myelofibrosis, high CD34 mRNA expression has previously been shown to be associated with high numbers of CFU-E.[12] We confirmed this finding in our series (Figure 3), with the exception of a subset of patients with polycythemia vera and chronic idiopathic myelofibrosis (3/27=11%) who did not show a significant number of endogenic colonies despite high CD34 mRNA expression. Unfortunately, false negative results are a well known limitation of clonogenic stem cell assays for the diagnosis of polycythemia vera.[11] Furthermore, in another subset of patients with clear endogenic colony growth (5/37=14%), CD34 mRNA expression was not detectable by PCR. The pathophysiologic explanation of these findings is not clear.

The nature of CD34+ cell trafficking in chronic idiopathic myelofibrosis

The increase of CD34+ cell trafficking in chronic idiopathic myelofibrosis raises several questions concerning the characteristics of circulating CD34+ cells.

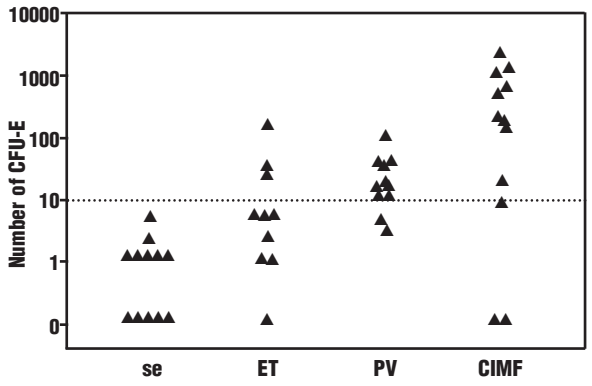

Figure 3. Clonogenic stem cell assays. Number of CFU-E for patients with secondary thrombocytosis or erythrocytosis (se), essential thrombocythemia (ET), polycythemia vera (PV) and chronic idiopathic myelofibrosis (CIMF). The dotted line indicates the *cut-off* value of our assay.[11]

Are these cells responsible for the clinical findings associated with the disease such as splenomegaly, extramedullar hematopoiesis and thrombosis? What is the function of circulating CD34⁺ cells? In a prospective study, we are systematically addressing the question of the function of circulating CD34⁺ cells in patients with myeloproliferative disease.

Finally, decreased CD34 mRNA expression was observed in some patients with ischemic cardiopathy (5/21), secondary thrombocytosis or erythrocytosis (3/14), essential thrombocythemia (2/10) and polycythemia vera (3/14) but not in normal controls or in patients with chronic idiopathic myelofibrosis. This finding supports the hypothesis of the role of CD34⁺ cell trafficking in organ homeostasis: both increased as well as diminished progenitor cell trafficking might be responsible for organ damage.

Quantitative PCR for measuring CD34 mRNA expression

Our PCR method was able to detect CD34 mRNA expression in all blood samples of the 21 healthy controls tested. It is a sensitive and reliable technique, particularly convenient for clinical studies as it can be easily implemented in diagnostic facilities and the results can be validated through external quality controls. We consider PCR a suitable method for the implementation of CD34 assessment in the diagnostic work-up (or follow-up) of patients with myeloproliferative diseases as it allows a reliable quantification of very low cell populations. We have described a PCR technique for the quantification of circulating CD34⁺ cells which allows CD34⁺ cell trafficking to be measured. This technique is particularly well suited for the quantification of very low ranges of cells and has some advantages over the gold standard flow cytometry. CD34 mRNA expression above the cut-off value of 0.13 was 100% predictive for polycythemia vera and chronic idiopathic myelofibrosis. Based on the prognostic significance of increased CD34-trafficking in patients with myeloproliferative diseases, and the good practicality of this method, we have now implemented CD34 quantification in the diagnostic work-up of patients referred to our center. The clinical outcome of the patients is being studied prospectively in the frame of a clinical study. CD34⁺ cells will be characterized to investigate the role of circulating progenitor cells in the pathophysiology of myeloproliferative disorders.

All authors meet the criteria for being contributing authors.

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