PATHOPHYSIOLOGICAL ASPECTS OF PLATELET-MEDIATED THROMBOSIS AND BLEEDING IN ESSENTIAL THROMBOCYTHEMIA

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PATHOPHYSIOLOGICAL ASPECTS OF PLATELET-MEDIATED THROMBOSIS AND BLEEDING IN ESSENTIAL THROMBOCYTHEMIA

Pathofysiologische aspecten van trombocyt-gemediëerde trombose en bloeding bij essentiële thrombocythemie

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INTRODUCTION TO ESSENTIAL THROMBOCYTHEMIA AND ITS HEMOSTATIC COMPLICATIONS

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Thrombocytosis, i.e. an elevation of the platelet count, is a common finding in clinical practice. In general, patients with thrombocytosis are distinguished into two main categories, primarily based on the cause underlying the increased platelet count. The term "reactive thrombocytosis (RT)" has commonly been applied to the condition of an increased platelet count after a period of bone marrow suppression associated with chronic or acute inflammation or infections, malignant diseases, hemorrhage, iron deficiency, or after splenectomy [1-4]. In contrast, the condition in those patients in whom there is a clonal proliferative drive to enhanced platelet production is termed "thrombocythemia" [1-4].

Thrombocythemia may occur in its primary form (also known as essential thrombocythemia) or may accompany any of the other myeloproliferative disorders polycythemia vera (PV), myeloid metaplasia with myelofibrosis (MMM), chronic myeloid leukemia (CML) as well as myelodysplastic syndromes [1-5]. The clinical course, treatment and prognosis vary considerably for patients with reactive thrombocytosis, myeloproliferative disorders and myelodysplastic syndromes. Therefore, a thorough analysis of the cause for the increased platelet count is warranted. This thesis will deal with essential thrombocythemia (ET), a myeloproliferative disorder characterized by a sustained elevation of the platelet count and a paradoxical predisposition to both thrombotic and bleeding complications [1-4].

1.1 DIAGNOSIS

In contrast to PV, MMM and CML, which may be identified by specific characteristics (increased red cell mass, collagen fibrosis of the marrow, and Philadelphia chromosome and bcr/abl rearrangements, respectively) the diagnosis of ET is still made by exclusion [2-4,6,7]. The diagnostic criteria of ET as proposed by the Polycythemia Vera Study Group [6] have been defined to distinguish ET from other myeloproliferative disorders and reactive causes of thrombocytosis (table 1.1), but lack - apart from an increased platelet count - positive criteria for the diagnosis ET.

In recent years much effort has been undertaken to define positive criteria for the diagnosis ET [8,9], taking into account data derived from studies on bone marrow morphology [10-13], DNA content of megakaryocytes (ploidy pattern) [14], spleen size [15] and *in vitro* cultures of hematopoietic progenitors [16-19]. These efforts have resulted in a proposal for new diagnostic criteria for ET (table 1.2). It remains to be evaluated whether ET can be diagnosed more reliably and specifically with these new criteria.

Table 1.1	Diagnostic criteria for essential thrombocythemia (adapted from [6]).
1.	Platelet count > 600 x 10^9/L.
2.	Hemoglobin ≤ 13 g/dL or normal red cell mass (males < 36 mL/kg; females < 32 mL/kg).
3.	Stainable iron in bone marrow or failure of iron trial (< 1 g/dL rise in hemoglobin after one month of iron therapy).
4.	No Philadelphia chromosome.
5.	Collagen fibrosis of bone marrow: a. absent or b. <1/3 biopsy area without both splenomegaly and leuko-erythroblastic reaction.
6.	No known cause for reactive thrombocytosis.

Table 1.2	Proposal for revised diagnostic criteria for ET (adapted from [8]).
Diagnostic	
A1.	Platelet count in excess of 400 x 10^9/L and no known cause of reactive thrombocytosis.
A2.	Increase and clustering of enlarged and mature megakaryocytes with hyperploid nuclei in bone marrow specimens.
A3.	No preceding or allied other subtype of myeloproliferative disorder or myelodysplastic syndrome.
Confirmative	
B1.	Normal or elevated leukocyte alkaline phosphatase score, normal ESR, and no fever or infection.
B2.	Normal or increased cellularity of bone marrow with or without the presence of reticulin fibers in biopsy material.
B3.	Splenomegaly on palpation or diagnostic imaging.
B4.	Spontaneous erythroid colony formation and/or spontaneous megakaryocyte colony formation in bone marrow culture.

1.2 EPIDEMIOLOGY

The true incidence of ET is unknown since extensive epidemiologic studies are not available. Based on data from the Mayo Clinic, Silverstein [20] estimated a frequency rate of 2 new ET patients per million in the population diagnosed each year. ET has usually been considered a disease of the middle aged, with its onset in fifth to sixth decades of life and a slight female preponderance. However, with the frequent inclusion of platelet counts in automated blood analysis, ET is also increasingly diagnosed in asymptomatic patients, young adults [21-25] and even children [26]. While it was initially suggested that ET might have a more benign course in younger patients [21], several studies since then [22-25] have demonstrated that ET in young patients may result in serious and life-threatening hemostatic complications. Genetic transmission of ET is unusual, although several families with multiple members having ET, occasionally in successive generations, have been described [27-29].

1.3 CLINICAL MANIFESTATIONS

In general, most clinical sequelae of ET are related to hemorrhagic and thrombotic episodes, which frequently complicate the clinical course of individual patients [30-40]. The presenting symptoms of patients with ET are quite variable. While early studies usually emphasized the bleeding complications of ET [41-43], more recent studies in ET have shown that thrombotic events pose a greater clinical hazard, particularly at lower degrees of an elevated platelet count [30,35,40,44 and table 1.3].

Table 1.3 **Hemogram of ET patients* with thrombotic and bleeding events at presentation. Data are given as median (interquartile range). Derived from reference [40].**

	Thrombosis (n=41)	Bleeding (n=5)	P-value
Hemoglobin (gmmol/L)	8.8 (8.2-9.9)	6.3 (5.7-8.8)	P= 0.016
Packed cell volume (L/L)	0.43 (0.40- 0.47)	0.33 (0.32-0.45)	P= 0.077
Platelet count ($x10^9$/L)	750 (575-1031)	1244 (969-2239)	P= 0.009
Leukocyte count ($x10^9$/L)	9.1 (7.6-10.8)	19.7 (9.3-23.4)	P= 0.028

* Two ET patients who presented with paradoxical thrombosis and bleeding were excluded from the statistical analysis.

As shown in table 1.4, 20 to 84% of the patients present with thrombotic complications as compared with 4 to 41% of the cases presenting with bleeding complications, respectively. Interestingly, 5 to 73% of the ET patients are asymptomatic at presentation, but may also remain free of hemostatic complications during long-term follow-up [4,32].

Thrombosis
Thrombotic events, involving both large arterial and venous vessels at virtually any site, may occur in ET, precipitating potentially life-threatening organ damage [30-40]. When involving the coronary arteries, myocardial ischemia and infarction may ensue, even in young individuals without concomitant risk factors for ischemic heart disease [32,45]. In contrast to other studies [2-4], deep vein thrombosis and pulmonary embolism are less frequently reported recently [30-40, table 1.4]. In addition, thrombotic occlusions of splenic, portal, hepatic and sagittal veins have occasionally been described in ET.

However, other - more characteristic - thrombotic manifestations, preferentially involving the arterial microvasculature, are observed in a high frequency (~60%) in ET [4,35,40]. These manifestations include the characteristic syndrome of erythromelalgia and transient neurologic symptoms. Erythromelalgia, derived from the Greek words *erythros* (redness), *melos* (extremity) and *algos* (pain), refers to a syndrome of redness and burning pain in the extremities, caused by a platelet-mediated arteriolar inflammation and occlusive thrombosis leading to acrocyanosis and even peripheral gangrene without evidence of pre-existing vascular disease [46-49]. Erythromelalgia may already be observed at platelet counts slightly above 350 x 10^9/l and therefore provide an early clue to the diagnosis of thrombocythemia [48-50]. There is considerable evidence that the microvascular disturbances may also involve the central nervous system [46,51,52,53]. In general, these symptoms consist of brief attacks of sudden cerebral or visual dysfunction, which can be either well localized or diffuse and non-specific. The neurologic symptoms are frequently accompanied by a dull and throbby headache lasting for several hours. The cerebral ischemic attacks in ET are rather atypical for TIAs as caused by atherosclerosis but a striking similarity to migraine accompaniments, in which spontaneous platelet aggregation and subsequent sludging and transient obstruction of the cerebral microvasculature have been incriminated, is noted [46,51-54].

Bleeding
Recurrent bleeding from mucous membranes and the digestive tract, easy bruising and platelet counts in excess of 1000 x 10^9/l, usually associated with splenomegaly may also characterize ET [30-43]. These symptoms basically reflect defective primary hemostasis (platelet plug formation). Joint and mus-

cle bleeding and petechiae are rarely observed in patients with ET. The spectrum of bleeding symptoms in ET closely resembles the hemorrhagic manifestations of patients with congenital von Willebrand disease [55]. In fact, several authors have described an acquired von Willebrand factor deficiency in thrombocythemia patients with platelet counts in excess of 1000 x 10^9/l with concomitant bleeding tendency [56]. Furthermore, patients with ET also frequently suffer from post-operative bleeding, especially after splenectomy [1,3,4].

Risk factors for thrombosis and bleeding
Although it may be anticipated that conventional risk factors for atherothrombosis may further enhance the risk for thrombotic events in ET, this association has been reported in only one study [158]. The other studies demonstrated that only a previous thrombotic event [32,40] and to a lesser extent age [32] represented significant risk factors for thrombosis in ET. In general, no significant risk factors for bleeding were identified in these studies probably because of the low incidence of bleeding complications in these studies. However, several studies [30,33,40,44] have indicated that the frequency of bleeding complications increased when platelet counts rose above 1000 x 10^9/L. In other studies bleeding complications were related to treatment with platelet anti-aggregants [3,4,40,72].

1.4 TREATMENT

The optimal therapy for ET and even the need of any therapy, remains an area of controversy and debate. Since the relationship between the degree of platelet count elevation and the risk of thrombosis or bleeding in ET is at best tenuous, the necessity of platelet count reduction in order to reduce morbidity or mortality is unknown. In addition, it is to be noted that many individuals with ET are asymptomatic at presentation (table 1.4). Further, they also remain free of hemostatic complications during long-term follow-up [4,32]. Moreover, reactive thrombocytosis has not been associated with an increased risk for hemostatic complications despite a comparable increase in circulating platelets. Therefore, the indications for suppression of the increased platelet count by drugs that carry a potential risk of inducing secondary malignancies, remain a subject of dispute.

Little controversy exists as to the need for lowering the platelet count in the symptomatic patient. Both thrombotic and bleeding symptoms in association with an increased platelet count usually improve after cytoreduction of platelets [7,14,24,35,37]. Most studies in ET [7,14,24,40,57] but not all [58] have shown a decrease in incidence of thrombotic and bleeding complications by cytoreduction of platelets, which corroborates the pathogenetic role of an

Table 1.4 General characteristics and incidence of thrombohemorrhagic complications at presentation of patients with essential thrombocythemia.

Author	n	Age	Sex ratio (f:m)	Platelet count (x10⁹/l)	Thrombosis (%)	Bleeding (%)	Asymptomatic (%)	Functional symptoms* (%)
van de Pette et al [37]	37	60.5 (30-89)	1.18	987 (615-2450)	70	41	5	NS
Bellucci et al [30]	94	49.5 (6-90)	1.76	1200	22	38	67	42
Grossi et al [34]	39	60 (21-82)	1.29	1200 (1000-3800)	62	18	31	NS
Hehlmann et al [35]	61	58 (10-82)	1.10	897 (300-4000)	84	13	11	NS
Lahuerta-Palacios et al [36]	19	55 (9-78)	0.90	1104 (740-2300)	63	21	11	NS
Cortelazzo et al [32]	100	50 (17-82)	1.56	1135 (600-3000)	20	10	34	36
Fenaux et al [33]	147	60 (18-83)	1.45	NS (700-2900)	27	18	36	34
Colombi et al [31]	103	59 (9-88)	1.34	NS	24	4	73	33
Wehmeier et al [39]	26	NS	NS	NS	24	20	56	NS
Randi et al [38]	97	54 (40-73)	1.43	1090	45	38	28	NS
van Genderen et al [40]	68	57	0.70	922	60	7	21	9

NS = not specified; Age and platelet count are expressed as means (range); *dysesthesias of hands and feet, headache and poorly localizing atypical neurological symptoms, relieved by aspirin

absolute increased number of platelets in the pathogenesis of these complications (table 1.5). Although the thrombocytosis associated with ET has successfully been treated with a variety of chemotherapeutic agents, including busulphan, melphalan, chlorambucil, pipobroman, thiotepa, radioactive phosphorus and CCNU (Lomustine), their use has been associated with a variable risk of (secondary) leukemia [20,59-61,69,70]. The leukemogenic potential of hydroxyurea remains unclear. Therefore, non-leukemogenic biologic response modifiers such as α-interferon [62-66] and more recently anagrelide [67,68] have also been employed to succesfully reduce the increased platelet count associated with myeloproliferative disorders. However, since their use has been hampered by drug availability, cost-benefit ratio and potential side-effects, it has yet not been possible to critically evaluate the long-term side-effects of these drugs.

The use of platelet anti-aggregating agents in ET remains an extremely controversial area [3,4]. On one hand, ET patients have an increased predisposition to bleeding. This risk of bleeding may be potentiated by the use of drugs that further affect platelet function [3,4,71,72]. On the other hand, ET patients who have recurrent thrombotic complications, particularly those with digital ischemia such as erythromelalgia or transient cerebrovascular ischemic symptoms, are likely to benefit from long-term low-dose aspirin as shown by several authors [40,46-48,53]. As shown in table 1.5, long-term treatment with low-dose aspirin (i.e. 100 mg/day) is also associated with a reduced frequency of thrombotic complications [40]. Erythromelalgia may effectively be treated by platelet cytoreduction and by inhibiting platelet cyclooxygenase [46-48]. Interestingly, drugs which do not inhibit platelet cyclooxygenase, i.e. coumadin derivatives, heparin, sulfinpyrazone, dipyridamole, sodium salicylate, acetaminophen, glafenin, are ineffective in the treatment of erythromelalgia [48].

A unique role for aspirin in ET may be found in the patient with recurrent abortions or intrauterine fetal demise. Given the apparent thrombotic origin of placental changes and the unknown teratogenicity of other treatment options aspirin seems a logical treatment option which has resulted in successful pregnancy [74,75]. Aspirin should probably not be used in patients with mixed histories of bleeding and thrombosis. These patients are likely candidates for platelet cytoreduction.

1.5 PROGNOSIS

A large study of 247 ET patients (median age 64 years), followed up for a median of 27 months (range 1 to 144 months) revealed no statistically significant survival difference as compared with the normal population [76]. This

Table 1.5 Incidence of thrombotic and bleeding complications in 68 patients with essential thrombocythemia, who had long-term follow-up, according to treatment strategy. Derived from [40].

Treatment	Duration of follow-up (person-yr)	Thrombotic complications		Bleeding complications	
		Events (n)	Events/100 person-yr	Events (n)	Events/100 person-yr
Watchful waiting	127	27	32.3	2	1.6
Low-dose aspirin	139	5	3.6[1]	10	7.2[4]
Cytoreduction	113	10	8.9[2]	2	1.8[5]
Low-dose aspirin and cytoreduction	40	0	0[3]	4	10.0[6]
Total	419	42		18	

Legend:
[1] = $p<.001$ ($\chi^2 = 17.3$, 1 df), for the comparison with watchful waiting (thrombosis)
[2] = $p=.014$ ($\chi^2 = 6.0$, 1 df),"""
[3] = $p=.003$ ($\chi^2 = 8.6$, 1 df) ,"""
[4] = $p=.032$ ($\chi^2 = 4.6$, 1 df), for the comparison with watchful waiting (bleeding)
[5] = $p=.92$ ($\chi^2 = 0.01$, 1 df),"""
[6] = $p=.014$ ($\chi^2 = 6.0$, 1 df),"""

is in agreement with several other studies indicating that life expectancy in ET is approximately unaltered despite a considerable morbidity due to frequent thrombohemorrhagic complications [3,4,31,35,40]. In rare cases of ET conversion to another myeloproliferative disorder may occur as part of the natural history of the disease [77]. Although all the other myeloproliferative disorders have the potential, to a greater or lesser degree, to spontaneously convert to acute leukemia, conversion of ET to acute leukemia has been rare and usually related to previous myelosuppressive therapy [3,4,69].

1.6 PATHOPHYSIOLOGY OF BLEEDING AND THROMBOSIS

A wide variety of platelet abnormalities, summarized in table 1.6, has been incriminated in the pathogenesis of bleeding and thrombosis in ET. In general, they represent either platelet hyper- or hypofunction [3,4]. Although it is reasonable to suspect that these platelet defects originate at the megakaryocyte level, this is far from established. Some have suggested that the platelet abnormalities in ET might be caused by modification of functional and biochemical characteristics of platelets as a result of hemostatic encounters in the circulation leading to *in vivo* platelet release and exhaustion. Alternatively, the platelet abnormalities in ET may be caused by aging of platelets in the circulation. Others have related the platelet abnormalities to progression of the underlying disease. Finally, the platelet abnormalities may also be influenced by treatment of the underlying disease [3,4].

Table 1.6 **Platelet abnormalities in essential thrombocythemia**
1. Platelet function in vitro a. hypoaggregation (~ 30%) with epinephrine, ADP and collagen [3,30,35,78]. b. hypoaggregation with arachidonic acid and prostaglandin peroxides [79,80] c. spontaneous platelet aggregation [35,46,81-84] d. circulating platelet aggregates [84,85] e. increased plasma levels of platelet-derived products [86-89] f. circulating activated platelets [90,91]
2. Platelet function in vivo a. prolonged bleeding time (~ 20%)[3,24,30-33,92-96] b. reduced platelet life span [97-100] c. platelet activation in vivo [88,89,101,102]
3. Platelet size and distribution increased platelet distribution width at normal or elevated mean platelet volume [103,104]

4. Platelet density and ultrastructural morphology

a. paucity of granular content and hypertrophy of dense tubular and open canalicular system [105,106]
b. reduction in platelet buoyant density [107,108]

5. Platelet secretory granular content

a. reduced dense body stores of adenine nucleotides and serotonin [95,109-115]
b. reduced α-granule stores of: ß-thromboglobulin, fibrinogen, von Willebrand factor, platelet-derived growth factor [87-89,116-118]
c. normal lysosomal granule stores [119]

6. Platelet glycoproteins and receptors

a. reduction of glycoprotein Ib and corresponding increase in cleavage product in plasma [120-123]
b. reduction in glycoprotein IIb/IIIa [123-125]
c. increase in glycoprotein IV (thrombospondin) [126,127]
d. decreased sialylation of glycoprotein Ib and IIIa [128]
e. loss of α-adrenergic receptors, resulting in lack of aggregation with epinephrine [129]
f. decreased number of prostaglandin D_2 receptors, resulting in increased resistance to inhibitory prostaglandins [130-132]
g. absence of glycoprotein Ia-IIa, resulting in lack of aggregation with collagen [133]
h. increased expression of F_c receptors [134]
i. heterogeneity of fibrinogen binding sites [125]

7. Platelet membrane and cytoskeleton

a. changes in fatty acid composition affecting membrane fluidity [135,136]
b. abnormalities in cytoskeleton protein composition upon platelet stimulation with thrombin [137]
c. increased resistance to digitonine lysis [138]
d. defective platelet procoagulant activity, including reduced factor X activation and decreased prothrombinase expression [96,139,140]

8. Platelet signal transduction

a. impaired receptor-respons coupling in epinephrine-insensitive platelets [141-143]
b. impaired thromboxane A_2 receptor-respons coupling via GTP-binding proteins [80,144]
c. defective cGMP-dependent signal transduction [145]
d. defects in calcium mobilization and exchange across platelet membranes [143,146,147]

9. Platelet metabolism

a. abnormalities in uptake and storage of serotinin [109,148,149]
b. abnormalities in arachidonate metabolism including lipoxygenase deficiency [112,150-154]
c. lactate overproduction in rest and after stimulation [155]
d. increased glyoxalase I activity [156]

Although the described platelet abnormalities are usually not seen in reactive thrombocytosis [3,4,159], the clinical significance of these abnormalities, apart from (differential) diagnostic purposes, remains uncertain for ET. Attempts to relate these various abnormalities to bleeding and thrombotic complications have failed to reveal any consistent pattern [93-95,159-163]. There are several possible explanations for these discrepancies. First, some of the platelet abnormalities described in ET may be secondary to platelet activation *in vitro* and modification of platelet morphology and size during blood sampling and processing, which thus might have no relevance to *in vivo* platelet function [164-166]. However, several authors have provided unequivocal evidence for platelet activation *in vivo* by measuring platelet-specific proteins or stable degradation products of platelet-derived thromboxane in urine, thereby avoiding artifacts from *ex vivo* platelet activation [88,89,102]. Second, thrombocythemic platelets may exhibit more than one platelet defect simultaneously, potentially exerting opposite effects on hemostasis [3,4]. Third, over the years various platelet function tests have been used, introducing several methodological variabilities which may affect the interpretation of the results of these experiments. For example, platelet aggregation studies in whole blood indicated hyperfunction but platelet aggregation was found to be normal or reduced when performed with platelet-rich plasma of the same ET patient [167]. Four, the abnormalities of circulating platelets may vary in patients with time as a consequence of progression of the underlying disease [160,162, 163] or therapeutic intervention [168-171].

The poor correlation between specific platelet abnormalities and thrombohemorrhagic events also raises the possibility that pathogenetic factors other than platelets may play a role in the hemostatic dysbalance in ET. Among these factors functional abnormalities of leukocytes [172-174], the fibrinolytic system [175,176] and vascular endothelium [177-179] might be considered as well as defects in cell-cell interactions [180,181]. However, the results of these studies have, for the time being, not established a generally consistent explanation.

1.7 RATIONALE FOR THE CLINICAL STUDIES

In general, most hypotheses on the mechanisms underlying the thrombotic diathesis of ET attribute a key role to platelet dysfunction. Indeed, the reproducible evidence of platelet activation *in vivo* in subjects with ET [88,89,102] as well as clinical evidence of the beneficial effect of in particular platelet cyclooxygenase inhibiting drugs [40,46-48,52,53,81,84] and cytoreduction of the increased platelet count [57] on the occurrence of thrombotic complications in ET are all in support of dysfunction of platelets playing a pivotal role in the pathophysiology of the thrombotic complications in ET ("platelet-medi-

ated thrombosis"). Since the majority of the thrombotic complications occur in the arterial microvasculature, further study of the pathognomonic thrombotic complication erythromelalgia may provide more insight in the pathophysiology of thrombotic complications in ET.

To that end, we have investigated whether platelets are involved in erythromelalgia by performing platelet survival studies in symptomatic and asymptomatic thrombocythemia patients as well as in symptomatic patients after treatment of erythromelalgia with aspirin [CHAPTER 2]. In addition, the absence of a beneficial effect of treatment with coumadin derivatives on erythromelalgia suggests that activation of the coagulation cascade and in particular fibrin formation is not a critical step in the pathogenesis of erythromelalgia. We therefore studied several parameters of coagulation and fibrinolysis to delineate the hemostatic profile of erythromelalgia [CHAPTER 3]. Furthermore, we investigated whether the observed increase in platelet thromboxane formation in ET, reflecting platelet activation *in vivo*, was actually related to development of (microvascular) thrombotic complications [CHAPTER 4].

Several clinical studies have suggested that bleeding complications occur more frequently at relatively higher platelet counts in ET [30,33,40,44]. The striking similarities between the bleeding tendency of ET and von Willebrand disease and the occasional description of an acquired deficiency of von Willebrand factor in ET disappearing after cytoreduction of the increased platelet count [55] suggest that dysfunctional platelets might also play a role in the pathophysiology of bleeding in ET ("platelet-mediated bleeding").

In a longitudinal observational study of one ET patient we noted that bleeding episodes were associated with the development of an acquired functional deficiency of von Willebrand factor in plasma. The latter deficiency was consistently related to the platelet count [CHAPTER 5]. In a subsequent study we therefore evaluated whether the inverse relationship between platelet count and decreased von Willebrand factor activity in plasma is a phenomenon specific of myeloproliferative disorders or whether it could be a consequence of the increased platelet count itself [CHAPTER 6]. In CHAPTER 7 we investigated the hypothesis that the decreased function of von Willebrand factor in plasma in ET associated with high platelet counts might be due to an increased turnover of large von Willebrand factor multimers by platelets. Finally, in CHAPTER 8 we addressed the question whether impaired activity of von Willebrand factor in plasma in ET might provide an explanation for the excessive prolongation of the bleeding time after aspirin.

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2

PLATELET CONSUMPTION IN THROMBOCYTHEMIA COMPLICATED BY ERYTHROMELALGIA: REVERSAL BY ASPIRIN

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SUMMARY

The involvement of platelets in the pathogenesis of erythromelalgia, a frequent and characteristic microvascular thrombotic manifestation in patients with essential thrombocythemia and polycythemia rubra vera, was investigated by measuring the survival and turnover of ^{51}Cr labeled autologous platelets in 10 patients with thrombocythemia complicated by erythromelalgia, in 10 asymptomatic thrombocythemia patients and in 6 subjects with reactive thrombocytosis. The mean platelet survival time of the erythromelalgia patients was 4.2±0.2 days, which is significantly decreased as compared with asymptomatic thrombocythemia patients (6.6±0.3 days, $p<0.001$) and patients with reactive thrombocytosis (8.0±0.4 days, $p<0.001$). The mean platelet survival time of asymptomatic thrombocythemia patients was significantly decreased ($p<0.01$) as compared with reactive thrombocytosis patients. Treatment of erythromelalgia with aspirin increased the mean platelet survival time from 4.0±0.3 days to 6.9±0.4 days ($p<0.001$) and was associated with an elevation of the platelet count of 216±30 x 10^9 platelets per liter ($p<0.001$). Coumadin failed to improve platelet survival or symptoms caused by erythromelalgia. The increased platelet consumption in erythromelalgia is attributed to the formation of platelet thrombi in the arterial microvasculature. This conclusion is supported by the ability of aspirin to interrupt platelet consumption and clinical features of erythromelalgia.

INTRODUCTION

Erythromelalgia, featured by red congestion and burning pain of the extremities, appears to be a characteristic and frequent microvascular thrombotic manifestation associated with essential thrombocythemia and polycythemia rubra vera. Erythromelalgia may already be documented at slightly increased platelet counts above 400 x 10^9/l. The disabling distress of erythromelalgia is usually preceded by acroparesthesias, e.g. tingling, "pin and needles" sensations and numbness in the toes or fingers. If left untreated, the symptoms of erythromelalgia, usually confined to the ball of the forefoot, toes, hand palm and finger tips, may progress to painfull acrocyanosis and even peripheral gangrene [1-3].

Histopathological examination of red congested erythromelalgic skin lesions demonstrated inflammation, intimal proliferation and thrombotic occlusions of the acral arterial microvasculature without evidence of pre-existing vascular disease [4]. In contrast to the inefficacy of oral anticoagulation with coumadin, treatment with aspirin characteristically relieves the erythromelalgic distress and restores the ischemic circulation disturbances of the acra, coin-

ciding with the period of inhibition of platelet cyclooxygenase activity and aggregation *in vitro* [1,5]. Furthermore, cytoreduction of the increased platelet count to normal abolishes erythromelalgic distress in patients with both essential thrombocythemia or polycythemia rubra vera [1]. These observations suggest that erythromelalgia results from a platelet-mediated thrombosis of preferably the arterial microvasculature of the extremities. In contrast, erythromelalgia and microvascular thrombotic events are rarely or not observed in patients with reactive thrombocytosis with a comparable rise in circulating platelets [6,7], suggesting that an intrinsic platelet defect gives rise to the arterial thrombotic predisposition in patients with thrombocythemia. To investigate the involvement of platelets in erythromelalgia platelet kinetics were prospectively studied in thrombocythemia patients, with and without erythromelalgia, and in control subjects with reactive thrombocytosis. Furthermore, because of the striking beneficial effect of aspirin on erythromelalgia platelet kinetics were also studied in patients with active erythromelalgia during curative treatment of erythromelalgia with aspirin.

PATIENTS AND METHODS

Patients

The diagnosis essential thrombocythemia (ET) or polycythemia rubra vera (PRV) was established according to the criteria of the Polycythemia Vera Study Group [8,9]. Platelet kinetics were prospectively studied since 1980 in 10 patients with thrombocythemia (6 ET, 4 PRV; 5 males and 5 females, ranging in age from 38 years to 71 years) at times of erythromelalgia (group E+) and in 10 asymptomatic patients (6 ET, 4 PRV; 7 males and 3 females, ranging in age from 41 to 79 years) with thrombocythemia (group E -). Erythromelalgia was diagnosed by its typical manifestations of red, warm, congested extremities and painful burning sensations with preferential involvement of the fore foot, sole and one or more toes and fingers [1-4]. The erythromelalgia patients included in this study all suffered from painful burning, red congested extremities. Six of them also had accompanying acrocyanosis, complicated by a necrotic spot on the right hallux in one. All PRV patients showed a persistent thrombocythemia after continued correction of the hematocrit (Ht) to normal (Ht<0.45). None of the patients included was treated with cytotoxic drugs in the foregoing year or ingested aspirin in the 14 days preceding the platelet survival study. The kinetic study was repeated in 7 E+ patients during treatment with 500 mg aspirin per day for more than two weeks. Two of these seven E+ patients were on adequate oral anticoagulation with coumadin during the whole period of study. Six asymptomatic patients (3 males and 3 females, ranging in age from 42 to 60 years) with reactive thrombocytosis (group RT) who had comparable elevated platelet counts due to pulmonary abcesses in 2, iron deficiency in 2, Crohn's disease in 1 and oesophagic carcinoma in 1,

respectively, served as control subjects. All patients and control subjects were newly diagnosed and cooperated in the study after informed consent.

Methods
Platelet counts were performed on peripheral blood collected in EDTA using the Platelet Analyzer 810 (Baker Instruments, Allentown, Pa.). Platelet survival studies were performed with sodium ^{51}Cr-chromate labeled autologous platelets according to the recommendation of the International Committee for Standardization in Hematology [10]. The ^{51}Cr labeling technique was used throughout the study in spite of the more recently recommended ^{111}In labeling technique for platelet survival studies [11] for reasons of continuity. Moreover, comparable results for platelet survival are published using either the ^{111}In or ^{51}Cr labeling technique [12-14]. Platelet recovery in the circulation at equilibrium was calculated from the total blood radioactivity extrapolated to time zero as a fraction of the injected platelet-bound radioactivity. The mean platelet survival was calculated according to the multiple hit model [15,16]. Platelet turnover, which under steady-state conditions is a measure of both platelet production and destruction, was calculated by the following formula: platelet turnover (platelets x 10^9/L/day) = blood platelet count per liter / platelet survival time (days) x recovery (decimal) [17,18].
Spleen lengths were estimated by ^{99m}Tc-scintigraphy or ultrasound examination.

Statistical analysis
Analysis of variance (ANOVA) followed by Student-Newman-Keuls test (SNK test) was used to identify pair-wise significant differences between group means of E+, E- and RT patients. Paired t-tests were used to analyze the effect of aspirin on platelet survival in erythromelalgia. The platelet survival curves were analyzed by least squares fitting to linear, exponential and multiple hit models. A comparison as to goodness of fit of these models within each group of patients was judged on the basis of residual sums of square through application of Friedman's rank test. Based on these analyses platelet survival curves were labeled either "linear" or "curvilinear". Data in the Results section are presented as mean ±S.E.M.

RESULTS

Patients
No significant differences with respect to age and platelet count were observed between RT, E- and E+ patients. All RT patients had a normal spleen size (spleen length ≤ 12 cm). In contrast, 5 E- patients and 5 E+ patients presented with splenomegaly.

Table 2.1	Platelet kinetic data of patients with reactive thrombocytosis (RT), asymptomatic thrombocythemia (E-) and thrombocythemia complicated by erythromelalgia (E+), expressed as mean±S.E.M. *=P<0.05; **=P<0.01; ***= P<0.001			
	RT (n=6)	E- (n=10)	E+n= (10)	p-value
Platelet count ($x10^9$/l)	1091±186	947±134	880±109	Global, p>0.05
Initial platelet recovery (%)	67±5	48±6	45±3	E+ vs RT:* E- vs RT:*
Mean platelet survival (days)	8.0±0.4	6.6±0.3	4.2±0.2	E+ vs RT:*** E+ vs E-:*** E- vs RT:**
Platelet turnover ($x10^9$/L/day)	202±38	313±26	545±108	E+ vs RT:* E+ vs E-:*
Survival curve pattern	linear	mixed	curvilinear	

Platelet kinetic data

Platelet survival curves. Statistical testing of the platelet survival curves with regard to the goodness of fit of linear relationships lead to the following results. The individual platelet survival curves of RT patients did not significantly deviate from a linear disappearance pattern. In 3 of 10 E- patients a statistically significant curvilinear disappearance pattern was observed. In E+ patients all individual platelet survival curves showed a clear curvilinear disappearance pattern. The platelet kinetic data are summarized in table 2.1.

Initial platelet recovery. The mean recovery of platelets in RT patients (67±5 %) was significantly increased as compared with both E- patients (48±6 %, $p<0.05$) and E+ patients (45±3 %, $p<0.01$), due to splenomegaly being present in 5 of 10 E- patients and in 5 of 10 E+ patients [19]. A significant linear correlation between platelet recovery and spleen size could be established in E- (n=10, r=-0.97, $p<0.001$) and E+ patients (n=10, r=-0.89, $p<0.001$, data not shown). Neither spleen size nor mean platelet recovery did significantly differ between E- and E+ patients, thereby excluding an effect of differences in splenic sequestration on platelet kinetics.

Mean platelet survival time. As shown in figure 2.1A, the mean platelet survival time of E+ patients (4.2±0.2 days) was significantly decreased as compared to both E- patients (6.6±0.3 days, $p<0.001$) and RT patients (8.0±0.4 days, $p<0.001$). The mean platelet survival of E- patients was significantly

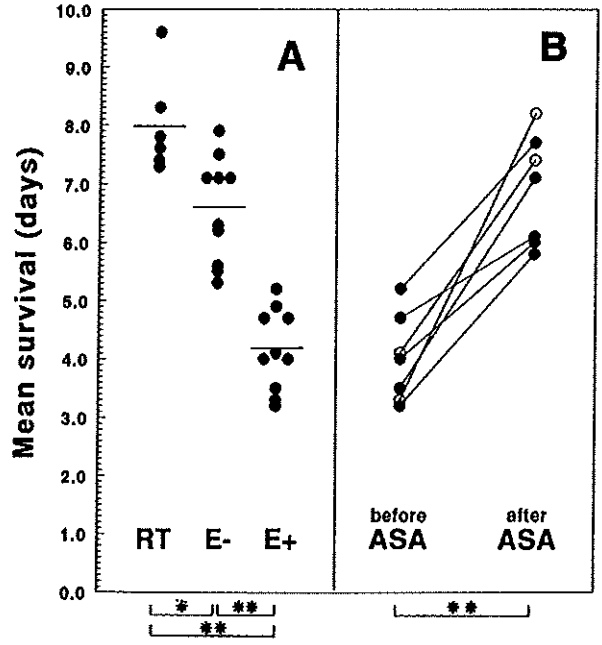

Figure 2.1

(A). Survival time of ^{51}Cr labeled autologous platelets in patients with reactive thrombocytosis (RT), asymptomatic thrombocythemia (E-) and in thrombocythemia patients suffering from erythromelalgia (E+). The solid line represents the mean platelet survival time. (*) denotes $p<0.01$; (**) $p<0.001$.

(B). Survival time of ^{51}Cr labeled autologous platelets in 7 thrombocythemia patients suffering from erythromelalgia before and after treatment of erythromelalgia with aspirin (ASA). Open symbols represent the patients with erythromelalgia who were on adequate oral anticoagulation with coumadin during both platelet kinetic studies. (**) denotes $p<0.001$.

Table 2.2 Effect of 500 mg aspirin (ASA) per day on platelet survival in 7 patients with thrombocythemia complicated by erythromelalgia. Data are given as mean ± S.E.M.

	before ASA (n=7)	after ASA (n=7)	P-value
Platelet count($x10^9$/L)	940±144	1157±161	P<0.001
Initial platelet recovery (%)	44±3	47±3	P>0.05
Mean platelet survival (days)	4.0±0.3	6.9±0.4	P<0.001
Platelet turnover ($x10^9$/L/day)	583±129	361±48	P<0.05
Platelet survival curve pattern	Curvilinear	Linear	

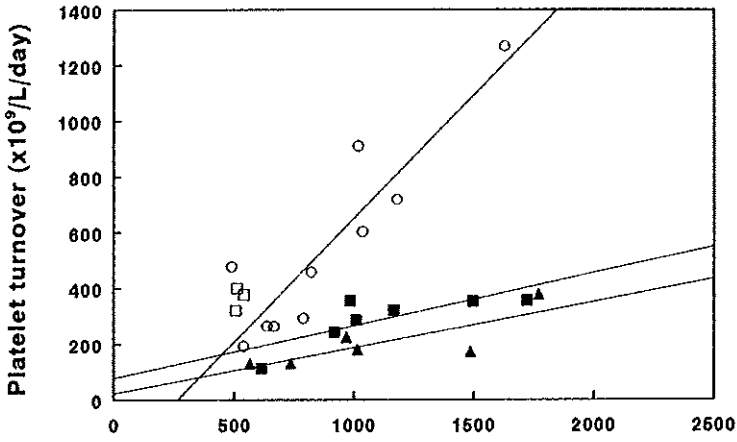

Figure 2.2

Relationships between platelet turnover and platelet counts. (▲) Patients with reactive thrombocytosis (n=6, r=0.80, p<0.05, best-fit linear regression is expressed by y=0.17x +22); (■) Asymptomatic thrombocythemia patients (n=7, r=0.79, p<0.05, regression line y=0.19x+77); (□) Asymptomatic thrombocythemia patients with curvilinear survival curves who were excluded from statistical analysis (whole group of asymptomatic thrombocythemia patients: n=10, r=0.19, p>0.05); (○) Thrombocythemia patients suffering from erythromelalgia (n=10, r=0.90, p<0.05, linear regression line y=0.89x-235).

(p<0.01) decreased as compared with RT patients. However, exclusion of the 3 E- patients with curvilinear survival curves resulted in a mean platelet survival time of 7.0±0.2 days with no significant differences (p>0.05) from the RT group.

Platelet turnover per day. The mean platelet turnover per day in E+ patients (545±108 x 10^9/L/day) was significantly increased compared with RT patients (202±38 x 10^9/L/day, p<0.05) and with E- patients (313±26 x 10^9/L/day, p<0.05). No significant differences in mean platelet turnover per day were observed between RT and E- patients.

When the platelet turnover was compared with platelet counts for each patient a significant correlation was found in the RT group (n=6, r=0.80, p<0.05) and the E+ group (n=10, r=0.90, p<0.05) but not in the E- group (n=10, r=0.19, p>0.05). However, a significant correlation between platelet turnover and platelet count was observed in the latter group after exclusion of the three E- patients who had curvilinear survival curves (n=7, r=0.79, p<0.05), as is shown in figure 2.2.

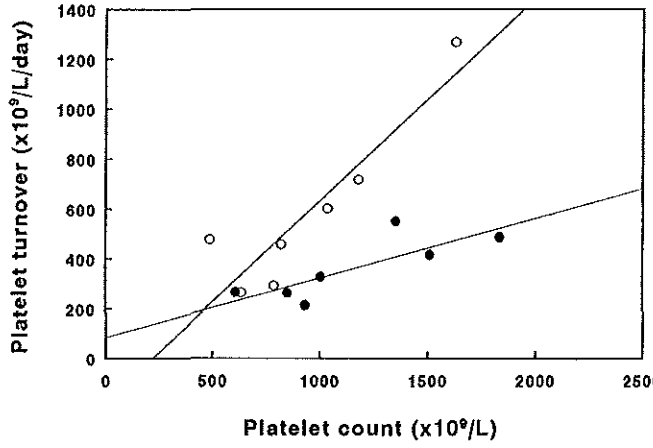

Figure 2.3

Relationships between platelet turnover and platelet counts of 7 thrombocythemia patients suffering from erythromelalgia who were studied before and after treatment of erythromelalgia with 500 mg of aspirin. (○) Thrombocythemia patients suffering from erythromelalgia studied before treatment with aspirin (n=7, r=0.91, p<0.05, linear regression line expressed by y=0.81x-181; (•) The same thrombocythemia patients studied after treatment of erythromelalgia with aspirin for at least two weeks (n=7, r= 0.81, p<0.05, linear regression line y=0.24x+84).

Effect of aspirin on platelet survival in erythromelalgia

In 7 E+ patients platelet kinetics were also studied during curative treatment of erythromelalgia with 500 mg aspirin per day for at least two weeks. The kinetic data are summarized in table 2.2. After aspirin therapy a significant increase of the platelet count of 216±30 x 10^9 platelets/L was noted ($p<0.001$). Treatment of erythromelalgia with aspirin did not influence the initial platelet recovery (44±3 % versus 47±3 %, $p>0.05$). As is depicted in figure 2.1B the mean platelet survival time dramatically increased from 4.0±0.3 days to 6.9±0.4 days ($p<0.001$), not significantly different from asymptomatic E- patients. After treatment of erythromelalgia with aspirin the mean platelet turnover per day decreased from 583±129 x 10^9/L/day to 361±48 x 10^9/L/day ($p<0.05$), the latter not being significantly different from asymptomatic thrombocythemia patients. Furthermore, after treatment of erythromelalgia with aspirin all individual platelet survival curves changed from a curvilinear to a linear disappearance pattern. In 2 of 7 E+ patients platelet kinetic studies were performed during persistence of erythromelalgic distress despite oral anticoagulation with coumadin. A normalization of the decreased platelet sur-

vival was obtained only after additional treatment with aspirin (figure 2.1B). The platelet turnover of the erythromelalgia patients before (ASA-) and after treatment of erythromelalgia with aspirin (ASA+) also showed a significant linear correlation with the matched platelet counts of these patients, as is shown in figure 2.3 (before aspirin: therapy n=7, r=0.91, $p<0.05$, regression line is expressed by y=0.81x-181; after aspirin: n=7, r =0.81, $p<0.05$, regression line y=0.24x+84). The relative increase in platelet turnover associated with erythromelalgia (ΔE) follows from the equation: $\Delta E=(0.81x_{ASA-}- 181)-(0.24x_{ASA+}+84)$, where x_{ASA-} denotes the platelet count before aspirin therapy and x_{ASA+} the platelet count after treatment of erythromelalgia with aspirin. Thus, the calculated increase in platelet turnover associated with erythromelalgia ("thrombotic mass") approximates 219±79 x 10^9/L/day, which accounts for 30±8 % of the total platelet turnover of thrombocythemia patients suffering from erythromelalgia.

DISCUSSION

In the present prospective study the survival time and turnover of ^{51}Cr labeled autologous platelets were systematically measured in both asymptomatic and symptomatic thrombocythemia patients and in control subjects with reactive thrombocytosis, in order to investigate the involvement of platelets in erythromelalgia, a frequent thrombotic complication of thrombocythemia [1]. It is demonstrated that in erythromelalgia the mean platelet survival time is shortened, indicating that radiolabeled platelets exhibit an accelerated removal from the circulation due to increased utilization [18] as is also evident from the increased platelet turnover per day. The relative increase in platelet turnover associated with erythromelalgia ("thrombotic mass") approximates 219±79 x 10^9/L/day, accounting for 30±8 % of the total platelet turnover in patients with erythromelalgia, and correlates well to the observed rise in the number of circulating platelets of 216±30 x 10^9/L after treatment of erythromelalgia with aspirin.

Inhibition of platelet cyclooxygenase activity and platelet aggregation by aspirin in thrombocythemia patients suffering from erythromelalgia also resulted in a complete relief of inflammation and pain coinciding with restoration of the acroischemic circulation disturbances, as has previously been described [1]. The decreased platelet survival time and increased platelet turnover were concordantly normalized in comparison with both patients with reactive thrombocytosis and asymptomatic thrombocythemia. Coumadin did not relieve erythromelalgia and failed to improve the decreased platelet survival in erythromelalgia, thus providing - besides the large body of clinical evidence [1-4] - also kinetic evidence for the inefficacy of coumadin derivatives in the treatment of erythromelalgia. These observations and the consistent rise

in circulating platelets after treatment of erythromelalgia with aspirin suggest that the consumption of platelets in erythromelalgia is abolished by inhibiting platelet aggregation. The finding that, after treatment of erythromelalgia with aspirin, all platelet survival curves changed from a curvilinear to a linear disappearance pattern, implying that platelet removal was largely by senescence [18], further supports this.

In the patients with reactive thrombocytosis this platelet kinetic study revealed a normal mean platelet survival time and linear platelet survival curves, consistent with the findings of others [6,7]. Although the platelet count and platelet turnover per day did not differ between patients with reactive thrombocytosis and patients with asymptomatic thrombocythemia, the mean platelet survival time in patients with asymptomatic thrombocythemia was decreased, the latter being consistent with the findings of Bautista and collegues [20]. The decrease in mean platelet survival time of asymptomatic thrombocythemia patients was attributed to three asymptomatic thrombocythemia patients who had curvilinear disappearance patterns. Exclusion of these patients resulted in a normal mean platelet survival time for the group of asymptomatic thrombocythemia patients as has been described by others [18]. The relative decrease in mean platelet survival time in these patients with curvilinear survival curves may be a reflection of the hyperaggregability of platelets in thrombocythemia [5], but, however, without giving rise to clinical signs of erythromelalgia or other thromboses.

In conclusion, this platelet kinetic study and the beneficial response to aspirin provide compelling evidence for an increase in platelet consumption in erythromelalgia which is attributed to the formation of platelet thrombi in the arterial microvasculature contributing to the signs and symptoms of erythromelalgia. However, the mechanism whereby some patients with thrombocythemia develop erythromelalgia under the prevailing high shear stress conditions of the end-arterial microvasculature remains to be determined. Elucidation of its mechanism will provide a valuable clue to the understanding of the arterial thrombotic predisposition in thrombocythemia as the thrombotic events in thrombocythemia are not confined to the acral microvasculature, but may also involve the cerebrovascular [21,22] and coronary arterial circulation [23].

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3

ERYTHROMELALGIA IN ESSENTIAL THROMBOCYTHEMIA IS CHARACTERIZED BY PLATELET ACTIVATION AND ENDOTHELIAL CELL DAMAGE BUT NOT BY THROMBIN GENERATION

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SUMMARY

Erythromelalgia, a characteristic aspirin-responsive microvascular thrombotic complication in essential thrombocythemia (ET), may develop despite oral anticoagulant treatment or treatment with heparin, suggesting that the generation of thrombin is not a prerequisite for its development. To study this, a cross-sectional comparison of the plasma levels of thrombomodulin (TM), platelet factor 4 (PF4), ß-thromboglobulin (ß-TG), prothrombin fragment 1+2 (F1+2) and total degradation products of fibrin(ogen) (TDP) was made between 5 ET patients suffering from erythromelalgia, 16 asymptomatic ET patients and 20 control subjects, and after treatment with aspirin, respectively. Furthermore, 2 ET patients with a history of erythromelalgia were studied at regular time intervals after discontinuation of aspirin until erythromelalgia recurred. As compared with asymptomatic ET patients and controls subjects erythromelalgia was characterized by significantly higher ß-TG and TM levels but no significant differences were detected in either F1+2 or TDP levels. Treatment of erythromelalgia with aspirin resulted in disappearance of erythromelalgic signs and symptoms, which was paralleled by a significant decrease of ß-TG and TM levels. Histopathologic and immunohistochemical analysis of biopsies derived from erythromelalgic skin areas of 2 ET patients showed that erythromelalgic thrombi stained positively for von Willebrand factor opposed to only a weak fibrin staining. Our data suggest that erythromelalgia is caused by the intravascular activation and aggregation of platelets with subsequent sludging or occlusion of the acral arterial microvasculature. The generation of thrombin appears not to be essential for the formation of these platelet thrombi, thereby giving a plausible explanantion for the inefficacy of coumadin derivatives and heparin in the prevention and treatment of erythromelalgia in essential thrombocythemia.

INTRODUCTION

Erythromelalgia, featured by red, congested extremities with painful burning sensations, is a characteristic complication in essential thrombocythemia (ET) [1-4]. Previous histopathological [4] and platelet kinetic studies [5] have incriminated a thrombotic occlusion and inflammation of the acral arterial microvasculature in the pathogenesis of erythromelalgia. In ET erythromelalgia may already develop at slightly increased platelet counts in excess of 400 x 10^9/L, but it is not observed in patients with reactive thrombocytosis, who have a comparable rise of the platelet count, suggesting that in ET hyperaggregable platelets give rise to erythromelalgia [1,3,6]. Indeed, inhibition of platelet function and aggregation by aspirin relieves erythromelalgia completely, restores the microvascular circulation disturbances and prevents recurrences of erythromelalgia [1,3,6]. Furthermore, cytoreduction of the increased

platelet count to normal values alleviates erythromelalgia [1,6] and recurrences of erythromelalgia usually coincide with relapses of thrombocythemia [1,6], suggesting that erythromelalgia and thrombocythemia are causally linked [3].

Interestingly, erythromelalgia may occur in ET patients despite oral anticoagulant therapy or treatment with heparin [1,3,6], suggesting that the generation of thrombin is not a prerequisite for the development of thrombi in erythromelalgia. In the present study we therefore characterized the hemostatic profile of erythromelalgia. To that end, we measured plasma ß-thromboglobulin and platelet factor 4, as markers for platelet activation; thrombomodulin as a marker for endothelial cell damage; and prothrombin fragment 1+2 and total degradation products of fibrin and fibrinogen as markers for activation of coagulation and fibrinolysis, respectively. In addition, because of the striking beneficial effect of aspirin on erythromelalgia, we also investigated the effect of intervention with aspirin on the aforementioned hemostatic parameters.

PATIENTS

Study population

Twenty-one consecutive patients with essential thrombocythemia (ET) entered the study after giving informed consent. The experimental protocol was approved by our Institutional Review Board. ET was diagnosed according to the criteria of the Polycythemia Vera Study Group [7]. Five ET patients suffered from erythromelalgia at the time of study (E+ group; 5 males, ranging in age from 45 to 68 years (median 65 yr)); the remaining 16 ET patients (E- group; 8 males, 8 females, ranging in age from 21 to 83 years (median 62 yr)) were asymptomatic. Erythromelalgia was diagnosed by its typical features of painful burning and red congested extremities, as has been described in detail elsewhere [1,3]. One E+ patient suffered from erythromelalgia while receiving adequate oral anticoagulation (Thrombotest™ INR 4.8). Twenty healthy volunteers, compatible for age and sex, served as control subjects (group N). No patient or control person suffered from hepatic or renal insufficiency which might influence the concentration of some biological parameters.

Design of the studies

In the first study, a cross-sectional comparison of the hemostatic parameters mentioned in the "Methods" section was made between 5 ET patients suffering from erythromelalgia, 16 asymptomatic ET patients and 20 control subjects. A second study was designed to evaluate the effect of intervention with 500 mg aspirin on the aforementioned hemostatic parameters. For that purpose, 5 ET patients suffering from erythromelalgia, 10 asymptomatic ET

patients and 10 healthy volunteers (these patients and controls had all participated in the cross-sectional study) were restudied after treatment with 500 mg aspirin for at least 7 days. In order to correlate hemostatic parameters with clinical symptoms of erythromelalgia a third study was performed, in which 2 ET patients with a history of erythromelalgia were studied at regular time intervals after discontinuation of aspirin until erythromelalgia recurred. Recurrences of erythromelalgia were treated with a platelet-specific aspirin regimen of 50 mg per day [8,9]. Finally, in a fourth study, skin punch biopsies were taken from 2 ET patients suffering from erythromelalgia to document erythromelalgia on a histopathologic and immunohistochemical level.

METHODS

Blood of each patient was collected between 8 and 10 am to avoid circadian variations. Blood was obtained by antecubital venipuncture and collected in 10 mL siliconized Vacutainer tubes (Becton Dickinson, New Jersey, USA), containing 1 mL of a 0.105 M buffered citrate solution and immediately placed on melting ice. Plasma was obtained by centrifugation (4 °C, 2500 x g, 10 minutes). Subsequently, the plasma was transferred to another tube and centrifuged for 15 minutes at 25000 x g at 4 °C to remove cellular components, and stored at -70 °C until analysis. For the measurement of plasma ß-thromboglobulin and platelet factor 4, blood was collected in special iced tubes (Diatube H, Diagnostica Stago, Asnières, France), containing citrate and the platelet antiaggregants theophylline, adenosine and dipyridamole, in order to prevent platelet activation *ex vivo*. Plasma was obtained after centrifugation at 2500 x g for 30 minutes at 4 °C. Platelets were counted in EDTA blood samples using the Platelet Analyzer 810 (Baker Instruments).

Platelet activation markers
Plasma ß-thromboglobulin (ß-TG) and platelet factor 4 (PF4) were measured using an enzyme-linked immunosorbent assay kit (Diagnostica Stago). A high platelet count itself may lead to elevated plasma ß-TG and PF4 levels [10]. To allow for between-groups comparisons the plasma ß-TG and PF4 values were divided by the whole blood platelet count, and expressed as the corrected ß-thromboglobulin value (ß-TG corr) and corrected platelet factor 4 value (PF4 corr), respectively.

Markers for activation of coagulation and fibrinolysis
Plasma prothrombin fragment 1+2 (F1+2) was measured by an enzyme-immunoassay (Enzygnost F1+2 micro, Behring, Marburg, Germany). Total degradation products of fibrin and fibrinogen (TDP) was determined in plasma with an ELISA (Fibrinostika TDP Microelisa system, Organon Teknika, Boxtel, The Netherlands).

Table 3.1 Hemostatic parameters in normal individuals (N), asymptomatic patients with essential thrombocythemia (E-) and essential thrombocythemia patients suffering from erythromelalgia (E+). Data are given as mean ± S.E.

	N (n=20)	E- (n=16)	E+ (n=5)	p-value	
Thrombomodulin (ng/mL)	39.8±2.1	72.5±4.0	90.2±10.2	N vs E- N vs E+ E- vs E+	p<0.001 p<0.001 p<0.05
Platelet count (x 10^9/L)	256±10	671±66	689±105	N vs E- N vs E+ E- vs E+	p<0.001 p<0.001 p>0.05
PF4 corr (IU/10^8 plts)	1.6±0.1	2.9±0.5	9.1±5.0	N vs E- N vs E+ E- vs E+	p>0.05 p<0.001 p<0.01
ß-TG corr (IU/10^8 plts)	15.6±1.2	36.7±6.6	127.9±33.4	N vs E- N vs E+ E- vs E+	p>0.05 p<0.001 p<0.001
F1+2 (nmol/L)	1.3±0.1	1.2±0.1	1.2±0.4	p>0.05	
TDP (ng/mL)	669±31	707±51	702±83	p>0.05	

Marker for endothelial cell damage
Thrombomodulin (TM) was measured with an ELISA kit (Asserachrom Thrombomodulin, Diagnostica Stago).

Immunohistochemical studies
Skin punch biopsies of 0.3 cm were fixed in 40 % formaldehyde, dehydrated in alcohol and embedded in paraplast for light microscopic immunohistochemical studies. To detect the presence of von Willebrand factor and fibrin in thrombi derived from erythromelalgic skin areas, sections of 2-4 µm were stained for von Willebrand factor with a streptavidin-biotin complex method using HRP conjugated antibodies to human von Willebrand factor (Dakopatts, Dako A/S, roskilde, Denmark). Staining for fibrin was performed as described by Lendrum [11].

Statistical analysis
Analysis of variance, followed by Student-Newman-Keuls (SNK-test) was used to identify pair-wise significant differences between normal individuals, asymptomatic ET patients and ET patients suffering from erythromelalgia. The paired t-test was used to analyse the effect of intervention with aspirin on the hemostatic parameters described in the "Methods" section. All data in the "Results" section are given as mean ± S.E.

RESULTS

Cross-sectional study and the effect of treatment with aspirin

The results of the cross-sectional study are shown in table 3.1. In comparison to asymptomatic ET patients and control subjects erythromelalgia is characterized by platelet hyperactivation and increased levels of endothelial cell damage markers. In contrast, there were no significant differences in either prothrombin fragment 1+2 levels or total degradation products of fibrin(ogen). A decrease of platelet activation and thrombomodulin levels was obtained by treatment of erythromelalgia with aspirin (table 3.2), which was accompanied by a significant rise of the platelet count, consistent with previous observations [5].

Follow-up after discontinuation of aspirin

Two ET patients, with a history of erythromelalgia, were studied after discontinuation of aspirin. In case 1 erythromelalgia developed in 2 fingers of the left hand 7 days after discontinuation of aspirin (data not shown). Erythromelalgia was preceded by "pins and needles" sensations from the third day on after cessation of ASA. The follow-up of case 2 is shown in detail in figure 3.1. This particular ET patient developed erythromelalgia of the left hallux and lateral edge of his left foot 3 days after discontinuation of ASA. Erythromelalgia was succesfully treated with 50 mg aspirin per day. A clear

Figure 3.1

Simultaneous study of clinical signs and symptoms of erythromelalgia, platelet activation (open symbols) and thrombin generation (closed symbols) in an ET patient with a history of erythromelalgia after discontinuation of aspirin. Three days after discontinuation this ET patient suffered from erythromelalgia of his left hallux and lateral foot edge, which was associated with prominent platelet activation but without excessive thrombin generation. Erythromelalgia was succesfully treated with a platelet-specific aspirin regimen of 50 mg per day. Treatment with 100 mg aspirin per day did even further decrease platelet activation.

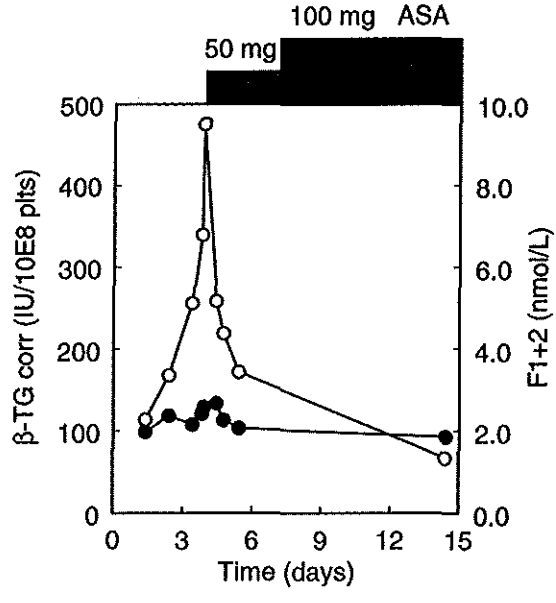

Table 3.2 Effect of intervention with aspirin on hemostatic parameters in normal individuals (N), asymptomatic thrombocythemia patients (E-) and essential thrombocythemia patients suffering from erythromelalgia (E+). Data are given as mean ± S.E.

	N (n=10)			E- (n=10)			E+ (n=5)		
	ASA-	ASA+	p-value	ASA-	ASA+	p-value	ASA-	ASA+	p-value
TM (ng/mL)	36.7±2.7	36.3±2.8	p>0.05	69.5±5.6	60.9±6.0	p>0.05	90.2±10.2	64.1±11.8	p<0.05
Platelet count (x 10^9/L)	237±11	250±13	p>0.05	689±77	794±71	p>0.05	689±105	857±52	p<0.05
PF4 (IU/10^8 plts)	1.8±0.2	1.2±0.1	p>0.05	3.3±0.8	3.4±1.1	p>0.05	9.1±5.0	4.3±3.3	p>0.05
ß-TG (IU/10^8 plts)	17.8±1.9	16.5±2.0	p>0.05	51.2±7.1	36.0±7.9	p<0.05	127.9±33.4	28.5±14.8	p<0.05
F1+2 (nmol/L)	1.2±0.1	1.2±0.1	p>0.05	1.2±0.1	1.1±0.1	p<0.01	1.2±0.4	1.1±0.3	p>0.05
TDP (ng/mL)	694±53	721±79	p>0.05	739±70	639±42	p>0.05	702±83	538±41	p<0.05

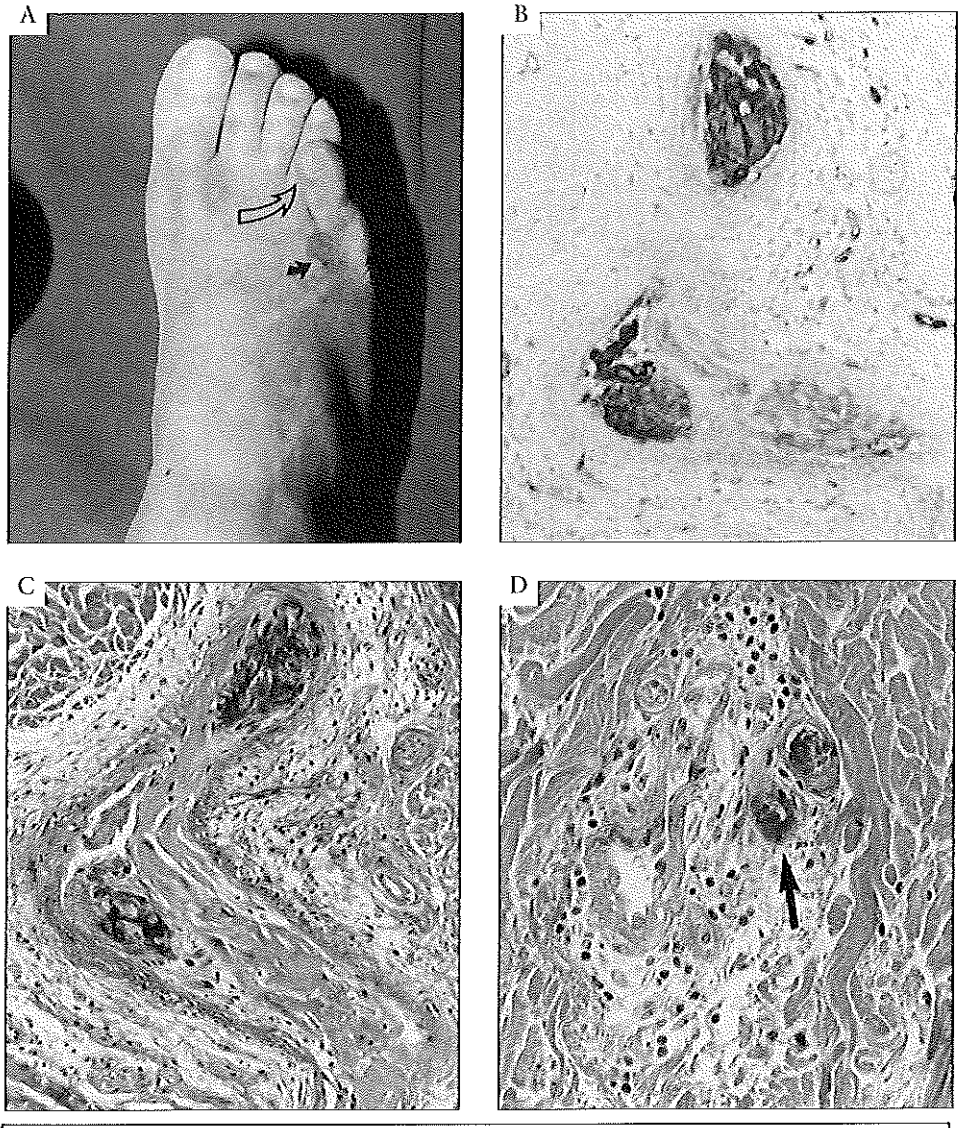

Figure 3.2

A. A typical example of erythromelalgia characterized by red-bluish discoloration of digit V and lateral edge of the right foot with mottled painful burning red spots on the dorsum of the right foot.
B. Biopsy taken from an erythromelalgic skin area showing arteriolar blood vessels in the skin occluded by thrombi revealing a strong staining for von Willebrand factor (magnification 280 x).
C. Fibrin staining of the same biopsy, showing a weak staining for fibrin (magnification 280 x)
D. Positive control fibrin staining, derived from a patient with a primary antiphospholipid syndrome with recurrent arterial thromboses (magnification 280 x).

relationship between erythromelalgia and platelet activation became evident, whereas no relevant changes in prothrombin F1+2 levels were noted (the laboratory data of patient 1 are comparable with those of patient 2). Thrombomodulin levels also decreased by treatment with 50 mg aspirin (data not shown). Increasing the ASA dose to 100 mg per day resulted in even further inhibition of platelet α-granule release, a finding also observed by Rocca and collegues [8].

Immunohistochemical studies

Skin punch biopsies were taken from erythromelalgic skin areas in two ET patients suffering from erythromelalgia, which showed the typical thrombotic occlusion of arterioles, as described in detail elsewhere [4]. A typical case of erythromelalgia is shown in figure 3.2A. Immunohistochemical studies of these biopsies revealed that thrombi in erythromelalgia stained strongly for von Willebrand factor (fig. 3.2B), opposed to only a weak staining for fibrin (fig. 3.2C). For comparison, a positive staining for fibrin, derived from a biopsy from a patient with a primary antiphospholipid syndrome with recurrent arterial thromboses, is shown in figure 3.2D.

DISCUSSION

Based on previous clinical studies we suggested that hyperaggregable platelets in ET play a pivotal role in the pathogenesis of erythromelalgia [1-6]. The present study further establishes the relationship between platelet activation and erythromelalgia by demonstrating that platelet activation markers (corrected for the platelet count) are significantly higher in patients suffering from erythromelalgia in comparison to asymptomatic ET patients and control subjects. Furthermore, disappearance of erythromelalgic symptoms, as is induced by treatment with 500 mg of aspirin, is paralleled by a decrease in platelet activation in association with a typical rise of the platelet count. We have previously shown that the rise of the platelet count after treatment of erythromelalgia with aspirin is due to inhibition of platelet consumption and a normalization of the survival time of platelets [5]. However, the most convincing evidence for the role of platelets in the pathogenesis of erythromelalgia is probably provided by the follow-up study of clinical and biochemical parameters of erythromelalgia after discontinuation of aspirin. Consistent with previous observations [1], at least 3 consecutive days without aspirin treatment are required for erythromelalgia to develop, which is associated with prominent platelet activation (figure 3.1). It seems conceivable that the observed time course between erythromelalgia and platelet activation after discontinuation of aspirin corresponds to the linear return of platelet cyclooxygenase activity in the systemic circulation as a consequence of renewal of the platelet population [8,9]. Moreover, erythromelalgia was even succesfully treated with a

platelet-selective aspirin regimen of 50 mg per day [8,9], which also points to a pivotal role of an intact platelet cyclooxygenase activity for the development of erythromelalgic signs and symptoms.

This study further demonstrates that erythromelalgia is associated with endothelial cell damage, as judged by elevated plasma thrombomodulin levels and its decrease after treatment of erythromelalgia with aspirin. Bellucci and collegues [12] concluded from their study in asymptomatic ET patients, that platelet activation does not neccessarily lead to endothelial cell damage, although thrombomodulin levels in their study tended to be increased in ET patients. In their study, however, 8 of 16 ET patients were studied while receiving platelet anti-aggregant treatment. Our data suggest that inhibition of platelet aggregation by treatment with ASA may result in a reduction of plasma thrombomodulin levels, probably accounting for the discrepant conclusions. Moreover, endothelial cell damage has been reported in several other clinical disorders associated with platelet activation such as disseminated intravascular coagulation and TTP [13], lupus erythematosus with antiphospholipid antibodies [14] and diabetic microangiopathy [15]. In the latter clinical disorder thrombomodulin levels are also reduced by treatment with ASA [16]. These observations and the observation that thrombomodulin levels in erythromelalgia are also reduced by a platelet-selective regimen of 50 mg of aspirin suggest that the endothelial damage in erythromelalgia is associated with platelet activation. However, thrombomodulin levels in both asymptomatic ET patients and ET patients suffering from erythromelalgia do not reach the normal range after treatment with aspirin (indicating some persistent endothelial damage), suggesting that an increased platelet count itself may lead to a rise of plasma thrombomodulin levels. Unfortunately, there are no data available on the effect of cytoreduction of an increased platelet count on thrombomodulin levels in ET.

Despite the limited population size of ET patients suffering from erythromelalgia, the present study provides several arguments that erythromelalgia is not predominated by systemic generation of thrombin. First, prothrombin fragment 1+2 levels in patients with erythromelalgia are not significantly elevated as compared with both normal individuals and asymptomatic ET patients. Second, one ET patient, who suffered from erythromelalgia despite oral anticoagulation, had a prothrombin fragment 1+2 level of only 0.3 nmol/mL, a value which is distinctly below the normal reference range. Third, in the follow-up study of 2 ET patients who developed erythromelalgia after discontinuation of aspirin, we did not observe a relevant rise in prothrombin fragment 1+2 levels. Four, erythromelalgia is associated with normal levels of total degradation products of both fibrin and fibrinogen, providing circumstantial evidence for the absence of excessive thrombin generation in erythromelalgia. The observed decrease of plasma prothrombin fragment 1+2 levels with resul-

tant decrease in TDP levels in the erythromelalgia patients after treatment with aspirin is more likely to be related to an effect of aspirin on thrombin generation, as has been reported [17,18], than being related to treatment of erythromelalgia, since a similar effect of aspirin was also noted in ET patients without symptoms.

Although speculative, our results suggest that erythromelalgia is caused by relatively unstable platelet thrombi because the thrombi lack the consolidating effect of thrombin-induced fibrin formation. This hypothesis, however, is supported by immunohistochemical studies of erythromelalgic thrombi, which showed a weak staining for fibrin opposed to a strong staining for von Willebrand factor, indicating a platelet-rich thrombus. Interestingly, similar immunohistochemical findings have been reported for thrombi derived from patients with thrombotic thrombocytopenic purpura (TTP) [19]. As in ET, the vaso-occlusive symptoms in TTP have been ascribed to intravascular platelet activation and clumping [20]. Interestingly, TTP patients may also suffer from erythromelalgia-like symptoms, which dramatically improve after treatment with ASA [21].

In conclusion, our data indicate that erythromelalgia is caused by the intravascular activation and aggregation of platelets with subsequent sludging or occlusion of the acral arterial microvasculature which may be reversed by treatment with aspirin. Thrombin generation appears not to be essential for the formation of the platelet thrombi in erythromelalgia, thereby giving a plausible explanation for the inefficacy of coumadin derivatives and heparin in the treatment and prevention of erythromelalgia in essential thrombocythemia.

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4

SPONTANEOUS PLATELET ACTIVATION IN VIVO AS A CAUSE OF PLATELET-MEDIATED ARTERIAL THROMBOSIS IN THROMBOCYTHEMIA

A rationale for the use of low-dose aspirin as an antithrombotic agent

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Manuscript submitted

SUMMARY

The clinical course of patients with polycythemia vera (PV) and essential thrombocythemia (ET) is frequently complicated by arterial thrombotic events. The pathogenesis is not clearly understood but usually attributed to abnormalities in platelet function. An increase in platelet thromboxane formation has been described in the majority of asymptomatic patients with thrombocythemia, probably reflecting spontaneous platelet activation *in vivo*. In the present study we investigated whether an increase in platelet thromboxane formation may actually lead to arterial microvascular thrombosis. In addition, we studied the effect of selective inhibition of platelet thromboxane formation on clinical outcome by reinstituting of low-dose aspirin. Six ET patients and 1 PV participated in this study. Within 10 days after withdrawal of aspirin, 3 patients developed arterial microvascular thrombosis of extremities (erythromelalgia), which was preceded by a 3 to 30-fold increase in urinary thromboxane excretion as compared with patients who remained asymptomatic. The increased urinary thromboxane excretion and clinical signs could be inhibited by a platelet-specific aspirin regimen of 50 mg per day without affecting vascular cyclooxygenase indicating that platelets were the source of the increased thromboxane generation. These data suggest that in symptomatic patients an enhanced formation of thromboxane by platelets, reflecting platelet activation *in vivo*, precedes the development of arterial microvascular thrombosis. These data provide a rationale for using low-dose aspirin as an antithrombotic agent in thrombocythemia.

INTRODUCTION

Up to 70% of the patients with the myeloproliferative disorders polycythemia vera (PV) and essential thrombocythemia (ET) may experience an arterial thrombotic complication, often in the absence of atherosclerotic vascular disease [1,2]. Although the thrombotic events may occur almost everywhere in the vascular bed, they frequently arise in the microvasculature of peripheral and cerebral arteries, leading to erythromelalgia [3] and transient ischemic attacks [4], respectively. Erythromelalgia is featured by red congestion and burning pain of toes and fingers and caused by platelet-mediated inflammation and thrombotic occlusions of the acral arterial microvasculature, which usually progresses to ischemic acrocyanosis or gangrenous necrosis if left untreated [3,5]. Neither oral anticoagulants nor heparin nor anti-platelet drugs which do not inhibit platelet cyclooxygenase (COX-1) are effective in the treatment of erythromelalgia [5]. In contrast, complete relief of pain and restoration of microvascular circulation disturbances may be obtained with drugs selectively inhibiting platelet COX-1 such as low-dose aspirin (ASA) and indomethacin [5,6], probably through prevention of platelet-dependent thromboxane formation.

The formation of thromboxane A_2, a potent vasoconstrictor and inducer of platelet aggregation, plays an important role in the amplification loop of platelet activation. In contrast to normal individuals, who lack the arterial thrombotic predisposition as is seen in patients with thrombocythemia, the majority of asymptomatic ET [7] and PV patients [8] have an increased biosynthesis of thromboxane, probably reflecting spontaneous platelet activation *in vivo*. Based on these observations we hypothesized that the thrombotic predisposition in thrombocythemia may be mediated by thromboxane-dependent routes of platelet activation. To examine this, a prospective study was designed linking the urinary excretion of thromboxane B_2 (TxB_2), a stable hydrolysis product of thromboxane A_2, in thrombocythemia patients after withdrawal of ASA, in temporal relation to clinical outcome, i.e. the occurrence of erythromelalgia. To determine whether the platelets were the major source for the increase in urinary thromboxane excretion in thrombocythemia, COX-1 of platelets from symptomatic and asymptomatic thrombocythemia patients was selectively inhibited by an ASA-regimen of 50 mg daily [6,9].

PATIENTS AND METHODS

Study population
Seven consecutive patients with thrombocythemia without overt atherosclerotic vascular disease participated in this pilot study, after giving informed consent. The experimental protocol was approved by our Institutional Review Board. The patient characteristics are shown in table 4.1. ET and PV were diagnosed according to established clinical and laboratory criteria [5].

Design of the studies
In the first part of the study, the development of arterial microvascular thrombosis of the extremities (erythromelalgia) after discontinuation of ASA was investigated in temporal relation to urinary TxB_2 excretion in 6 thrombocythemia patients (patients #1-#6). At entry, all patients were asymptomatic while receiving aspirin. If erythromelalgia did not occur within 7-10 days after withdrawal of ASA, treatment with ASA was reinstituted. In case erythromelalgia did develop, treatment with ASA was reinstituted as soon as the diagnosis was established on clinical grounds. Erythromelalgia was diagnosed by its typical features of painful burning and red congested extremities and confirmed histopathologically by skin punch biopsies, as described previously [3,5]. Asymptomatic thrombocythemia patients, i.e. patients who did not develop erythromelalgia after discontinuation of ASA, served as control subjects for those thrombocythemia patients who became symptomatic.

The purpose of the second part of the study, in which 7 patients participated

Table 4.1 General characteristics of thrombocythemia patients

Patient #	Sex/Age	Underlying disorder	Year of diagnosis	Previous thrombosis	Previous treatment regimen	Plasma creatinine (μmol/L)	Hemoglobin (mmol/L)	Packed Cell Volume (L/L)	Leukocytes ($x10^9$/L)	Platelets ($x10^9$/L)	Development of erythromelalgia after withdrawal of ASA
#1	M/60	ET	1988	TIAs	OAC, ASA	104	8.5	0.43	11.3	729	No
#2	F/43	ET	1994	-	ASA	48	8.2	0.38	4.2	742	No
#3	M/57	ET	1980	E	B, ASA	59	9.1	0.44	4.2	444	No
#4	M/70	ET	1992	DVT, TIAs, MI	OAC, ASA	98	10.2	0.50	10.2	628	Yes
#5	M/61	ET	1995	E	HU, ASA	92	9.5	0.45	12.6	941	Yes
#6	M/48	ET	1992	E	ASA	97	9.7	0.45	6.6	851	Yes
#7	F/71	PV	1996	DVT	OAC	57	10.6	0.59	46.8	542	#
Normal range						60-110	♀7.3-9.3 ♂8.2-10.2	♀0.35-0.45 ♂0.40-0.50	4.0-10.0	140-360	

Legend: F=female; M=male; PV=polycythemia vera; ET=essential thrombocythemia; DVT=deep vein thrombosis; TIAs=transient ischemic attacks; E=erythromelalgia; MI=myocardial infarction; OAC=oral anticoagulants; ASA=aspirin; B=busulphan; HU=hydroxyurea; # Patient 7 presented with erythromelalgia and participated only in the second study

(patient #7 presented with erythromelalgia despite adequate oral anticoagulation and participated only in the second part of the study), was two-fold: first, to evaluate the effect of inhibition of TxB_2 generation on erythromelalgia by different doses of ASA (50 mg - 100 mg - 150 mg per day) and second, to evaluate the relative contribution of blood platelets to enhanced urinary TxB_2excretion, by the administration of a platelet-specific ASA regimen of 50 mg per day for 7 days [9].

METHODS

Plasma creatinine, hemoglobin level, packed cell volume, leukocyte and platelet count were measured using routine procedures. All urine samples were collected in 8-hours portions during the first study and in 24-hours portions during the second study. They were frozen immediately and stored at -80°C until analysis. Plasma and urinary levels of creatinine were measured by Jaffe's method without deproteinization. TxB_2 levels were determined in serum and urine samples by radioimmunoassay without further purification steps, as described [10]. Extraction of TxB_2 from urinary samples was not performed to avoid the use of solvents which may result in destruction of cell membranes and in potential release of arachidonic acid. Antiserum against TxB_2 showed a 60% cross-reactivity against 2,3-dinor-TxB_2 [10]. In addition, we also measured urinary 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$), the stable hydrolysis product of prostacyclin, as an estimate of vascular cyclooxygenase activity. All data are given as mean±SEM.

RESULTS

The development of erythromelalgia after discontinuation of ASA in relation to urinary TxB_2 excretion

Three of 6 ET patients developed erythromelalgia 4, 5 and 8 days after discontinuation of ASA, respectively. A progressive increase in urinary TxB_2 excretion, preceding the development of arterial microvascular thrombosis of the extremities (erythromelalgia), was noted in these patients (figure 4.1). At time of clinical diagnosis of erythromelalgia, a 3 to 30-fold (mean 7794±3697 pg TxB_2/mg creatinine) increase in urinary TxB_2 levels was present (including patient #7 who presented with erythromelalgia) as compared with asymptomatic thrombocythemia patients. The urinary TxB_2 excretion in asymptomatic ET patients after discontinuation of ASA never exceeded 900 pg TxB_2 /mg creatinine (figure 4.1). The differences in urinary TxB_2 excretion between symptomatic and asymptomatic thrombocythemia patients cannot be explained by differences in platelet count since urinary TxB_2 excretion values remained substantially increased in erythromelalgia patients if corrected for platelet count (1163±583 vs 63±17 pg TxB_2/mg creatinine/10^{11} platelets for

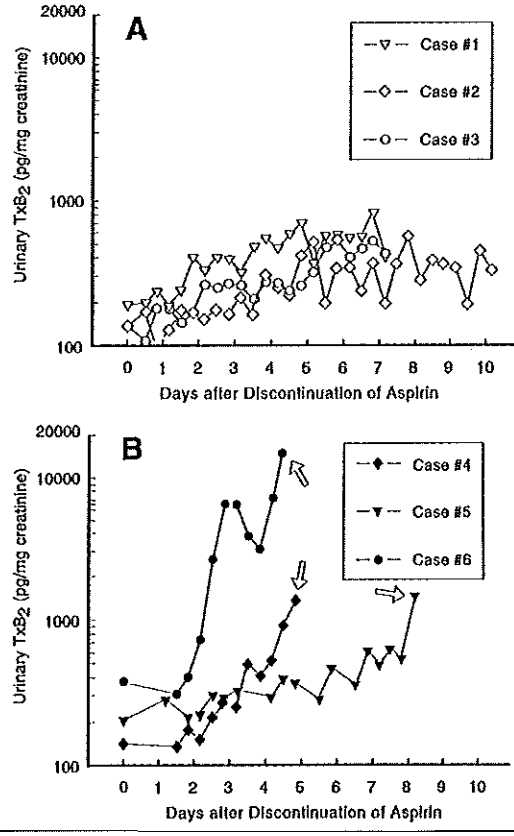

Figure 4.1

Temporal relationship between urinary TxB_2 excretion and development of erythromelalgia after discontinuation of aspirin (ASA). Figure 4.1A: Urinary TxB_2 excretion after discontinuation of ASA in asymptomatic patients. Figure 4.1B: development of erythromelalgia is associated with a progressive increase in urinary TxB_2 excretion. Arrows indicate clinical diagnosis of erythromelalgia and reinstitution of ASA.

symptomatic and asymptomatic patients, respectively). In addition, measurements of TxB_2 during whole blood clotting, which reflects the maximum capacity of platelets to generate thromboxane, made differences in biosynthetic capacity of platelets from symptomatic and asymptomatic thrombocythemia patients rather unlikely (data not shown).

Erythromelalgia was also associated with a 2-fold increase in urinary 6-oxo-$PGF_{1\alpha}$levels (mean 842 pg 6-oxo-$PGF_{1\alpha}$ /mg creatinine) as compared with

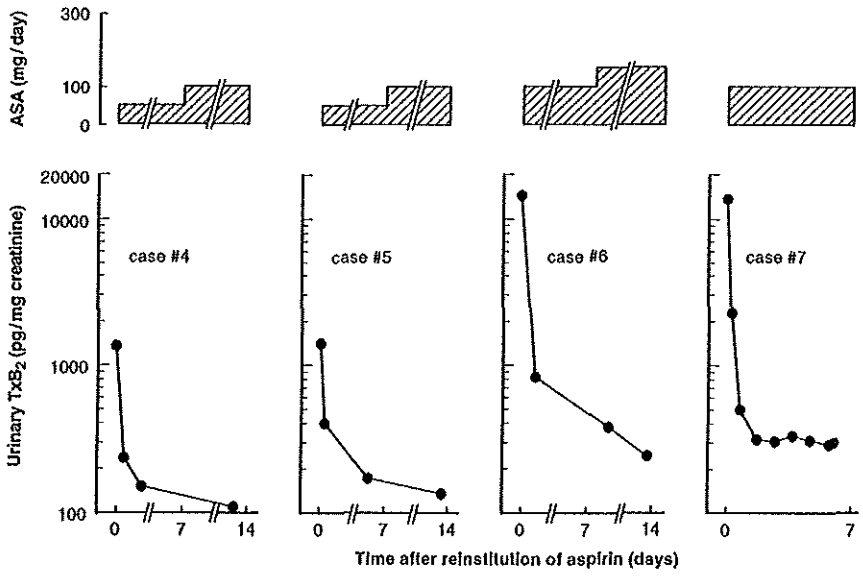

Figure 4.2

The effect of inhibition of urinary TxB_2 generation by different doses of ASA in patients suffering from erythromelalgia.

asymptomatic patients (mean 356 pg 6- oxo-$PGF_{1\alpha}$/mg creatinine), probably as a consequence of platelet hyperreactivity [11] or upregulation of vascular cyclo-oxygenase [12] since the increase in urinary 6-oxo-$PGF_{1\alpha}$ excretion associated with erythromelalgia was rapidly reversed by treatment of erythromelalgia.

Effect of inhibition of TxB_2 generation by ASA on erythromelalgia

Treatment with ASA doses as low as 50 mg/day for 7 days resulted in a rapid and profound decline in urinary TxB_2 excretion, in parallel with relief of erythromelalgic distress and microvascular circulation disturbances (figure 4.2). With higher ASA doses (100 mg/day and 150 mg/day for 7 days) even more inhibition of urinary TxB_2 excretion was observed, although the extent to which the urinary TxB_2 excretion was inhibited slightly varied between the different symptomatic patients (figure 4.2). Urinary 6-oxo-$PGF_{1\alpha}$ excretion in 3 asymptomatic thrombocythemia patients while receiving 0 mg, 50 mg ASA and 100 mg ASA per day for 7 days was 356±13, 387±34 and 362±14 pg/mg creatinine, respectively, indicating that these ASA doses did not affect vascular cyclooxygenase.

DISCUSSION

Previous clinical and laboratory studies indicated that erythromelalgia is caused by the intravascular activation and aggregation of platelets with subsequent sludging or transient occlusion of the arterial microcirculation of the extremities [3,5,13,14]. The results of present study are in support of a primary causautive role of platelets in the development of this thrombotic complication. First, thrombocythemia appears to be associated with a powerful thrombogenic drive as is illustrated by the observation that, in the absence of conventional risk factors for ischemic heart disease, half of the thrombocythemia patients did actually develop thrombosis of the arterial microcirculation of the acra, within only 4 to 8 days after discontinuation of ASA. Second, simultaneous collection of 24-hours urinary samples showed that the development of erythromelalgia was preceded by a progressive increase in urinary TxB_2 excretion. Third, this increase in urinary TxB_2 excretion occurred only in those patients who became symptomatic. Although urinary thromboxane metabolites do not necessarily reflect a specific site of eicosanoid biosynthesis, several arguments are consistent with a role for platelets as the major source of this enhanced thromboxane biosynthesis.

By exploiting the capacity of ASA to acetylate platelet COX-1 selectively when administered daily in low doses (i.e. 50 mg per day) [9,15] we were able to show a prominent inhibition of urinary TxB_2 excretion, with concurrent relief of erythromelalgic distress and acral circulation disturbances. Other studies showed that 50 mg ASA per day did not affect other sites of cyclooxygenase activity such as the kidney, that may be involved in enhanced thromboxane production under pathophysiologic circumstances [16]. Furthermore, at least 3 to 4 days without ASA treatment are required for erythromelalgia to develop [3,5,13]. The observed time course of recovery of thromboxane synthesis after withdrawal of ASA corresponds to the return of unacetylated platelet COX-1, with a normal capacity to synthesize thromboxane, in the systemic circulation as a consequence of renewal of the platelet population [7,13]. In addition, platelet survival studies showed that the shortened platelet survival time associated with erythromelalgia normalized after treatment with ASA in conjunction with relief of erythromelalgic distress and peripheral circulation disturbances, but not after treatment with coumadin derivatives [14].

Although the results of this study do not provide a specific clue to the trigger mechanisms for enhanced platelet activation *in vivo* in thrombocythemia, they suggest that *in vivo* the thrombotic drive in thrombocythemia patients is exerted by thromboxane-dependent routes of platelet activation eventually resulting in the formation of platelet-rich thrombi. The results of the present study provide a rationale for using low-dose ASA as an antithrombotic agent in thrombocythemia.

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5

ACQUIRED VON WILLEBRAND DISEASE AS A CAUSE OF RECURRENT MUCOCUTANEOUS BLEEDINGS IN ESSENTIAL THROMBOCYTHEMIA: RELATIONSHIP WITH PLATELET COUNT

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SUMMARY

We present a 4-year follow-up of a 42-year-old patient with essential thrombocythemia, whose clinical course was complicated by two major mucocutaneous bleeding episodes. On both occasions an acquired functional von Willebrand factor deficiency could be demonstrated. In contrast to what is reported in the literature, an inverse relationship between platelet number and plasma high-molecular-weight multimers of von Willebrand factor could be established.

INTRODUCTION

Essential thrombocythemia (ET), a clonal myeloproliferative disorder characterized by a persistent elevation of the platelet count, paradoxically predisposes to both thrombosis and hemorrhages [1,2]. Despite the identification of a broad array of specific structural, biochemical and metabolic platelet defects in patients with ET, a convincing correlation between these defects and the clinical complications of thrombosis and bleeding has never been established [1,2].

Several authors have described a decrease or absence of plasma high-molecular-weight multimers of von Willebrand factor (vWF) in patients with myeloproliferative disorders and associated bleeding tendency [3-5]. We present here a 4-year follow-up of a patient with ET whose clinical course was complicated by two bleeding episodes associated with an acquired von Willebrand factor deficiency. An inverse relationship between platelet number and plasma high-molecular-weight multimers of von Willebrand factor was demonstrated.

CASE REPORT

A 42-year-old woman was referred to our department in December 1989 with upper abdominal complaints due to massive splenomegaly. Ultrasound examination of the abdomen revealed an enlarged spleen of 20 cm and thrombosis of the splenic vein. Subsequent endoscopy showed esophageal varices. A slight thrombocytosis of 431 x 10^9/L was established (figure 5.1). Hemoglobin levels, white cell count and differentiation, creatinine, urea and liver function tests were all normal. Extended hemostatic analysis excluded the presence of a lupus anticoagulant antibody and a deficiency of antithrombin III, protein C and protein S. Bone marrow smear and biopsy material showed an increased cellularity of normal hematopoiesis, a pronounced increase of clustered megakaryocytes and the presence of fine reticulin fibers, but the absence of collagen fibers and osteosclerosis, compatible with the diagnosis ET [2]. The

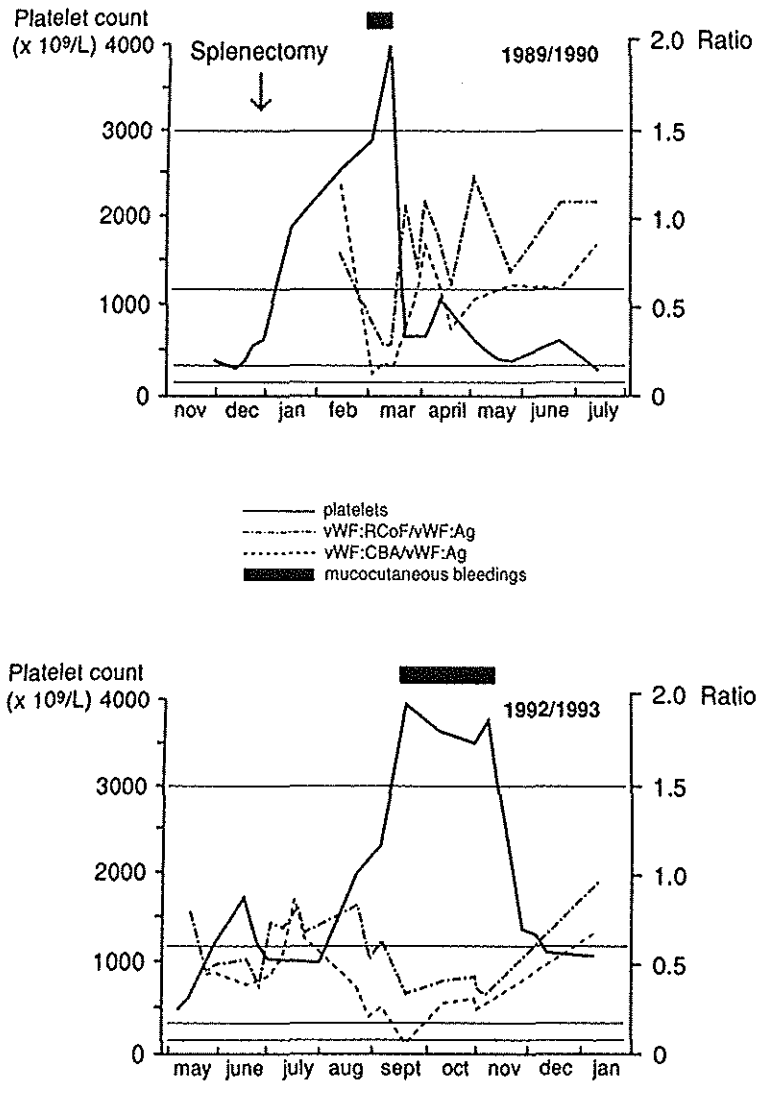

Figure 5.1

The clinical course of a patient with essential thrombocythemia was complicated by two mucocutaneous bleedings episodes at platelet counts above 2000 x 10⁹/L. On both occasions a functional von Willebrand factor deficiency could be demonstrated; this disappeared after reduction of the platelet count with hydroxyurea. Von Willebrand factor activities are expressed as the ratio of vWF:RCoF/vWF:Ag or vWF:CBA/vWF:Ag to denote the amount of functionally active von Willebrand factor. The upper two horizontal lines represent the normal range for the vWF:RCoF/vWF:Ag and vWF:CBA/vWF:Ag ratios. The lower two horizontal lines indicate the normal range for the platelet count.

score for the leukocyte alkaline phosphatase stain was 129 (normal 20-100 U/mL). Splenectomy in December 1989 (spleen weight 788 gram) was uneventful. In March 1990, the platelet count rose to about 3500 x 10^9/L; this was associated with spontaneous mucocutaneous bleeding consisting of easy bruising, recurrent ecchymoses after minor injuries, and melena, causing a fall of the hemoglobin level from 7.3 to 4.6 mmol/L. Extended coagulation tests revealed a functional deficiency of plasma von Willebrand factor [von Willebrand factor antigen (vWF:Ag) 1.36 U/mL; ristocetin cofactor activity (vWF:RCoF) 0.40 U/mL, and collagen binding activity (vWF:CBA) 0.22 U/mL, normal values for all vWF-related activities 0.60-1.40 U/mL] and a prolonged Ivy bleeding time (>15 min; normal ≤ 4 min). Platelet aggregation tests were normal.

From that time on we prospectively studied plasma von Willebrand factor activities in relation to the platelet count. Reduction of the platelet count with hydroxyurea resulted in disappearance of the bleeding symptoms and the functional von Willebrand factor defect and in a normalization of the Ivy bleeding time. At platelet counts between 700 and 1650 x 10^9/L for more than 1 year neither the bleeding nor the functional von Willebrand factor defect (vWF:Ag varied from 1.22 to 2.00 U/mL; vWF:RCoF from 0.64 to 1.72 U/mL and vWF:CBA from 0.67 to 1.26 U/mL) recurred.

Discontinuation of hydroxyurea because of side effects (headache and nausea) in August 1992 resulted in a rapid increase in circulating platelets (platelet counts 3700 to 4000 x 10^9/L) and recurrence of the mucocutaneous bleeding tendency, which was characterized by easy bruising, prolonged bleeding after minor cuts, and menorrhagia, causing a fall of the hemoglobin level from 7.0 to 3.7 mmol/L. Again, a functional von Willebrand factor defect (vWF:Ag 1.31 U/mL; vWF:RCoF 0.46 U/mL, and vWF:CBA 0.11 U/mL) and a prolongation of the bleeding time (> 15 min) were demonstrated. The bleeding symptoms and the functional von Willebrand factor defect disappeared after reduction of the platelet count with hydroxyurea. The hemoglobin level completely recovered with oral iron administration.

MATERIALS AND METHODS

All hemostatic tests were performed using routine procedures. Platelets were counted electronically in blood anticoagulated with EDTA. Bleeding times were measured according to Ivy [6]. Von Willebrand factor antigen was assayed by an ELISA using rabbit anti-human vWF and rabbit horseradish peroxidase-conjugated anti-human vWF polyclonal antibodies [7]. Ristocetin cofactor activity was assayed with formalin-fixed platelets [8] (intra-assay variation 10%; inter-assay variation 11%). The binding of vWF to collagen

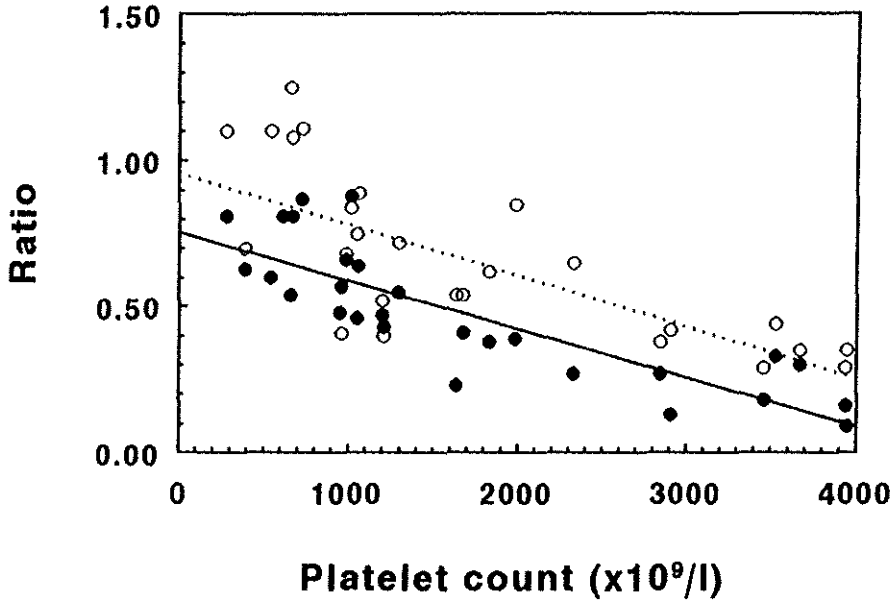

Figure 5.2

Intrapatient relationship between platelet count and either vWF:RCoF/vWF:Ag ratio (open symbols) or vWF:CBA/vWF:Ag ratio (closed symbols) and corresponding linear curve fits (broken and solid lines, respectively).

was measured according to the ELISA-based method of Brown and Bosak [9], with slight modifications (intra-assay variation 5%; inter-assay variation 9%). Microtiter plate wells (A/S Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 µL of a 0.2 mg/mL suspension of collagen (Bovine achilles tendon, type I, Sigma, St. Louis, USA) in 20 mM acetic acid. After washing with PBS-Tween, 100 µL of a 1/40 dilution of the test plasmas in PBS-Tween-albumin were added to the wells and incubated for 2 h at room temperature. After rinsing the wells were incubated with 1/200 dilution in PBS-Tween-albumin of horseradish peroxidase-conjugated rabbit polyclonal immunoglobulins to human vWF (Dakopatts, Denmark) for another 2 h at room temperature. After washing, 100 µL ABTS substrate solution was added. After 15 min incubation the color development was stopped by the addition of 10 µL concentrated acetic acid and the extinction was measured at 414 nm. The multimeric pattern of vWF was visualized according to Brosstad et al [10].

The relationship between platelet count and plasma levels of von Willebrand factor was analyzed by linear regression and Spearman's rank correlation test. Statistical significance was accepted at $p<0.05$.

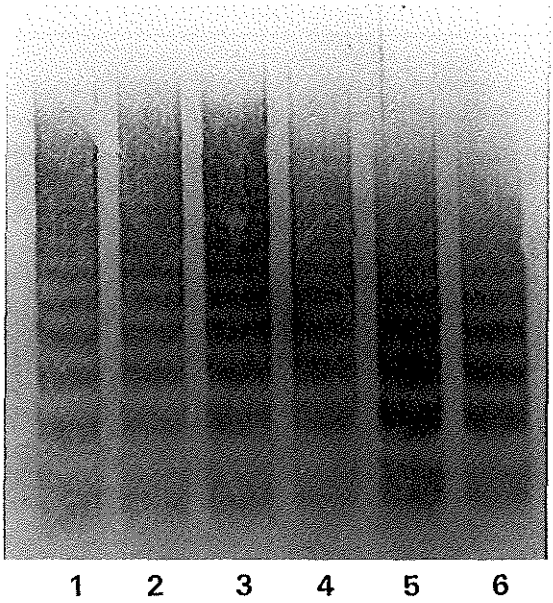

Figure 5.3

Multimeric pattern of plasma von Willebrand factor of normal pooled plasma and patient's plasma at different levels of thrombocytosis. Lane 1, normal pooled plasma; lanes 2-6, multimeric pattern of plasma vWF of the patient at platelet counts of 390 x 10^9/L, 727 x 10^9/L, 1058 x 10^9/L, 2905 x 10^9/L and 3938 x 10^9/L, respectively. Note the progressive decrease of the high-molecular-weight multimers of von Willebrand factor in the plasma of the patient with increasing platelet counts.

RESULTS

The clinical course of the described patient was complicated by two major bleeding events. On both occasions an acquired von Willebrand defect and a prolonged Ivy bleeding time could be demonstrated, occurring at platelet counts above 2000 x 10^9/L (figure 5.1). At times of manifest bleeding, the vWF:RCoF/vWF:Ag ratios and vWF:CBA/vWF:Ag ratios were decreased; 0.40±0.13 and 0.19±0.08, respectively, indicating a functional deficiency of vWF. Reduction of the platelet count with hydroxyurea resulted in disappearance of the bleeding symptoms as well as of the acquired functional von Willebrand defect (figure 5.1) and in a normalization of the Ivy bleeding time. A significant inverse relationship between the platelet count and the ratios of vWF:RCoF/vWF:Ag ($r=-0.73$, $p<0.001$) and vWF:CBA/vWF:Ag ($r=-0.83$, $p<0.001$) was demonstrated (figure 5.2). Analysis of the vWF multimers at different levels of thrombocytosis (figure 5.3) revealed a progressive decrease of the high-molecular-weight multimers of vWF with increasing platelet counts.

DISCUSSION

In the reported patients with ET and an acquired von Willebrand factor deficiency, bleedings usually became apparent at platelet counts in excess of 1000 x 10^9/L and disappeared or improved after reduction of the platelet count to below 1000 x 10^9/L, suggesting that platelets play a pivotal role in the etiology of such bleeding [3-5]. However, a clear relationship between platelet count and clinical complication of bleeding could never be established [1,2].

In the patient presented here an inverse relationship between platelet number and plasma vWF was demonstrated, resulting in a functional von Willebrand factor defect at platelet counts above 1000 x 10^9/L. With increasing platelet counts both the vWF:RCoF/vWF:Ag and vWF:CBA/vWF:Ag ratios progressively decreased, suggesting a deficiency of the high-molecular-weight multimers of vWF, the most potent forms of vWF in securing hemostasis. The decrease of high-molecular-weight multimers of vWF at platelet counts above 1000 x 10^9/L was also demonstrated by visualization of the multimeric distribution of vWF. However, clinical bleedings became manifest only at platelet counts in excess of 2000 x 10^9/L. Laboratory investigations at times of bleeding showed a deficiency of vWF:CBA activity, in contrast to only subnormal values of vWF:RCoF activity, suggesting that the vWF:CBA activity is a more reliable parameter for *in vivo* vWF function than the ristocetin co-factor activity.

This report and previous studies [3-5] indicate that cytoreduction of the platelet count below 1000 x 10^9/L favors prevention of bleeding complications in ET, which is consistent with recent epidemiological findings that hemorrhages in ET occur more frequently at platelet counts in excess of 1000 x 10^9/L [2,11-13]. However, in patients with reactive thrombocytosis hemorrhagic complications occur rarely or not at all, which suggests that more than a rise in the number of circulating platelets is needed to explain the observed hemorrhagic predisposition in essential thrombocythemia [1,2].

The pathogenesis of the von Willebrand factor deficiency in ET is unclear. It has been demonstrated that the deficiency of the high-molecular-weight multimers of vWF is a phenomenon occurring *in vivo* and is not due to proteolysis *in vitro* [4,14]. The observed increase in proteolytic cleavage fragments of the vWF subunit in ET might be attributed to *in vivo* degradation of von Willebrand factor by platelet-derived calcium-activated neutral proteases (calpains) and human leukocyte elastase [4]. However, others suggest that the decrease of the high-molecular-weight multimers of vWF is the result of binding of vWF to an increased number of circulating activated platelets at high platelet counts [14,15]. Although the exact mechanism operative in ET remains to be determined, our data imply that the decrease of the high-mole-

cular-weight multimers of von Willebrand factor is related to an elevated number of circulating platelets. Interestingly, Budde and collegues [16] have recently shown that the decrease of the high-molecular-weight multimers of von Willebrand factor is also demonstrable in the plasmas of patients with reactive thrombocytosis. Therefore, an elevated number of circulating platelets is more likely to be responsible for the observed decrease in high-molecular-weight multimers of von Willebrand factor than the presence of presumed functionally abnormal platelets derived from clonal proliferation. However, further studies in larger patient groups are indicated to clarify the correlation of vWF abnormalities and the occurrence of a bleeding tendency in the course of myeloproliferative disorders, as is evident from the presented case, whereas such a correlation has not been documented in patients with reactive thrombocytosis.

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6

THE REDUCTION OF LARGE VON WILLEBRAND FACTOR MULTIMERS IN PLASMA IN ESSENTIAL THROMBOCYTHEMIA IS RELATED TO THE PLATELET COUNT

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SUMMARY

We have investigated the relationship between platelet count and large vWF (von Willebrand factor) multimers in plasma in 36 patients with essential thrombocythemia (ET) and 26 patients with reactive thrombocytosis (RT). In both ET and RT patients an inverse relationship could be established between platelet count and large vWF multimers in plasma as well in relatively decreased ristocetin cofactor/von Willebrand factor antigen and collagen binding activity/von Willebrand factor antigen ratios. A normalization of the platelet count was accompanied by restoration of a normal plasma vWF multimeric distribution. Our data suggest that increasing numbers of platelets circulating in blood result in increased removal of large vWF multimers from plasma.

INTRODUCTION

Several authors have incriminated an acquired decrease or absence of large von Willebrand factor (vWF) multimers in plasma as a potential cause of bleeding in patients with myeloproliferative disorders (MPDs) associated with high platelet and/or leukocyte counts [1,2]. Recently, we have reported a patient with essential thrombocythemia (ET), in whom the decrease of large vWF multimers was persistently related to the number of platelets circulating in blood, actually resulting in mucocutaneous bleedings at platelet counts > 2000 x 10^9/L [3]. This observation suggested that an increase in the number of platelets circulating in blood may be a risk factor for bleeding in ET. In the present study we have further explored the relationship between platelet count and large vWF multimers in plasma in 36 patients with ET. Furthermore, to investigate whether the decrease of large vWF multimers in plasma with increasing platelet counts is a phenomenon restricted to MPDs, we have also studied 26 patients with reactive thrombocytosis (RT).

PATIENTS

The protocol was approved by the local ethics committee. After informed consent, 36 patients with ET and 26 patients with RT were included in the study from January 1992 to October 1994. The diagnosis ET was based on established criteria [4]. One ET patient was splenectomized. All 26 RT patients (4 carcinoma, 1 pyelonephritis, 2 iron-deficient anemia, 2 psoriasis, 1 arteriitis temporalis, 1 pancreatitis, 1 abdominal abcess, 1 Behçet's disease, 2 Crohn's disease, 1 amyloidosis and 10 patients following splenectomy [due to carcinoma in 3, idiopathic thrombocytopenia in 2 and abdominal trauma in 5] had a normal or almost normal platelet count after 6 months follow-up. At the time

of blood sampling 25 of 36 ET patients and 6 of 26 RT patients were treated with aspirin; 2 ET patients were studied while receiving hydroxyurea. At time of study 2 ET patients had bleeding symptoms, at platelet counts of 1137 x 10^9/L and 3120 x 10^9/L, respectively.

METHODS

Platelet and leukocyte counts were obtained routinely in whole blood anticoagulated with EDTA. For all other studies, blood was collected in one-tenth final volume of 3.8% trisodium citrate solution containing aprotinin, EDTA and N-ethylmaleimide to give final concentrations of 500 KIU/mL, 5 mmol/L and 6 mmol/L, respectively. Plasma von Willebrand factor antigen (vWF:Ag), ristocetin cofactor activity (vWF:RCoF) and the ability of plasma vWF to bind to bovine collagen type I (vWF:CBA) were assayed, as described in detail elsewhere [5]. Multimeric analysis of plasma vWF and the quantification of plasma large vWF multimers were performed as described [6].

STATISTICS

Mann-Whitney-U test was applied for comparison of ET and RT group medians. Correlations were studied using Spearman rank order tests. Fisher's exact test was applied to determine differences in sex distribution between ET and RT groups. Slope differences of regression lines for ET and RT group were tested for significance using an appropriate Student test. Statistical significance was accepted at $p<0.05$.

RESULTS

The general patient characteristics and haematological data are shown in table 6.1. For both ET and RT groups no significant differences in vWF-related parameters were found between patients receiving aspirin or not (data not shown). No significant differences were observed in age, sex distribution and platelet count between ET and RT patients (table 6.1). RT patients, however, had significantly higher leukocyte counts and plasma vWF:Ag, vWF:RCoF and vWF:CBA levels than ET patients. A decreased vWF:RCoF activity was observed in 10 (28%) ET and 2 (8%) RT patients, whereas the vWF:CBA was decreased in 17 (47%) ET and 6 (23%) RT patients, respectively. Quantification of the large vWF multimers revealed deficient values in 12 (33%) ET and 8 (31%) RT patients, respectively. As the large vWF multimers are essential for vWF function, we evaluated whether the decrease of large vWF multimers in plasma was also reflected in a relatively decreased

Table 6.1 Patient characteristics and hematological data				
	Normal range	ET	RT	p-value
No. of patients		36	26	
Age (yr)		63 (21-86) [55.2-64.7]	57.5 (23-88) [45.5-61.0]	p>0.05
Sex		19 M; 17 F	17 M; 9 F	p>0.05
Platelets (x10⁹/L)	130-350	750 (401-3120) [657-999]	746 (401-2329) [774-1116]	p>0.05
Leukocytes (x10⁹/L)	4.0-10.0	8.6 (4.2-15.8) [6.8-10.0]	13.0 (8.7-23.8) [11.8-16.4]	P<0.005
vWF:Ag (U/mL)	0.60-1.40	1.10 (0.67-3.26) [1.03-1.40]	2.19 (0.57-4.86) [1.79-2.61]	p<0.0001
vWF:RCoF (U/mL)	0.60-1.40	0.92 (0.10-1.61) [0.76-1.03]	1.88 (0.38-3.86) [1.39-2.15]	p<0.0005
vWF:CBA (U/mL)	0.60-1.40	0.61 (0.16-1.94) [0.60-0.84]	1.28 (0.09-2.79) [1.02-1.66]	P<0.005
RCoF/Ag ratio	0.60-1.50	0.75 (0.11-1.30) [0.66-0.84]	0.85 (0.18-1.26) [0.72-0.90]	p>0.05
CBA/Ag ratio	0.60-1.50	0.60 (0.16-1.24) [0.53-0.70]	0.61 (0.03-1.07) [0.50-0.71]	p>0.05
Large vWF multimers (%)	15.0-25.0	16.4 (0.8-26.1) [14.2-17.8]	20.6 (4.6-29.7) [15.4-21.0]	p>0.05

Data are represented as median (range) [95% CI]. Abbreviations: ET = essential thrombocythemia; RT = reactive thrombocytosis; M = male; F = female; vWF:Ag = von Willebrand factor antigen; vWF:RCoF = ristocetin cofactor activity; vWF:CBA = collagen binding activity.

vWF:RCoF and vWF:CBA activity of plasma vWF. To that end, the function of vWF was expressed as either the vWF:RCoF/vWF:Ag ratio or as the vWF:CBA/vWF:Ag ratio, respectively. As might be anticipated, the percentage of large vWF multimers in plasma showed a significant correlation with the vWF:RCoF/vWF:Ag ratios (ET: r=0.65, p=0.0001; RT: r=0.60, p<0.05) and with the vWF:CBA/vWF:Ag ratio (ET: r=0.49, p<0.05; RT: r=0.68, p<0.005) in both patient groups. Multimeric analysis of plasma vWF showed a decrease of large vWF multimers at high platelet counts, which improved after normalization of the platelet count (figure 6.1). The decrease in percentage of large vWF multimers in plasma correlated well with the platelet count in both ET patients (Figure 6.2A, r=-0.73, p<0.0001) and RT patients (Figure 6.2A, r=-0.67, p<0.01). In both ET and RT patients the vWF:RCoF/vWF:Ag ratio decreased with increasing platelet counts (Figure 6.2B, ET: r=-0.65, p<0.0005; RT: r=-0.63, p<0.001), as did the vWF:CBA/vVWF:Ag ratio (Figure 6.2C, ET: r=-0.52, p<0.0005; RT: r=-0.68, p<0.0001). The slopes of the corresponding

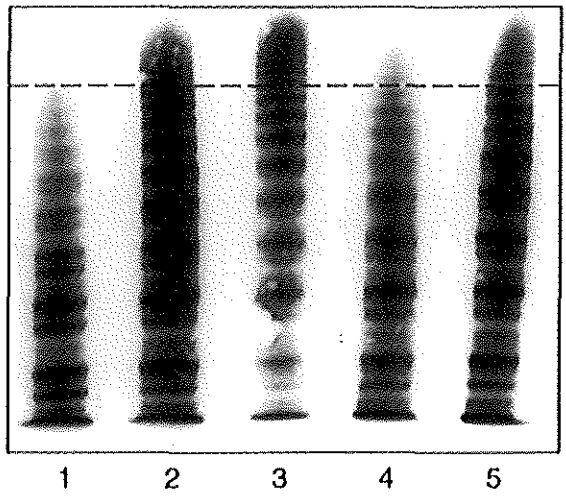

Figure 6.1

Representative multimeric distributions of plasma vWF in patients with ET and RT at various platelet counts and after normalization of the platelet count. Lanes 1 and 2: ET patient before (platelet count 3201 x 10^9/L) and after reduction of the increased platelet count with hydroxyurea (platelet count 666 x 10^9/L). Lane 3: normal pooled plasma. Lanes 4 and 5: RT patient after (platelet count 1480 x 10^9/L) and before splenectomy (platelet count 429 x 10^9/L), respectively. Dotted line across the gel indicates the position of the tenth multimer. Large vWF multimers were arbitrarily defined as those above the tenth multimer.

regression lines of ET and RT patients did not differ significantly in case of both the vWF:RCoF/vWF:Ag ratio (Figure 6.2B, p=0.90) and the vWF:CBA/vWF:Ag ratio (Figure 6.2C, p=0.14). No significant correlation was observed between the leukocyte count and the percentage of large vWF multimers in the plasmas of either ET patients (r=-0.34, p>0.05) or RT patients (r=-0.08, p>0.05).

DISCUSSION

The present study demonstrates that clinical conditions with an increased platelet count are associated with qualitative abnormalities of plasma vWF, particularly at platelet counts above 1000 x 10^9/L. The reduction of plasma large vWF multimers, which is also reflected in relatively decreased vWF:RCoF and vWF:CBA activities as compared with vWF:Ag, is directly related to the platelet count, but not to the leukocyte count, consistent with previous observations [3,6]. Furthermore, the vWF abnormalities are observed in the plasmas of both patients with ET and RT patients, even in non-splenec-

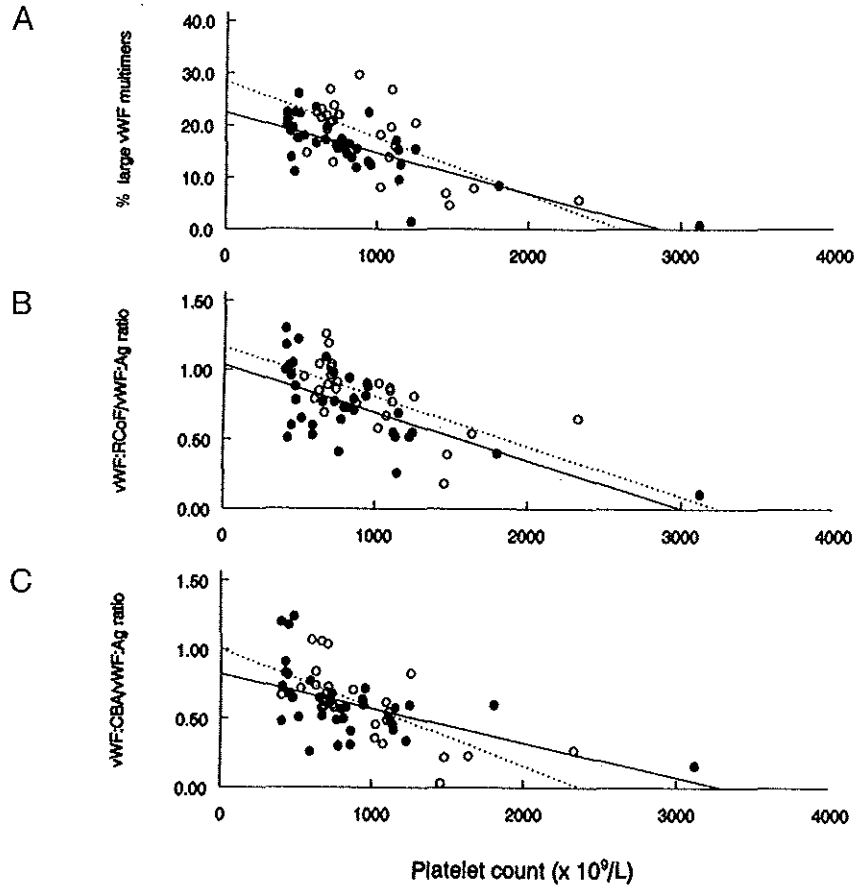

Figure 6.2

Relationship between platelet count and either large vWF multimers in plasma (Figure 6.2A), vWF:RCoF/vWF:Ag ratio (Figure 6.2B) or vWF:CBA/vWF:Ag ratio (Figure 6.2C) in 36 patients with essential thrombocythemia (closed symbols) and 26 patients with reactive thrombocytosis (open symbols). The solid and broken lines in each graph represent the linear regression lines of ET and RT patients, respectively.

tomized RT individuals. This suggests that an increased number of circulating platelets in blood, irrespective of the underlying clinical disorder, may lead to a reduction of large vWF multimers in plasma. This finding is supported by the observation that the multimeric pattern of plasma vWF is improved after normalization of the platelet count [1-3,6]. Moreover, the relationship between the number of platelets circulating in blood and plasma vWF is further stressed by the observation that patients with thrombocytopenia have increased plasma factor VIII/vWF levels [7].

However, our findings contrast with the findings of Fabris and collegues [2], who did not observe a relationship between platelet count and decrease of large vWF multimers in 13 ET patients and 3 RT patients. The discrepant results between their study and ours are not immediately apparent, but might be due to differences in (arbitrary) definition of large vWF multimers, to methodological differences in visualization and/or quantification of large vWF multimers or to the heterogeneity of the patient populations studied.

Although the exact mechanism causing this reduction of large vWF multimers in plasma in high platelet count states remains to be elucidated, our data are in line with several recent epidemiological studies in ET showing an increased frequency of bleedings when the platelet count exceeds the level of 1000 x 10^9/L [8-10]. Whether a reduction of large vWF multimers in plasma is a determinant factor for bleeding in ET, however, needs to be established in appropriate clinical trials.

The observation that large vWF multimers are reduced in patients with an increased platelet count may also have clinical implications for the use of platelet anti-aggregants. Since aspirin is frequently administered to patients with MPDs to prevent thrombosis [4,11], particularly when the platelet count is increased, bleedings may be precipitated when concurrent plasma vWF abnormalities are present. Actually, excessive prolongation of the bleeding time [12] and even severe bleedings [1,13] have been observed after administering aspirin to MPD patients with high platelet counts.

Patients with RT and high platelet counts may also have reduced levels of large vWF multimers in plasma. However, the clinical course of RT patients is generally not complicated by bleedings [4]. This may be explained by several factors. First, patients with RT have significantly higher plasma vWF levels than ET patients due to the behavior of vWF as a reactive protein. Although the large vWF multimers in plasma may be relatively decreased in RT patients, their absolute concentration is probably sufficient to secure normal hemostasis. Second, the elevation of the platelet count in RT patients lasts only for a limited period of time and the abnormalities of plasma vWF are reversible with a normalization of the platelet count. Third, in contrast to patients with ET where abnormalities of platelet function and aggregation are frequently encountered, it is generally assumed that platelet function is normal in RT [4].

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7

DECREASED HALF-LIFE TIME OF PLASMA VON WILLEBRAND FACTOR COLLAGEN BINDING ACTIVITY IN ESSENTIAL THROMBOCYTHEMIA: NORMALIZATION AFTER CYTOREDUCTION OF THE INCREASED PLATELET COUNT

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SUMMARY

Patients with essential thrombocythemia (ET) exhibit a decrease of large von Willebrand factor multimers in plasma, which is inversely related to the platelet count. In the present study we investigated whether the decrease of large vWF multimers in plasma with increasing platelet counts is the consequence of increased turnover of large vWF multimers *in vivo*. To that end we measured the half-life times of endogeneously released vWF:Ag and vWF:CBA (collagen binding activity) after intravenous administration of DDAVP to 9 ET patients and 9 control subjects (N). In addition, the half-life times of vWF:Ag and vWF:CBA were also measured in 4 ET patients after cytoreduction of the increased platelet count to normal or nearly normal values. Estimated half-life times of vWF:Ag did not differ between ET patients and normals (11.0±4.0 h vs 12.4±2.5 h, p>.05). Estimated half-life times of vWF:CBA were significantly lower in ET patients as compared with normal individuals (6.1±2.0 h vs 8.4±2.5 h, p<.05). After cytoreduction of the increased platelet count to (nearly) normal values in all 4 ET patients the half-life time of vWF:CBA significantly (p=.014) increased from 5.2±1.2 h to 8.7±2.0 h. Our data suggest that platelets may play a role in the homeostasis of circulating von Willebrand factor, which may compromise normal hemostasis at fairly increased platelet counts.

INTRODUCTION

Essential thrombocythemia (ET) is a myeloproliferative disorder characterized by a persistent increase in the number of circulating platelets in blood and a paradoxical predisposition to both thrombotic and bleeding complications [1]. Several studies have shown that bleedings occur more frequently at platelet counts exceeding 1000 x 10^9/L [2-5]. In previous studies [4,6,7] it was demonstrated that high platelet count states are associated with a decrease of large von Willebrand factor (vWF) multimers in plasma, which show an inverse relationship with the increased platelet count. The decrease of large vWF multimers in plasma becomes particularly apparent at platelet counts exceeding 1000 x 10^9/L [6,7] and may eventually lead to spontaneous bleeding tendency due to an acquired von Willebrand factor deficiency at even higher platelet counts [4]. After reduction of the increased platelet count large vWF multimers reappear in plasma [6-9], suggesting that an increase in circulating platelets in blood results in increased removal of large vWF multimers from plasma. To test this, we estimated the half-life times of endogenously released vWF in plasma after a single intravenous dose of DDAVP in 9 ET patients with an increased platelet count and in 9 control subjects. In 4 ET patients the DDAVP infusion study was repeated after reduction of the increased platelet count with hydroxyurea. Since large vWF multimers in plasma bind preferen-

tially to collagen [10] and their function can reliably be measured *in vitro* with the collagen binding assay [4,7,11,12], we were particularly interested in the half-life time of the collagen binding activity of von Willebrand factor in plasma in patients with an increased platelet count and the effect of cytoreduction on its half-life time.

PATIENTS AND METHODS

Patients

The experimental protocol was approved by our Institutional Review Board. After giving informed consent, 9 asymptomatic ET patients (5 males, 4 females; ranging in age from 22 to 73 years (mean 51 years) entered the study. The diagnosis of ET was based on established criteria [1]. Nine healthy volunteers served as control subjects. No patient or control subject suffered from hepatic or renal insufficiency or had a contraindication for administration of DDAVP. All patients and controls were treated with 100 mg aspirin for 3 days before DDAVP infusion. Four ET patients were restudied within several weeks after cytoreduction of the increased platelet count with hydroxyurea.

Methods

DDAVP (Minrin®, Ferring, Hoofddorp, The Netherlands) (4 μg/mL) was dissolved in sterile saline to a total volume of 50 mL and infused intravenously over a 30 min period at a dosage of 0.4 μg/kg. In the majority of ET patients and control subjects infusion of DDAVP resulted in facial flushing and a slight decrease in systolic and diastolic blood pressure (< 20 mm Hg), which was associated with a slight increase in heart rate. No other side effects, in particular no significant hemodilution (as evidenced by serial measurements of packed cell volume), were noted.

Blood samples were obtained before and immediately, 1, 2, 3, 4 and 6 h after DDAVP infusion. Hemoglobin levels, packed cell volume (hematocrit), leukocyte and platelet counts were obtained routinely in whole blood anticoagulated with EDTA. Plasma von Willebrand factor antigen (vWF:Ag) and the ability of plasma vWF to bind to bovine collagen type I (vWF:CBA) were assayed in citrated plasma containing aprotinin, EDTA, and N-ethylmaleimide in a final concentrations of 500 kIU/mL, 5 mmol/L and 6 mmol/L, respectively, as described in detail elsewhere [4]. In previous studies a highly significant correlation between percentage plasma large vWF multimers and plasma vWF:CBA/vWF:Ag ratio was observed [4,7], as well as with vWF:RCoF/vWF:Ag ratios. For this study we preferred to use the collagen binding assay over the more traditionally used ristocetin cofactor assay to analyze vWF function since the collagen binding assay is ELISA-based with a lower coefficient of variation in direct comparison with the ristocetin cofactor

Table 7.1 General characteristics of ET patients and control subjects before DDAVP infusion. Data are given as median (range) [95% CI]

	ET (n=9)	N (n=9)	P-value
Hemoglobin (mmol/L)	8.6 (6.9 - 10.0) [7.8 - 9.5]	9.1 (7.2 - 10.1) [8.4 - 9.6]	p>0.05
Packed cell volume (L/L)	0.42 (0.36 - 0.48) [0.39 - 0.45]	0.41 (0.32 - 0.46) [0.38 - 0.44]	p>0.05
Leukocytes (x 10^9/L)	9.4 (4.0 - 27.5) [4.9 - 17.3]	6.0 (4.4 - 10.9) [5.0 - 8.0]	p>0.05
Platelets (x 10^9/L)	817 (386 - 1057) [641 - 958]	208 (158 - 379) [184 - 289]	p<0.0001
vWF:Ag (U/mL)	1.06 (0.59 - 2.13) [0.74 - 1.45]	1.16 (0.66 - 1.68) [0.91 - 1.46]	p>0.05
vWF:CBA (U/mL)	0.76 (0.34 - 1.84) [0.51 - 1.28]	1.52 (0.59 - 2.04) [0.98 - 1.77]	p>0.05
vWF:CBA/vWF:Ag ratio	0.82 (0.54 - 1.19) [0.62 - 0.94]	1.19 (0.88 - 1.31) [1.01 - 1.25]	p<0.001

assay [4] and its apparently higher sensitivity in the laboratory monitoring of DDAVP therapy [11].

Post-DDAVP infusion values were expressed as a percentage of pre-infusion values, which were arbitrarily set at 100%. Plasmin-α2-antiplasmin (PAP) complexes and total degradation products of both fibrin and fibrinogen (TDP) were measured in citrated plasma with commercially available kits from Behring (Enzygnost PAP® micro, Behringwerke, Marburg, Germany) and Organon (Fibrinostika® TDP Microelisa system, Organon Teknika, Boxtel, The Netherlands), respectively.

Half-life times of plasma vWF:Ag and plasma vWF:CBA were calculated with the formula [13]:

$$C(t) = C_0 \, e^{-k.t}$$

where C(t) = concentration of vWF in plasma as a function of time
C_0 = concentration of vWF at time zero
e = base for natural logaritms
k = first-order rate constant for the elimination phase (ß phase)
t = time

Table 7.2 Kinetic studies of vWF:Ag and vWF:CBA in ET patients and normal subjects. Data are given as median (range) [95% CI].

	ET patients (n=9)	Normal subjects (n=9)	P-value
Platelets (x 10^9/L)	817 (386 - 1057) [641 - 958]	208 (158 - 379) [184 - 289]	p<0.0001
peak vWF:Ag* (%)	275 (160 - 463) [205 - 365]	287 (248 - 495) [260 - 403]	p>0.05
peak vWF:CBA* (%)	397 (175 - 812) [265 - 595]	507 (276 - 840) [350 - 628]	p>0.05
$T_{1/2}$ vWF:Ag (hours)	10.8 (5.5 - 16.9) [7.9 - 14.1]	12.5 (8.7 - 15.5) [10.5 - 14.3]	p>0.05
$T_{1/2}$ vWF:CBA (hours)	5.1 (4.2 - 9.1) [4.6 - 7.7]	8.6 (5.1 - 12.3) [6.5 - 10.4]	p<0.05

Legend:*=peak vWF:Ag and vWF:CBA levels are expressed as a percentage of pre-DDAVP treatment value.

Since vWF follows first-order kinetics after being released in the circulation by DDAVP [14], the half-life time (t) equals ln2 / k. For practical reasons, half-life times of plasma vWF:Ag and plasma vWF:CBA were estimated with a curve fit, constructed with at least 4 time points within 1 h of DDAVP infusion. Given the available evidence in the literature where peak vWF levels are normally reached between 30 and 60 min after DDAVP infusion [15], a distribution phase (α phase) of 1 h seemed appropiate.

STATISTICS

All comparisons between ET patients and normal subjects were made with the use of Student's t-test. Paired t-test was used to evaluate the effect of cytoreduction on half-life times in ET patients and to evaluate the fibrinolytic response generated by DDAVP within either ET patients or normal individuals. For calculations of the half-life times linear regression analysis and Pearson correlation coefficients were used.

RESULTS

As shown in table 7.1, platelet counts were significantly higher in ET patients as in normal controls. VWF:CBA/vWF:Ag ratios were significantly lower in ET patients, as has previously been reported [4,7]. There were no significant

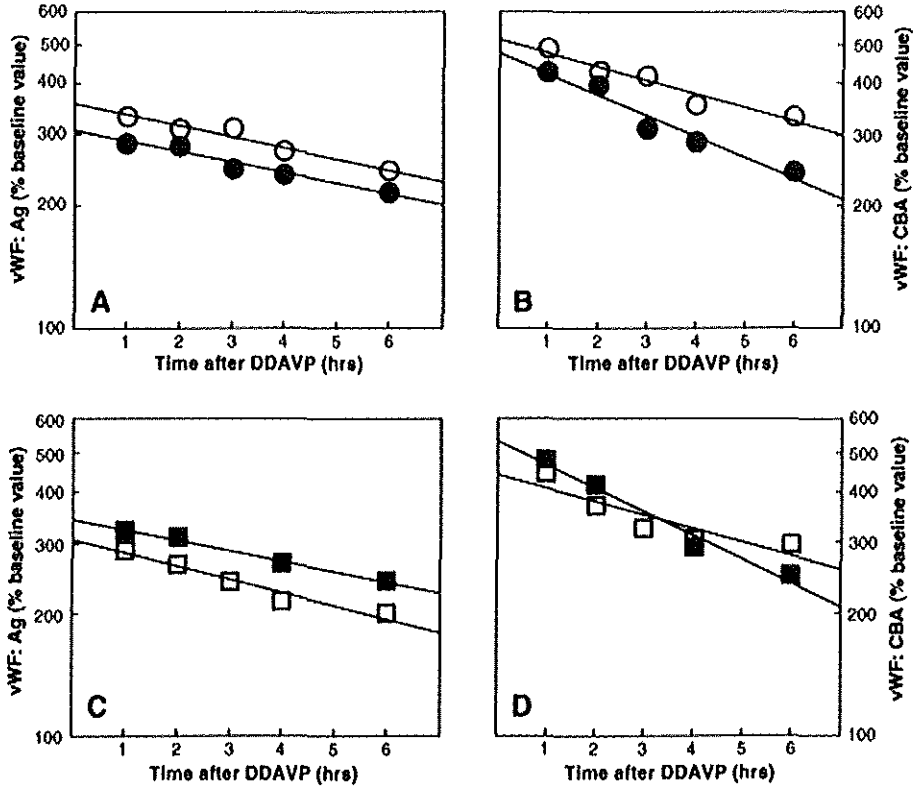

Figure 7.1

Kinetics of mean vWF:Ag and vWF:CBA activities after DDAVP infusion in ET patients and normal individuals. Correlation coefficients always exceeded 0.90.
Figures A-B: kinetics of vWF:Ag (fig 7.1A) and vWF:CBA (fig 7.1B) in 9 normal individuals (open symbols) and 9 patients with essential thrombocythemia (closed symbols).
Figures C-D: kinetics of vWF:Ag (fig 7.1C) and vWF:CBA (fig 7.1D) in 4 patients with essential thrombocythemia before (closed symbols) and after cytoreduction of the increased platelet count (open symbols).

differences in hemoglobin levels, packed cell volume, leukocyte counts, plasma vWF:Ag and plasma vWF:CBA between normal individuals and ET patients. As shown in table 7.2, there were no significant differences in peak vWF:Ag and vWF:CBA after DDAVP infusion between ET patients and normal controls. In addition, administration of DDAVP generated comparable peak vWF:Ag and vWF:CBA levels in ET patients before and after cytoreduction of the increased platelet count (table 7.3). The half-life times of vWF:Ag did neither significantly differ between ET patients and normal individuals on the one hand nor between ET patients before and after cytoreduction on the other. In contrast, half-life times of vWF:CBA were significantly shorter in ET

Table 7.3	Kinetic studies of vWF:Ag and vWF:CBA in 4 ET patients before and after cytoreduction of the increased platelet count		
	ET (n=4) before cytoreduction	ET (n=4) after cytoreduction	P-value
Platelets (x 10^9/L)	904 (817 - 1057) [746 - 1095]	369 (266 - 469) [212 - 524]	p<0.0005
Peak vWF:Ag (%)	329 (160 - 463) [111 - 530]	297 (258 - 306) [255 - 324]	p>0.05
Peak vWF:CBA (%)	491 (259 - 649) [189 - 756]	415 (382 - 566) [310 - 579]	p>0.05
$T_{1/2}$ vWF:Ag (hours)	10.5 (5.7 - 12.9) [5.1 - 14.7]	10.5 (5.6 - 13.1) [4.1 - 15.7]	p>0.05
$T_{1/2}$ vWF:CBA (hours)	4.9 (4.2 - 6.9) [3.4 - 7.1]	8.9 (6.5 - 10.7) [5.6 - 11.8]	p<0.02

patients as compared with normal controls (table 7.2) and cytoreduction of the increased platelet count resulted in a normalization of the half-life time of vWF:CBA (table 7.3). The decrease of vWF:Ag and vWF:CBA activities after DDAVP infusion in relation to time is shown in figure 7-1.

Although DDAVP may also release t-PA [15], it seems unlikely that the differences in vWF:CBA half-life times are mediated by DDAVP-generated t-PA since measurements of total degradation products of fibrin and fibrinogen revealed no significant fibrin(ogen)olysis in both ET patients and normal individuals after DDAVP (data not shown). In addition, in both groups a significant and comparable rise of plasmin-α2-antiplasmin complexes after administration of DDAVP was observed (data not shown), indicating rapid inactivation of t-PA generated plasmin, consistent with the observation of others [16].

DISCUSSION

ET patients exhibit a decrease of large vWF multimers in plasma, which appears to be inversely related to the platelet count [6,7]. The normal recovery of vWF-related parameters after DDAVP infusion, as compared with normal individuals, excludes the possibility of a decreased synthesis and/or defective release of vWF from endothelial storage sites as an alternative explanation for the decrease of large vWF multimers in plasma. The present study, however, shows that this decrease of large vWF multimers in ET patients is likely to be caused by an increased turnover of circulating large vWF multimers in association with an increased platelet count, as is reflected by the shortened

half-life times of vWF:CBA in ET patients and its normalization after cytoreduction of the increased platelet count.

The observation of an increased turnover of large vWF multimers in ET with an associated increase in circulating platelets is interesting, since it is generally assumed that vWF in solution has no measurable affinity for platelets. However, although speculative, some reversible interactions between plasma vWF and platelets may be anticipated, for instance during the passage of blood through the microcirculation, in which the prevailing shear stress may be sufficient to promote vWF binding to platelets [17,18]. Hypothetically, binding of large vWF multimers to platelets may result in a depletion of large vWF multimers in plasma. Platelet-bound vWF may subsequently be rapidly internalized and/or degraded.

Interestingly, increased amounts of proteolytic fragments of vWF have been found *in vivo* in patients with reactive thrombocytosis, as well as in patients with essential thrombocythemia [6,9,19]. In the latter group of patients, cytoreduction of the increased platelet count resulted in an abatement of proteolysis of vWF. As in normal individuals, in both patient groups the proteolysis of vWF was characterized by specific increases of the 140 kD (amino-terminus) and 176 kD (carboxy-terminus) proteolytic fragments of vWF, which derive from a single proteolytic cleavage at the peptide bond between residues Tyr 842 and Met 843 [20], a cleavage site tentatively reflecting the specificity of calpains. However, results of epitope mapping studies *in vitro* showed that calpains from porcine erythrocytes and kidney, which are mainly intracellular enzymes, failed to generate the vWF fragments produced *in vivo* [21]. Recently, both Tsai [22] and Furlan et al [23] independently described a partially purified protease, originating from plasma [23], which degrades vWF to the proteolytic vWF fragments observed *in vivo*, under experimental conditions in which vWF has undergone a conformational change. It is tempting to speculate that *in vivo* this conformational change of vWF may be accomplished by binding of vWF to platelets, thus making the proteolytic cleavage site on vWF accessible for the plasma protease. In this respect, a beneficial effect of cytoreduction of platelets on the proteolysis of plasma vWF may be anticipated. Further studies investigating the mechanisms underlying the increased turnover of large vWF multimers in plasma in association with high platelet count states are indicated.

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8

THE EXCESSIVE PROLONGATION OF THE BLEEDING TIME BY ASPIRIN IN ESSENTIAL THROMBOCYTHEMIA IS RELATED TO A DECREASE OF LARGE VON WILLEBRAND FACTOR MULTIMERS IN PLASMA

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SUMMARY

Patients with essential thrombocythemia (ET), who frequently have bleeding complications, may manifest an excessive prolongation of the bleeding time (BT) after ingestion of aspirin (ASA). The reason for this excessive prolongation of the BT is unknown, but it is attributed to qualitative platelet defects. Since patients with ET may also have acquired abnormalities of plasma and platelet von Willebrand factor (vWF), we studied whether the excessive prolongation of the BT by ASA was related to changes in either plasma or platelet vWF. To that end, we studied BT and plasma and platelet vWF in 10 ET patients, 10 patients with reactive thrombocytosis (RT), and 10 normal individuals, both before and after administration of 500 mg ASA for 7 days. In a second study, the effect of DDAVP infusion on plasma vWF in relation to the BT was studied in 10 normal individuals and 10 ET patients after treatment with 100 mg ASA for 3 days. In the first study, treatment with ASA resulted in a significant prolongation of the BT in normal subjects, RT patients and ET patients. However, in 5 ET patients an excessive (> 2 SD) prolongation of the BT by ASA was observed. Although ASA induced no direct changes in either plasma or platelet vWF levels in either normal subjects, RT patients or ET patients, all five ET patients who showed an excessive prolongation of the BT by ASA had significantly decreased levels of large vWF multimers in plasma. In the second study, infusion with DDAVP resulted in a significant increase in plasma large vWF multimers, paralleled by a normalization of (excessively) prolonged BT. Our data suggest that in ET inhibition of platelet function by ASA in the presence of concurrently decreased levels of large vWF multimers in plasma may have provoked the excessive BT prolongation.

INTRODUCTION

Excessive bleeding time (BT) prolongation and even severe bleeding have been observed after administering aspirin (ASA) to patients with essential thrombocythemia (ET) and polycythemia vera (PV) [1-5]. The reason for the excessive BT prolongation by ASA is not known, but it is currently attributed to qualitative platelet defects [1]. However, abnormal BT prolongation after ingestion of ASA is also observed in patients without dysfunctional platelets. For instance, the BT after ingestion of ASA has been used to unmask patients with asymptomatic von Willebrand disease [6]. Furthermore, in patients with congenital von Willebrand disease type 3 who lack the ability to synthesize vWF, a normalization of the prolonged BT may be obtained with transfusion of normal platelets after prior cryoprecipitate infusion, suggesting an important role of platelet vWF in platelet-vessel wall interactions [7]. Since ET patients may exhibit abnormalities of plasma vWF (in particular, reduction of large vWF multimers) [2,8-10], as well as abnormalities of platelet vWF [11],

a study was designed to find out whether the excessive BT prolongation after treatment with ASA in ET patients was related to abnormalities of plasma or platelet vWF.

PATIENTS AND METHODS

Design

The study protocol was approved by the local ethics commitee. The first study was designed to identify ET patients with an excessive BT prolongation after ingestion of 500 mg ASA (i.e., prolongation of BT exceeding mean + 2SD of BT of normal individuals after ingestion of ASA) and to investigate the relationship with changes in plasma or platelet vWF. To that end, a cross-sectional comparison of BT, plasma vWF and platelet vWF-related parameters was made between 10 ET patients, 10 RT patients and 10 control subjects, both before and after treatment with 500 mg ASA for 7 days. The group of RT patients was used as a control group to evaluate changes which might be attributed to an increased platelet count. In a second study, DDAVP (Minrin®, Ferring BV, Hoofddorp, the Netherlands) was administered intravenously in a dose of 0.4 μg/kg bodyweight to manipulate plasma vWF and its multimeric distribution and to evaluate the response of ASA-induced (excessively) prolonged BT to changes in plasma vWF. For this purpose, 10 ET patients and 10 control subjects received 100 mg ASA for 3 days before DDAVP infusion to effectively inhibit platelet cyclooxygenase. BT and plasma vWF-related parameters were measured before, and 1 and 6 hour after DDAVP infusion, respectively.

Patients

After informed consent, 10 asymptomatic patients with ET (6 males and 4 females; ranging in age from 54 to 83 years, median 62 years) and 10 patients with RT (5 males and 5 females; ranging in age from 35 to 88 years, median 63 years) participated in the first study. The diagnosis ET was based on commonly accepted clinical and laboratory criteria [5]. The underlying disorder associated with RT was pyelonefritis in 1, leprosy and iron deficiency in 1, psoriasis arthropathica in 1, arteriitis temporalis in 1 and splenectomy (due to gastric carcinoma in 2, abdominal trauma in 3 and idiopathic thrombocytopenia in 1) in 6 cases, respectively. Ten asymptomatic ET patients (5 males and 5 females; ranging in age from 21 to 73 years, median 57 years) with a platelet count of 651±240 x 10^9/L participated in the second study; 2 of them had also participated in the first study.

Methods

Platelet counts were determined routinely in whole blood anticoagulated with EDTA. For all studies involving vWF, blood was collected in one-tenth final

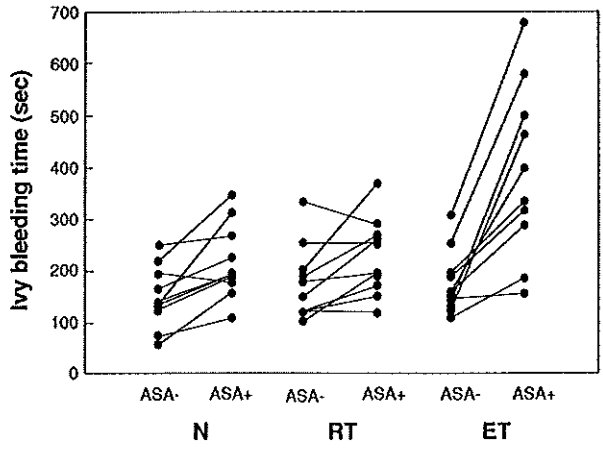

Figure 8.1

Ivy BT in 10 patients with essential thrombocythemia (ET), 10 patients with reactive thrombocytosis (RT) and 10 normal subjects (N), before (ASA-) and after treatment (ASA+) with 500 mg ASA for 7 days.

volume of 3.8 % trisodium citrate solution containing aprotinin, EDTA and N-ethylmaleimide to give final concentrations of 500 KIU/mL, 5 mmol/L and 6 mmol/L, respectively. Ivy BT, plasma von Willebrand factor antigen (vWF:Ag), ristocetin cofactor activity (vWF:RCoF) and collagen binding activity (vWF:CBA) were measured as described previously [9,12]. Multimeric analysis of plasma and platelet vWF and the quantification of plasma large vWF multimers was performed as reported in detail elsewhere [8]. Platelet vWF was assayed in supernatants of platelet lysates. In brief, platelets were isolated from platelet-rich-plasma. This was followed by 2 washings with PBS-EDTA and lysis with 1/10 (v/v) of 10% Triton X-100 in the presence of 500 KIU/mL aprotinin, 5 mmol/L EDTA and 6 mmol/L N-ethylmaleimide (final concentration), followed by precipitation of cellular fragments by centrifugation (10' at 4°C; 25.000 g). VWF:Ag and vWF:CBA in platelet lysates were measured as described for plasma vWF, apart from a standard curve constructed with 1% Triton X-100 (final concentration). Malondialdehyde (MDA) production was measured in platelet-rich plasma after stimulation of platelets by arachidonic acid (AA).

Statistics

Analysis of Variance (ANOVA), followed by Student-Newman-Keuls test (SNK test), was used to identify pair-wise significant differences in group means or differences between the post- and pre-ASA treatment values (ΔASA) of normal controls, RT and ET patients. Correlations between Ivy BT (or ΔBT) and plasma or platelet vWF parameters (or ΔASA) were analyzed by linear regression and Spearman rank order tests. Paired t-tests were used in the first study to analyze the effect of ASA within each patient or control group and in the second study to evaluate the effect of DDAVP within ET patients or normal subjects. In the second study, plasma vWF-parameters and BT of ET

Table 8.1 The effect of aspirin (ASA) on Ivy bleeding time, platelet count and von Willebrand factor-related parameters in plasma in normal individuals, patients with reactive thrombocytosis and essential thrombocythemia. Data are expressed as mean (SD).

	Normal individuals (n=10)		Reactive thrombocytosis (n=10)		Essential thrombocythemia (n=10)	
	ASA-	ASA+	ASA-	ASA+	ASA-	ASA+
Ivy BT (s)	149(60)	218(72)**	177(73)	228(75)*	178(62)	391(167)***
Platelets (x 10^9/L)	237(36)	250(40)	800(198)	729(264)	689(243)	794(223)
plasma vWF:Ag (U/mL)	1.36(0.44)	1.38(0.58)	2.57(1.24)	2.31(1.15)	1.52(0.81)	1.23(0.52)
plasma vWF:RCoF (U/mL)	0.98(0.30)	1.15(0.43)	1.48(1.10)	1.48(0.95)	1.15(0.46)	1.06(0.49)
plasma vWF:CBA (U/mL)	1.31(0.42)	1.42(0.71)	2.47(1.19)	2.00(1.06)	1.19(1.11)	0.77(0.50)
RCoF/Ag ratio in plasma	0.74(0.16)	0.87(0.25)	0.54(0.24)	0.60(0.13)	0.89(0.47)	0.88(0.38)
CBA/Ag ratio in plasma	0.97(0.17)	1.01(0.14)	0.96(0.34)	0.83(0.20)	0.74(0.37)	0.62(0.28)
plasma large vWF multimers (%)	23.1(2.5)	22.0(4.3)	16.4(6.3)	17.5(3.7)	16.6(3.4)	14.2(5.9)

Legend:Ivy BT = Ivy bleeding time; vWF:Ag = von Willebrand factor antigen; vWF:RCoF = ristocetin cofactor activity; vWF:CBA = collagen binding activity. *= p<0.05; **= p<0.005; ***= p<0.001 as compared with pre-ASA treatment value (paired t-test)

patients were compared with those of normal individuals by Student's t-test. Statistical significance was accepted at $p<0.05$. All data are expressed as mean±SD.

RESULTS

Pre-ASA treatment values

As shown in table 8.1, mean platelet counts were comparable in ET and RT patients, and both significantly increased as compared with normal subjects (RT vs N: $p<0.001$; ET vs N: $p<0.001$). Plasma vWF:Ag and vWF:CBA levels were significantly higher in RT patients as compared with both ET patients ($p<0.05$) and normal individuals ($p<0.05$), respectively. In contrast, percentages of plasma large vWF multimers were higher in normal individuals as compared with both RT patients ($p<0.01$) and ET patients ($p<0.01$), consis-

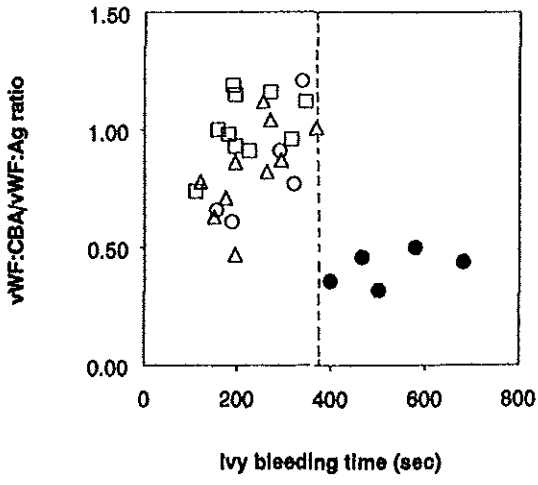

Figure 8.2

BT in relation to vWF:CBA/vWF:Ag ratios after administration of 500 mg ASA for 7 days to 10 normal individuals (open squares), 10 RT patients (open triangles) and 10 ET patients. Five ET patients had a normal BT prolongation (open circles) whereas an excessive BT prolongation (closed circles) was noted in 5 ET patients. Broken vertical line denotes the upper limit (=mean + 2 SD) of normal BT prolongation after 500 mg ASA.

tent with previous observations [8,10]. There were no significant differences in pre-ASA values of Ivy BT, plasma vWF:RCoF, plasma vWF:RCoF/vWF:Ag ratio, plasma vWF:CBA/vWF:Ag ratio, MDA-AA, platelet vWF:Ag and vWF:CBA (data not shown) between ET, RT patients and normal subjects. The multimeric distribution of platelet vWF was normal in all investigated cases, either with or without treatment with ASA (data not shown).

Effect of treatment with ASA

Treatment with ASA resulted in a significant inhibition of MDA-AA generation, consistent with an effective inhibition of platelet cyclooxygenase activity by ASA (data not shown). In both patient groups and controls, Ivy BT were significantly prolonged by ASA (figure 8.1). However, the BT prolongation was significantly more in ET patients (ΔBT 213±132 s) as compared with both RT patients (ΔBT 51±63 s, $p<0.001$) and normal subjects (ΔBT 69±55 s, $p<0.01$), respectively. In contrast, there were no significant differences between normal subjects, RT patients and ET patients in effects of ASA on either plasma or platelet vWF. In addition, in each patient or control group, no significant correlations were observed between changes in plasma or platelet vWF (or ΔASA) on one hand and Ivy BT (or ΔBT) on the other hand, indicating that it is rather unlikely that treatment with ASA had caused changes in plasma or platelet vWF resulting in an excessive BT prolongation. However, as shown in figure 8.2, treatment with ASA unmasked a subgroup of 5 ET patients, who had an excessive BT prolongation after ASA (524±109 s) in comparison to ET patients who had a normal prolongation of BT after ASA (258±82 s, $p<0.001$), RT patients (228±75 s, $p<0.001$) and normal individuals (218±72 s, $p<0.001$). All ET patients who showed an excessive ASA-induced BT prolongation had significantly decreased plasma

Table 8.2 Comparison of hemostatic parameters between ET patients with a normal prolongation (BT normal) and excessive prolongation (BT excessive) of the bleeding time after treatment with aspirin. Data are expressed as mean (SD).

	BT normal (n=5)	BT excessive (n=5)	p-value (t-test)
Ivy BT (s)	258(82)	524(109)	p=0.0024*
Platelets (x 10^9/L)	722(223)	866(223)	p=0.34
MDA-AA (nmol/10^9 plts)	0.11(0.17)	0.12(0.19)	p=0.93
plasma vWF:Ag (U/mL)	1.07(0.36)	1.38(0.65)	p=0.39
plasma vWF:RCoF (U/mL)	1.09(0.57)	1.03(0.46)	p=0.86
plasma vWF:CBA (U/mL)	0.95(0.61)	0.59(0.33)	p=0.27
RCoF/Ag ratio in plasma	1.00(0.49)	0.75(0.18)	p=0.32
CBA/Ag ratio in plasma	0.83(0.24)	0.42(0.07)	p=0.0061*
plasma large vWF multimers (%)	17.4(4.3)	10.9(5.7)	p=0.08*
platelet vWF:Ag ($U/10^{11}$ plts)	16.1(3.7)	15.9(7.9)	p=0.96
platelet vWF:CBA ($U/10^{11}$ plts)	14.3(5.0)	12.2(10.1)	p=0.69
CBA/Ag ratio in platelets	0.88 (0.14)	0.70(0.19)	p=0.13

Legend: see table 8.1; *= also (highly) significant in 4-group analysis N vs RT vs ET BT normal vs ET BT excessive

vWF:CBA/vWF:Ag ratios (n=5; 0.42±0.07), as compared with the ratios of ET patients with a normal ASA-induced BT prolongation (n=5; 0.83±0.24, p<0.001), RT patients (n=10; 0.83±0.20, p<0.001) and normal subjects (n=10; 1.01±0.14, p<0.001). In addition, the 5 ET patients with an excessive ASA-induced BT prolongation also had significantly decreased percentages of large vWF multimers in plasma (10.9±5.7 %), in comparison to ET patients with a normal BT prolongation (17.4±4.3 %, p<0.05), RT patients (17.5±3.7 %,

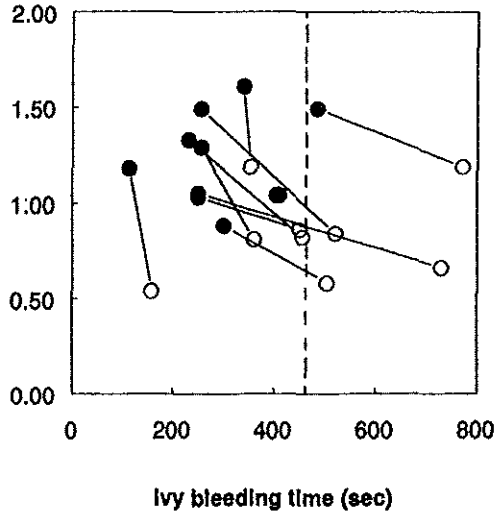

Figure 8.3

The effect of DDAVP-induced changes in large vWF multimers in plasma, given as vWF:CBA/vWF:Ag ratio, on ASA-induced prolonged BT in 10 patients with ET. Four of them had an excessive BT prolongation after treatment with 100 mg ASA for 3 days. Open symbols indicate values before administration of DDAVP; closed symbols values 1 hour after administration of DDAVP. Broken vertical line denotes the upper limit of a normal BT prolongation after 100 mg ASA.

$p<0.05$) and normal individuals (22.0±4.3 %, $p<0.001$). These data suggest that inhibition of platelet function by ASA in the presence of decreased levels of large vWF multimers in plasma may have provoked the excessive BT prolongation. Details of the hemostatic parameters of ET patients with and without an excessive ASA-induced BT prolongation are shown in table 8.2.

Effect of DDAVP on ASA-induced prolongation of the bleeding time.
In the second study, the deprivation of large vWF multimers in plasma of ET patients observed in the first study was corrected *in vivo* by DDAVP infusion, in parallel with BT measurements. Consistent with the findings of the first study, before DDAVP infusion ET patients had significantly lower plasma vWF:CBA/vWF:Ag ratios and significantly longer ASA-induced BT prolongations; 4 of 10 ET patients even fulfilled the criteria of an excessive BT prolongation (figure 8.3). After DDAVP infusion vWF:Ag rose from 1.14±0.31 to 3.42±0.82 U/mL ($p<0.0001$) in normal individuals, and from 1.32±0.59 to 2.79±0.59 U/mL ($p<0.0001$) in ET patients, respectively. In normal individuals, vWF:CBA rose from 1.31±0.46 to 5.52±2.40 U/mL ($p=0.0002$) after DDAVP, whereas in ET patients vWF:CBA rose from 1.15±0.61 to 3.40±0.72 U/mL ($p<0.0001$). As shown in table 8.3, both ET patients and control subjects showed significant increases of vWF:CBA/vWF:Ag ratios after DDAVP. In parallel with a rise of plasma vWF:CBA/vWF:Ag ratio, a normalization of BT was observed in all ET patients (figure 8.3), comparable to BT of normal individuals before DDAVP infusion. Six hours after DDAVP infusion a prolongation of BT in ET patients was observed, which again was associated with

Table 8.3	Effect of DDAVP on ASA-induced prolongation of bleeding time and plasma vWF:CBA/vWF:Ag ratio in ET patients (ET) and control subjects (N). Data are given as mean(SD).					
	Ivy bleeding time(s)			vWF:CBA/vWF:Ag ratio		
	before	after 1 hr	p-value*	before	after 1 hr	p-value
N (n=10)	292(85)	223(67)	p=0.007	1.12(0.15)	1.57(0.41)	p=0.004
ET (n=10)	470(179)	289(103)	p=0.004	0.85(0.23)	1.24(0.24)	p=0.0002
p-value**	p=0.011	p=0.11		p=0.006	p=0.04	
Legend:*= p-value of paired t-test (before vs 1 hr after DDAVP); **= p-value of Student's t-test (N vs ET)						

reduced plasma vWF:CBA/vWF:Ag ratios (data not shown).

DISCUSSION

The BT is frequently used as the method to evaluate the hemostatic effectiveness of platelets (i.e. primary hemostasis). Multiple mechanisms of platelet activation normally assure the efficiency of primary hemostasis. As a consequence, blockade of one of these pathways, for instance by ASA, results in only a slight disturbance of primary hemostasis, as is evidenced by a slight BT prolongation of normal individuals after ASA. If, however, potential compensatory pathways of primary hemostasis are also affected, for instance by a reduction of large vWF multimers in plasma, excessive BT prolongations and even bleedings may ensue after ingestion of ASA.

Although the population size of ET patients was limited, the present study showed that those patients who had an excessive BT prolongation after ingestion of ASA had a concurrent decrease of large vWF multimers in plasma. The decrease of large vWF multimers in plasma was not attributable to treatment with ASA, since treatment with ASA had no effect on either plasma or platelet vWF. These presented data, however, suggest that the inhibition of platelet function by ASA in the presence of concurrently decreased levels of large vWF multimers in plasma may have provoked the excessive BT prolongation.

The decrease in large vWF multimers in plasma was demonstrated by two independent assays, i.e., direct quantification of large vWF multimers in plasma after SDS gel electrophoresis of vWF and by ELISA-based measurements of the plasma vWF:CBA/vWF:Ag ratio. In a previous study we showed that plasma vWF:CBA/vWF:Ag ratios are tightly correlated to the percentage of plasma large vWF multimers [10]. The ristocetin cofactor activity, which is

usually used to test vWF function *in vitro*, appeared to be a less sensitive parameter for detecting modest reductions of large vWF multimers in plasma, consistent with previous observations [9,10,13].

The influence of a decreased level of large vWF multimers in plasma on the excessive ASA-induced BT prolongation was stressed by manipulating the plasma levels of large vWF multimers by the infusion of DDAVP. DDAVP releases vWF, including large vWF multimers, from endothelial storage sites into the circulation [14]. After administering DDAVP to ET patients with an ASA-induced (excessive) BT prolongation, plasma vWF:CBA/vWF:Ag ratios rose significantly 1 h after DDAVP infusion, indicating a rise of plasma levels of large vWF multimers, in parallel with a significant shortening of the BT, and vice versa 6 h after DDAVP. These data suggest that an excessive BT prolongation by ASA in ET may be corrected by normalization of plasma levels of large vWF multimers.

In previous studies we found that the reduction of large vWF multimers in plasma in ET is directly related to the number of circulating platelets [8-10]. The reduction of large vWF multimers in plasma becomes particularly apparent at platelet counts exceeding 1000 x 10^9/L, which even may result in a spontaneous bleeding tendency due to an acquired von Willebrand factor deficiency in plasma at higher platelet counts [2,9]. In accordance with this concept, several epidemiological studies in ET have actually shown an increased incidence of (mucocutaneous) bleedings when the platelet count exceeds the level of 1000 x 10^9/L [15-17].

However, as is apparent from the present study, the reduction of large vWF multimers in plasma is also demonstrable, albeit modest, at lower platelet counts, i.e. below 1000 x 10^9/L. These patients generally do not suffer from overt bleeding but they may have a latent bleeding tendency. An excessive BT prolongation after treatment with ASA may identify patients at risk for bleedings. Although these data need confirmation in appropriate clinical trials, they suggest that the reduction of large vWF multimers in ET may be an important determinant factor for bleeding, in particular when treatment with platelet antiaggregants is considered at increased platelet counts.

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9

GENERAL DISCUSSION AND SUMMARY

Despite extensive laboratory investigations resulting in the identification of a great variety of platelet abnormalities, the pathophysiology of the hemostatic dysbalance in ET resulting in an increased frequency of both thrombotic and hemorrhagic complications still remains obscure [CHAPTER 1]. The clinical significance of the described platelet abnormalities in ET remains uncertain since they appear to correlate poorly with clinical events of bleeding and thrombosis [1-3].

Is the thrombotic tendency in ET caused by platelet dysfunction or by the platelet number?

Most clinical studies provide arguments for the presence of dysfunctional platelets in ET [CHAPTER 1]. Clinical studies in ET invariably show a high frequency of thrombotic complications [1-3]. However, these complications do not occur or only rarely in patients with reactive thrombocytosis with comparable elevations of the platelet count [1-3]. A significant proportion of ET patients is asymptomatic at presentation and remains free of hemostatic complications during long-term follow-up [CHAPTER 1.3], suggesting that not all ET patients are naturally prone to developing thrombotic sequelae. The frequency of thrombotic complications appears to be reduced following treatment with anti-platelet agents such as aspirin or after platelet cytoreduction [CHAPTER 1.4]. In fact, in one prospective placebo-controlled study in which cytotoxic treatment in high-risk ET patients (i.e. age > 65 years and/or previous thrombotic complication) has been examined, a favourable effect of cytoreduction on the occurrence of major thrombotic events was demonstrated [4]. However, in this study a relative lack of effect of cytoreduction on the incidence of microvascular thrombotic complications was observed. This could have been due to the fact that the target level for cytoreduction was a platelet count of 600 x 10^9/L. A platelet count between 400 and 600 x 10^9/L does not neccessarily prevent the occurrence of microvascular thrombotic events such as erythromelalgia, as has been demonstrated in several studies [5,6]. However, the occurrence of these microvascular thrombotic events is abolished by cytoreduction of platelets to counts within the normal range [5], suggesting that in patients with ET a given number of dysfunctional platelets is needed to develop thrombotic complications. In studies described in this thesis we investigated several aspects of erythromelalgia in more detail as a representative model for arterial microvascular thrombosis in ET.

The further characterization of erythromelalgia as a model for platelet-mediated thrombosis in ET

In CHAPTER 2, platelet kinetic studies were performed in thrombocythemia patients suffering from erythromelalgia and control subjects. Mean platelet survival times were significantly shortened in erythromelalgia as compared with subjects with thrombocythemia without overt thrombosis indicating the involvement of platelets in erythromelalgia. Subjects with reactive thrombocytosis also showed normal platelet survival *in vivo*. The increased platelet consumption in erythromelalgia is most likely to be attributed to the formation of platelet thrombi in the acral microvasculature. In support of this explanation, the use of aspirin restores platelet survival and corrects the clinical features of erythromelalgia simultaneously. Interestingly, in contrast to aspirin, treatment with coumadin failed to improve platelet survival times in erythromelalgia. These observations provide evidence for a direct relationship between platelet kinetics, erythromelalgia and therapeutic efficacy of aspirin. The observed decreased platelet survival time in some asymptomatic ET patients might be a reflection of the dysfunction of thrombocythemic platelets leading to a shortened survival in the circulation.

The absence of a beneficial effect of oral anticoagulation on erythromelalgia suggests that thrombin generation and thereby the formation of fibrin is not a critical step in the pathogenesis of erythromelalgia. This issue has been further addressed in CHAPTER 3. Based on a cross-sectional study of symptomatic and asymptomatic ET patients and normal controls, erythromelalgia was characterized by platelet activation and endothelial cell damage but not by thrombin generation or lysis of fibrin(ogen). Treatment of erythromelalgia with aspirin could reverse platelet activation and in part endothelial cell damage. These findings were confirmed in a prospective study of 2 ET patients who developed erythromelalgia after withdrawal of aspirin. Interestingly, skin punch biopsies of erythromelalgic skin areas showed that erythromelalgia was associated with thrombi occluding small arterial vessels. Immunohistochemical analysis of these thrombi revealed platelet-rich thrombi with weak staining for fibrin. These data suggest that erythromelalgia is caused by platelet-rich thrombi that may occlude and potentially damage small acral arteries. Furthermore, the lack of significant thrombin generation in plasma and the weak fibrin staining of erythromelalgic thrombi would suggest that these thrombi are not or only minimally consolidated by fibrin and may therefore be relatively unstable. The observed "waxing and waning" of the initial symptoms of erythromelalgia [5] might be caused by transient occlusion or sludging of acral blood vessels by unstable thrombi. Although speculative, the observation that ET patients also frequently suffer from TIAs [7-9] may be related to similar events occurring in the cerebrovascular circulation. In agreement with this, TIAs in ET usually respond beneficially to

aspirin and cytoreduction [10].

The importance of platelet cyclooxygenase in the pathophysiology of microvasculair thrombosis in ET

The presence of dysfunctional platelets in the pathogenesis of thrombosis in ET is further corroborated by the characteristic pharmacological features of the treatment of erythromelalgia. Of the drugs known to interfere with platelet function, only agents which inhibit platelet cyclooxygenase alleviate erythromelalgia and prevent recurrences of erythromelalgia if administered continuously. These observations suggest a role for an active platelet prostaglandin synthesis in the pathophysiology of thrombosis in ET. In CHAPTER 4 the importance of platelet prostaglandin metabolism for the development of erythromelalgia has been evaluated. After discontinuation of aspirin, all thrombocythemia patients who developed erythromelalgia had a preceding progressive increase in platelet thromboxane formation, conceivably reflecting spontaneous platelet activation and aggregation *in vivo*, whereas patients who remained asymptomatic did not. Treatment with 50 mg of aspirin per day, which selectively inhibits platelet cyclooxygenase without affecting vascular cyclooxygenase activity [11], resulted in substantial inhibition of platelet thromboxane formation and in parallel a gradual disappearance of erythromelagic symptoms. These data might suggest a direct link between thromboxane-dependent platelet activation and the occurrence of microvascular arterial thrombosis in thrombocythemia.

Upon platelet activation, arachidonic acid is released from the intracellular platelet membrane by phospholipase A_2 [12,13]. Liberated arachidonic acid is converted to thromboxane A_2 via cyclooxgenase. Thromboxane A_2 is a potent agonist for platelet activation. Its rapid release from the activated platelet amplifies the platelet activation signal and subsequent platelet aggregation through activation of surface membrane thromboxane A_2 receptors on other platelets ('platelet recruitment'). However, it should be noted that a variety of agonists (e.g. ADP, 5-hydroxyptryptamine, vasopressin, platelet activating factor and thrombin) have the capacity to initiate similar activation pathways, eventually resulting in the formation of thromboxane A_2 [13]. Therefore, the observed increase in platelet thromboxane formation may not necessarily yield a specific clue to the trigger responsible for platelet activation *in vivo*. This question is also raised by the observation that dazoxiben, a reversible thromboxane synthase inhibitor, does neither alleviate erythromelalgia nor improves platelet survival in erythromelalgia despite adequate inhibition of formation of thromboxane [14]. These observations might indicate that not thromboxane itself but rather another stimulator, e.g. one (or more) of its metabolic precursors is involved in the development of erythromelalgia. Prostaglandins and thromboxane are formed by metabolism of arachidonic

acid by cyclooxygenase localised in the intracellular membrane [15]. The alternative route of arachidonic acid metabolism in platelets follows the lipoxygenase pathway localised in the cytosol, which results in the formation of 12-hydroxyeicosatetraenoic acid (12-HETE) [15]. Although the effects of 12-HETE on platelet function are not completely clear, several studies suggest that 12-HETE and its precursor 12-HPETE feeds back to inhibit cyclooxygenase and thus block the synthesis of thromboxane whereas others suggest that 12-HETE is required for irreversible platelet aggregation and increases platelet adhesiveness [15,16]. Interestingly, a number of studies have indicated a lipoxygenase deficiency in myeloproliferative disorders such as ET, sometimes associated with a shift towards an increase in thromboxane formation [17]. On theoretical grounds a lipoxygenase deficiency may be anticipated to result in a greater risk for developing thrombotic complications. However, the described patients tended to have bleeding episodes instead. We recently provided a possible explanation for this paradox through observations on a thrombocythemia patient with erythromelalgia who also appeared to have a lipoxygenase deficiency [18]. After treatment of erythromelalgia with aspirin, 12-HETE concentrations were found to be normal, indicating a normal lipoxygenase activity. These results indicate that a lipoxygenase deficiency may be secondary to platelet activation *in vivo*, probably as a consequence of differences in substrate availability for the two competitive metabolic pathways of arachidonic acid.

Altogether, these data suggest that the dysfunction of thrombocythemic platelets leading to the observed thrombotic tendency may be mediated by activation of platelets through the cyclooxygenase pathway, whereas the precise trigger for platelet activation *in vivo* remains unknown. Further studies to identify this intrinsic platelet defect in thrombocythemia will probably be hampered by the occurrence of platelet activation both *in vivo* and *ex vivo* and await the development of a model in which platelet function can conveniently be studied without the risk of activation. In addition, thrombosis should not be considered as a single cellular event but the hemostatic resultante of an interplay of multicellular events between endothelial cells, erythrocytes, leukocytes and platelets, which eventually will determine the development and progression of thrombi *in vivo* [19-22].

The importance of the platelet number in the pathophysiology of bleeding in ET

Early studies usually emphasized the bleeding complications of ET ("hemorrhagic thrombocythemia") [23]. Of note is that these bleeding complications usually occurred in patients with high platelet and/or leukocyte counts [1-3]. This suggested an association between risk of hemorrhage in ET with a more advanced stage of myeloproliferative disease, or perhaps the more frequent use

of platelet anti-aggregants. In more recent series, however, the reportred frequency of bleeding complications appears to be substantially less [CHAPTER 1]. It is tempting to speculate that the lower incidence of bleeding complications nowadays might be related to an earlier detection of a myeloproliferative disorder, e.g. as the result of the introduction of automated blood sampling and a concomitantly earlier start of (cytoreductive) treatment. This might have prevented the occurrence of extremely high platelet counts and associated bleeding complications.

In this thesis we focused on the role of von Willebrand factor in the pathophysiology of bleedings in ET (see CHAPTER 1 and CHAPTERS 5 to 8). Von Willebrand factor (vWF) is a large, multimeric glycoprotein in plasma, synthesized in megakaryocytes and vascular endothelium. It plays a crucial role in the early stages of hemostasis by mediating the shear-rate dependent adhesion of platelets at sites of vascular injury and by cross-linking platelets [24]. These processes are essential for stable clot formation. The hemostatic potency of vWF depends on its degree of multimerization, with the largest multimers being most effective in securing hemostasis. As is described in CHAPTER 5 bleeding complications may be associated with a deficiency of large vWF multimers in plasma ("acquired von Willebrand disease"). The decrease in large vWF multimers appeared to correlate inversely to the platelet count. At platelet counts of more than 1000 x 10^9/L the concentration of large vWF multimers declines and the risk of bleeding probably rises significantly. In CHAPTER 6 we demonstrated that this reduction of large von Willebrand factor multimers was directly related to the platelet count irrespective of the cause leading to an increased platelet count. Moreover, this study also provided several arguments for the absence of bleeding complications in patients with reactive thrombocytosis. Due to its behaviour as a reactive protein, the plasma vWF levels in patients with reactive thrombocytosis are significantly increased, thus probably compensating for the relative reduction of large von Willebrand factor multimers.

The direct relation between platelet count and large von Willebrand factor multimers is further supported by the observation that patients with thrombocytopenia have increased factor VIII/von Willebrand factor levels [25]. Hence, these data are consistent with a role of platelets in the homeostasis of plasma von Willebrand factor. This hypothesis is further substantiated with the experiments described in CHAPTER 7. Kinetic studies of von Willebrand factor in plasma in ET after infusion of DDAVP demonstrated a shortened half-life time of the collagen-binding activity of von Willebrand factor in plasma, suggesting an increased turnover of large vWF multimers. The decrease in half-life time normalized after platelet cytoreduction. Thus, in myeloproliferative disorders high platelet counts may potentially endanger normal hemostasis. These data are supported by the observation that majority of thrombo-

cythemia patients reported in the literature presenting with spontaneous bleeding (i.e. in the absence of platelet anti-aggregant therapy) associated with an acquired vWF deficiency show platelet counts > 2000 x 10^9/L [26,27]. In addition, it should be reminded that ET patients, by definition, lack the acute phase reaction which accompanies patients with reactive thrombocytosis.

The therapeutic use of platelet anti-aggregant agents may provide a second independent factor in the pathogenesis of hemorrhage in ET. In particular, evidence is accumulating that bleeding associated with the use of aspirin may be dose-related [28,29]. In those ET cases presenting with bleeding in association with platelet-antiaggregant therapy, platelet counts may be lower. As shown in CHAPTER 8, a modest decrease in large von Willebrand factor multimers in plasma may already be associated with an excessive prolongation of the bleeding time in individuals receiving aspirin. In these conditions, aspirin might inhibit potential compensatory pathways of primary hemostasis. Current evidence therefore would suggest that a reduction of large vWF multimers in plasma in combination with treatment with platelet anti-aggregants would enhance the risk of hemorrhage in ET. However, prospective studies investigating whether a reduction of large von Willebrand factor multimers may predict bleeding in ET are desired.

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APPENDIX

PREVENTION AND TREATMENT OF THROMBOTIC COMPLICATIONS IN ESSENTIAL THROMBOCYTHEMIA: EFFICACY AND SAFETY OF LOW-DOSE ASPIRIN

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SUMMARY

The efficacy and safety of aspirin in the prevention and treatment of thrombosis in patients with essential thrombocythemia (ET) was retrospectively analyzed in a cohort of 68 ET patients. Forty-one (60%) patients presented with thrombosis; five (7%) ET patients presented with bleeding; two (3%) patients had a paradoxical combination of bleeding and thrombosis at presentation. At presentation, patients with bleedings had significantly higher platelet and leukocyte counts than patients with thrombosis. A previous thrombotic event was identified as the only risk factor for thrombosis at presentation. During long-term follow-up the incidence of thrombosis was significantly reduced in patients receiving aspirin, either as monotherapy or in combination with cytoreduction. However, treatment with aspirin (500 mg/d) was associated with an increase in (minor) bleeding complications. In patients receiving aspirin, bleeding complications occurred particularly at platelet counts exceeding 1000 x 10^9/L. The overall 5- and 10-years survival probability was 93% and 84%, respectively, indicating that life expectancy in ET is close to normal. Although our data need confirmation in prospective clinical trials, they suggest that aspirin, particularly in lower doses (100 mg/d), may be a safe antithrombotic agent in ET with an acceptable risk for bleeding, if applied to patients with a platelet count < 1000 x 10^9/L and/or absence of a bleeding history.

INTRODUCTION

Although aspirin is beneficial in a wide range of patients at high risk for developing vascular occlusive events, the use of aspirin in myeloproliferative disorders (MPDs) has long been considered controversial [1]. On the one hand, treatment with high-dose aspirin in MPDs may be associated with an increased risk for bleeding [2]. On the other hand, microcirculatory thrombotic disturbances typical of the myeloproliferative disorder essential thrombocythemia (ET) such as erythromelalgia and transient nonlocalizing neurological symptoms have shown to be extremely sensitive to treatment with aspirin [3-7]. Since evidence is accumulating that, for control of the increased platelet count in ET, continuous treatment with hydroxyurea may also carry a risk of inducing secondary leukemia [8], the efficacy and safety of particularly low-dose aspirin obviously needs to be reassessed in the setting of myeloproliferative disorders [9]. Since the beneficial effect of aspirin was recognized for the prevention and treatment of erythromelalgia in ET in the 1970s, ET patients at our institution have preferably been treated with aspirin. As appropiate prospective studies aimed at evaluating the antithrombotic efficacy of aspirin in such a relatively rare disease as ET may be expected to take several years and would require a large number of patients, we performed a

retrospective follow-up study of a cohort of 68 ET patients diagnosed over a 20-year period at our institution, in an attempt to evaluate the efficacy and safety of aspirin in ET.

MATERIAL AND METHODS

Diagnosis

Between January 1974 and December 1993, ET was diagnosed in 68 patients according to accepted clinical and laboratory criteria, i.e platelet count ≥ 600 x 10⁹/L; no evidence or indication of raised red cell mass, taking into account the possibility of 'masked' pocythemia vera by concomitant iron deficiency; no Philadelphia chromosome; no support for a primary diagnosis of myelofibrosis or myelodysplastic syndrome; no demonstrated cause for reactive thrombocytosis [10]. The patient records of these 68 ET patients were analysed using a detailed questionnaire. Information was collected on date of birth, sex, date of diagnosis of ET and, if applicable, date and cause of death, treatment before, at, or after diagnosis; thrombotic events before, at, or after diagnosis; type of thrombosis (major arterial: stroke, myocardial infarction; minor arterial: transient ischemic attack, unstable angina pectoris, acute peripheral arterial acral occlusion (erythromelalgia); venous thromboembolic events); bleeding symptoms before, at, or after diagnosis; type of bleeding (cerebral bleeding; mucocutaneous bleeding including epistaxis, bruising and gum bleeding; gastrointestinal bleeding, including hematemesis and melena; muscle- and joint bleeding; petechiae; bleeding secondary to surgery). Bleeding was classified as major if hospitalization and/or blood transfusions were required. In addition, at presentation other risk factors for thrombosis were evaluated, which included smoking, hypertension, hypercholesterolemia and/or hypertriglyceridemia, diabetes mellitus and alcohol. Initial peripheral blood and bone marrow analysis included hemoglobin level, packed cell volume, erythrocyte, leukocyte and thrombocyte count, and bone marrow smear, biopsy and karyotyping.

Treatment strategies after diagnosis

In general, asymptomatic ET patients were not treated but followed until they became symptomatic. Exposure to potentially leukemogenic cytoreductive agents was always minimized as much as possible. Bleeding symptoms were a clear indication for cytoreductive treatment. Major arterial thrombotic events and microvascular thrombotic circulation disturbances such as erythromelalgia and nonlocalizing transient ischemic attacks were treated with cytoreductive treatment and/or aspirin. Initially, patients were treated with 500 mg/d aspirin. Since the late 1980s, however, patients have been treated with a lower aspirin dose (100 mg/d). Initial cytoreductive regimens consisted mainly of treatment with busulphan (BU), given in short courses of 2-4 mg daily, accord-

ing to the hematological response, under weekly control of peripheral blood. After induction of a complete remission (i.e., a platelet count < 350 x 10^9/L [6]), treatment with BU and, if applicable, aspirin was discontinued. Thereafter, patients were followed by careful observation. Because of the leukemogenic potential of BU, not more than 2 courses were normally given. Since the mid-1980s, however, hydroxyurea (HU) has been the drug of choice for reduction of the platelet count [11]. The starting dose of HU was 15-20 mg/kg/d. Thereafter, a maintenance dose of the drug was continuously administered to maintain the platelet count < 600 x 10^9/L without lowering the white cell count < 4.0 x 10^9/L.

During follow-up a total of 57 (84%) ET patients received aspirin; 37 patients received aspirin in combination with a cytoreductive regimen. A total of 44 (65%) ET patients were treated with a cytoreductive regimen. Thirty-two (47%) ET patients received BU; twenty (29%) patients were treated with HU, 9 of whom had received BU in the past; in another ET patient treatment with HU was stopped because of side-effects and treatment was continued with α-interferon. One ET patient was treated with radioactive phosphorus (1 course). Four ET patients did not receive any treatment during the whole follow-up period.

Follow-up related to treatment-regimen
Because of our specific interest in the efficacy and safety of aspirin as an antithrombotic drug in ET, the total follow-up time of each patient was divided into treatment-specific follow-up times according to the treatment regimen(s) the patient had received: I. careful observation; II. aspirin; III. cytoreduction; IV. combination of cytoreduction and aspirin. Each treatment-specific follow-up time ended with either the occurrence of a thrombotic or bleeding complication, or a change of treatment modality. In the cases where follow-up time was related to treatment with cytoreductive agents, the follow-up time ended with a relapse of ET (i.e., platelet count ≥ 500 x 10^9/L) after an intially complete remission. Censoring events were conversion of ET into another myeloproliferative or myelodysplastic disorder, death or end of study (31 December 1993). The total treatment-specific follow-up time of all patients and number of thrombotic or bleeding events within each treatment modality were counted; treatment-related incidence rates of thrombotic and bleeding events were calculated as the ratio of the number of events to the total treatment-specific follow-up time.

STATISTICS
All data were stored in a dBASE IV database and analysed using SPSS/PC+ 5.0.2. software.

Table A.1 Characteristics of 68 patients with essential thrombocythemia at presentation. Data are expressed as mean±SD or percentage.

		Patients	Normal range
Age (years)		57±15	
Sex ratio		40:28 (♂: ♀)	
Hemoglobin level (mmol/L)		8.8±1.3	8.2 - 10.2 (♂) 7.3 - 9.3 (♀)
Packed cell volume (L/L)		0.43±0.06	0.40 - 0.50 (♂) 0.35 - 0.45 (♀)
Platelet count (x 10^9/L)		922±423	140 - 360
Leukocyte count (x 10^9/L)		10.0±4.2	4.0 - 10.0
Splenomegaly (spleen length>12cm)		13/68 (19%)	
Presentation:			
Thrombosis	major arterial	4 (6%)	
	minor arterial	36 (53%)	
	venous	1 (1%)	
Bleeding	major	1 (1%)	
	minor	4 (6%)	
Paradoxical thrombosis and bleeding		2 (3%)	
Functional symptoms		6 (9%)	
Asymptomatic		14 (21%)	
Total		68 (100%)	

Diagnosis

Mann-Whitney-U tests were applied to compare the hemogram between ET patients with thrombosis and ET patients with bleeding at presentation. Risk factors for thrombosis at diagnosis were analysed initially by step-wise logistic regression analysis. Each risk factor was also tested separately using a Chi-square test or Fisher's exact test.

Follow-up

The treatment-specific number of events was assumed to have a Poisson distribution with expectation proportional to the treatment-specific follow-up time. Comparison of each treatment modality with careful observation ("no treatment"-arm) was made by comparing observed numbers of events with expected numbers of events. The expected numbers of events were calculated under the null hypothesis by partitioning the total number of events of the two treatment modalities over both treatment modalities proportionally to their numbers of person-years of follow-up. Given the total number of events of watchful waiting and another treatment modality, the number of events in

Table A.2 Hemogram of ET patients* with thrombotic and bleeding events at presentation. Data are given as median (interquartile range).

	Thrombosis (n=41)	Bleeding (n=5)	p-value
Hemoglobin (mmol/L)	8.8 (8.2 - 9.9)	6.3 (5.7 - 8.8)	p=0.016
Packed cell volume (L/L)	0.43 (0.40 - 0.47)	0.33 (0.32 - 0.45)	p=0.077
Platelet count (x 10^9/L)	750 (575 - 1031)	1244 (969 - 2239)	p=0.009
Leukocyte count (x 10^9/L)	9.1 (7.6 - 10.8)	19.7 (9.3 - 23.4)	p=0.028

*= Two ET patients who presented with paradoxical thrombosis and bleeding were excluded from the statistical analysis

either treatment modality is known to have a binomial distribution. Observed and expected numbers of events can then be compared in a 2x2 table using a Chi-square goodness-of-fit- test with 1 df.
An overall survival curve of all 68 ET patients was estimated according to the Kaplan-Meier method using the EGRET software package.

RESULTS

Characteristics of ET patients at presentation
As shown in table A.1 a total of 68 ET patients, 40 males and 28 females, ranging in age from 19 to 86 years (mean 57 years), were diagnosed. Forty-one ET patients (60%) presented with a thrombotic complication. Four patients (6%) suffered a major arterial thrombotic event (3 strokes, 1 myocardial infarction), 36 patients (53 %) presented with microcirculatory disturbances of peripheral, cerebral or coronary arteries (erythromelalgia in 21, transient nonlocalizing ischemic attacks (TIAs) in 6, unstable anginal pectoris in 4; 3 (4%) patients presented with both erythromelalgia and TIAs, whereas 1 (1%) patient suffered from TIAs and unstable angina at presentation; 1 patient presented with placental insufficiency due to recurrent placental infarction). Three ET patients presented with erythromelalgia despite adequate oral anticoagulation. One (1%) patient suffered from a deep venous thrombosis of the leg. Five (7%) patients presented with bleedings; 1 of them had a major gastrointestinal tract bleeding, whereas the other 4 ET patients suffered from minor mucocutaneous bleeding. Two (3%) ET patients had a paradoxical combination of bleeding and thrombosis at presentation; ery-

Table A.3 **Analysis of risk factors for thrombosis at time of diagnosis in 68 patients with essential thrombocythemia.**

Presence of risk factor		Fraction	p-value*
Smoking	thrombosis	11/33	p=0.85
	no thrombosis	8/20	
Hypertension	thrombosis	6/38	p=0.71
	no thrombosis	2/19	
Hyperlipidemia	thrombosis	8/24	p=0.68
	no thrombosis	2/10	
Diabetes	thrombosis	2/33	p=0.13
	no thrombosis	3/13	
Alcohol	thrombosis	4/32	p=0.27
	no thrombosis	5/19	
Sex (male)	thrombosis	27/43	p=0.54
	no thrombosis	13/25	
Age (years)	thrombosis	58±13	p=0.40
	no thrombosis	54±18	
Previous thrombosis	thrombosis	22/42	p=0.018
	no thrombosis	5/25	

*= Chi-square tests are used (or Fisher's exact test if expected cell frequencies are less than 5). The risk factor age was analyzed using a Mann-Whitney-U test.

thromelalgia and bruises in 1 ET patient, erythromelalgia and melena in the other. Six (9%) ET patients had functional symptoms such as dizziness, tinnitus, unexplainable headache and dysesthesias of hand or feet. Fourteen (21%) ET patients had no symptoms at diagnosis. As shown in table A.2, patients who presented with bleeding had significantly higher platelet and leukocyte counts and lower hemoglobin levels than patients who presented with a thrombotic event.

Analysis of risk factors for thrombosis at presentation
Stepwise logistic regression analysis (using the backwards elimination method), in which a number of risk factors were introduced simultaneously, revealed a previous thrombotic event as the only significant risk factor for thrombosis at presentation in ET. Neither stepwise logistic regression analysis nor testing single factors using a Chi-square test or Fisher's exact test identified smoking, hypertension, hyperlipidemia, diabetes, alcohol, sex or age as a significant risk factor for thrombosis in ET (table A.3).

Thrombotic and bleeding events during long-term follow-up
After diagnosis, the 68 ET patients were followed for a total of 419 person-years (mean follow-up 6.2 years). As shown in table A.4, 42 thrombotic events, mainly consisting of erythromelalgia and TIAs, were observed in 19 (28%) ET patients. The majority of these thrombotic complications occurred

Table A.4 Incidence of thrombotic and bleeding complications in 68 patients with essential thrombocythemia, who had long-term follow-up, according to treatment strategy. Derived from [40].

Treatment	Duration of follow-up (person-yr)	Thrombotic complications		Bleeding complications	
		Events (n)	Events/100 person-yr	Events (n)	Events/100 person-yr
Watchful waiting	127	27	32.3	2	1.6
Low-dose aspirin	139	5	3.6[1]	10	7.2[4]
Cytoreduction	113	10	8.9[2]	2	1.8[5]
Low-dose aspirin and cytoreduction	40	0	0[3]	4	10.0[6]
Total	419	42		18	

Legend:
[1] = $p<.001$ ($\chi^2 = 17.3$, 1 df), for the comparison with watchful waiting (thrombosis)
[2] = $p=.014$ ($\chi^2 = 6.0$, 1 df),""
[3] = $p=.003$ ($\chi^2 = 8.6$, 1 df) ,""
[4] = $p=.032$ ($\chi^2 = 4.6$, 1 df), for the comparison with watchful waiting (bleeding)
[5] = $p=.92$ ($\chi^2 = 0.01$, 1 df),""
[6] = $p=.014$ ($\chi^2 = 6.0$, 1 df),""

in patients without treatment (careful observation). In patients receiving cytoreductive treatment 10 thrombotic complications occurred during follow-up, at platelet counts of 624±255 x 10^9/L, respectively, indicating an inadequate control of thrombocytosis. The incidence rate of thrombotic complications was significantly reduced in patients receiving aspirin, either as monotherapy or in combination with cytoreduction (table A.4).

During follow-up 18 bleeding complications were observed in 14 (21%) patients. These bleeding episodes were usually minor in nature (mainly epistaxis and skin bleeding) and occurred in the majority of the cases in association with aspirin treatment, either as monotherapy or in combination with cytoreduction. In patients receiving aspirin as monotherapy 10 bleedings were observed at platelet counts ranging from 661 to 3460 x 10^9/L (mean 1737 x 10^9/L). One ET suffered a fatal hemorrhagic stroke whilst receiving 2 g/d of aspirin at an increased platelet count of 2200 x 10^9/L. The other - all minor - bleeding complications associated with aspirin occurred mainly at a dose of 500 mg/d. Only 4 of the initially 14 asymptomatic ET patients remained asymptomatic after at least 30 person-years of follow-up.

Survival analysis
During long-term follow-up 7 (10%) ET patients, 6 males and 1 female, ranging in age from 59 to 79 years (mean 72 years), died 6±6 years after diagnosis. Causes of death were pulmonary embolism, pneumonia, prostatic cancer in 3 cases; cardial insufficiency after recurrent myocardial infarctions while on oral anticoagulant treatment in 1 case; hemorrhagic stroke during treatment with high-dose aspirin in 1 patient; end-stage myelofibrosis with leukemic transformation in 1 patient with a treatment history of busulphan, respectively. In 1 case the cause of death was unknown. The overall 5 and 10 years survival probability (independent of treatment regimen) of all 68 ET patients was 93% and 84%, respectively.

Conversion of ET
In 12 (18%) ET patients a progression towards another myeloproliferative disorder or myelodysplastic disorder was observed. Six patients developed polycythemia vera after a mean of 4.9 years, 2 progressed to a myelodysplastic syndrome after 5.4 years and 4 developed myelofibrosis after 5.6 years after diagnosis of ET, respectively.

DISCUSSION

The present study emphasizes that thrombotic complications pose a greater clinical hazard than bleeding complications in ET. Particularly microvascular thrombotic complications such as erythromelalgia and transient ischemic

attacks dominated the clinical picture at presentation. Although the high frequency of these microvascular thrombotic complications in our study may be due partly to a referral bias because of our specific interest in erythromelalgia, other studies have also found a high frequency of particularly microvascular thrombotic complications, both in retrospective [12-16] and prospective studies [17]. The latter study also demonstrated the benificial effect of cytoreduction of an increased platelet count by HU on the occurrence of thrombotic complications in ET, thus stressing the thrombogenic potential of even a slightly increased platelet count in ET. This concept is further corroborated by the finding in our study where those patients who developed thrombosis while receiving cytoreductive treatment all had platelet counts in excess of 400 x 10^9/L, indicating an inadequate control of thrombocytosis. This is in line with the finding of Cortelazzo et al [17] where 2 ET patients who were treated with HU and suffered a thrombotic complication still had increased platelet counts (490 and 632 x 10^9/L, respectively). Additional clinical evidence is provided by our observations in patients with thrombocythemia that erythromelalgia may develop at slightly increased platelet counts, i.e. > 400 x 10^9/L [6]. Furthermore, in this study risk factors for thrombosis were also analysed. Consistent with the findings of Cortelazzo et al [15] and Colombi et al [16], a previous thrombotic event was identified as an important risk factor for thrombosis in ET, presumably reflecting the arterial thrombophilia in ET. Some authors have also reported age as a risk factor for thrombosis in ET [15]. Although ET patients who presented with thrombosis tended to be older in our study, age was not identified as a risk factor for thrombosis.

Interestingly, thrombotic complications are rarely or not at all observed in patients with reactive thrombocytosis who have a comparable rise in the platelet count [10]. This observation suggests that, besides the increase in the number of circulating platelets, abnormal platelet function may also be an important determining factor for the observed arterial thrombotic predisposition in ET. Tests of platelet function are usually of little help in accurately predicting either thrombotic or bleeding complications in ET [10,18,19], although platelet function in ET may change during the course of the disease [20]. However, several studies have shown that microcirculatory disturbances typical of ET such as erythromelalgia and nonlocalizing transient ischemic attacks are extremely sensitive to inhibition of platelet cyclooxygenase activity and aggregation [6,7,21]. Drugs which do not affect platelet cyclooxygenase activity, including oral anticoagulants, appear to be ineffective in the treatment of erythromelalgia [6]. Moreover, the results of this study suggest that aspirin is not only effective in the treatment of microvascular disturbances but also reduces recurrences of thrombotic events in symptomatic ET patients after long-term follow-up. The finding that also asymptomatic ET patients may have an enhanced biosynthesis of thromboxane, most likely reflecting platelet activation *in vivo*, which may be suppressed by low-dose aspirin, pro-

vides further arguments for using aspirin as an antithrombotic agent in ET [22]. In this respect, it is certainly noteworthy that only 4 of the initially 14 asymptomatic ET patients in our study remained asymptomatic without treatment during long-term follow-up.

Unfortunately, treatment with aspirin is associated with bleeding side-effects. In our study we observed 18 bleeding episodes in the follow-up period, the majority of which occurred in patients receiving aspirin either as monotherapy or in combination with cytoreduction. Although we observed 1 fatal cerebral hemorrhage associated with aspirin use, conceivably related to its extremely high dose used at that time, bleeding is usually minor. Consistent with the finding of others [12,23,24], bleedings in ET were associated with platelet counts exceeding 1000 x 10^9/L. In addition, the majority of the ET patients who had bleeding complications during follow-up whilst receiving aspirin had platelet counts in excess of 1000 x 10^9/L, suggesting that aspirin should probably not be prescribed or used with caution in patients with high platelet counts. The finding of an acquired von Willebrand factor defect in MPD patients associated with high platelet counts is in this respect certainly noteworthy [25-27].

The overall 5- and 10-years survival probability in our study of 93 and 84%, respectively, of 68 ET patients with a mean age of 57 years at diagnosis, indicates that life expectancy in ET can be considered normal or nearly normal. These survival probabilities are comparable with the survival of ET patients reported elsewhere [16]. The (nearly) normal life-expectancy in ET suggests that the use of agressive myelosuppressive treatment should probably restricted to those patients in whom the treatment indication outweights the risk of inducing leukemia. If cytoreduction is indicated for the young ET patient, treatment with potentially non-leukemogenic agents such as α-interferon or anagrelide may be attractive alternatives for hydroxyurea, although the experience with these treatment options is still limited and hampered by their frequent side-effects, drug availability and cost-benefit ratio [10].

In conclusion, our data suggest that aspirin is a safe and effective antithrombotic agent in patients with ET. Although the indiscriminate use of aspirin in patients with ET is contraindicated, aspirin, particularly in lower doses (i.e., 100 mg/d) may be a safe antithrombotic agent with an acceptable risk for bleeding if applied to selected cases, i.e., platelet count < 1000 x 10^9/L and absence of a bleeding tendency. Uncontrollable thrombocytosis (platelet count exceeding 1000 x 10^9/L) and a bleeding tendency are probably rational indications for cytoreductive treatment. Prospective studies in ET evaluating the antithrombotic efficacy of low-dose aspirin within the given frame are indicated.

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SAMENVATTING

Essentiële thrombocythemie (ET) is een chronische aandoening van het beenmerg leidend tot een aanhoudende verhoging van het aantal bloedplaatjes in het perifere bloed. De belangrijkste eigenschappen van deze ziekte worden in HOOFDSTUK 1 weergegeven. Het klinisch beloop wordt vooral gekenmerkt door het herhaaldelijk optreden van trombose (bloedstolsel) in met name de kleinere slagaders van handen, voeten, hart en hersenen. Opvallend is dat deze complicaties niet of nauwelijks optreden bij patienten met een reactieve trombocytose (RT), die om een andere reden (bijv. infectie of miltverwijdering) een verhoogd aantal bloedplaatjes hebben. Deze waarneming suggereert dat de reden waarom ET patienten zo vaak een trombose ontwikkelen waarschijnlijk niet alleen door het aantal bloedplaatjes verklaard kan worden maar dat ook andere factoren een rol spelen, zoals bijvoorbeeld de funktie van de bloedplaatjes.

Om de rol van bloedplaatjes bij het ontstaan van trombose van de handen en voeten (erythromelalgie), de complicatie die het meest voorkomt bij patienten met ET, te onderzoeken is in HOOFDSTUK 2 de overleving van gelabelde bloedplaatjes bij ET patienten met en zonder klachten van erythromelalgie bepaald. In tegenstelling tot ET patienten zonder klachten bleek bij patienten met erythromelalgie de overleving van gelabelde bloedplaatjes sterk verkort te zijn, waarschijnlijk omdat de bloedplaatjes door de stolselvorming verbruikt worden. Dit trad ook op indien deze patienten behandeld werden met bloedverdunners (Marcoumar, Sintrom). Als patienten met erythromelalgie hierna echter behandeld werden met aspirine, dat het samenklonteren van bloedplaatjes tegengaat, bleek de overleving van gelabelde bloedplaatjes weer normaal te worden.

De waarneming dat erythromelalgie niet goed te behandelen is met bloedverdunners, hetgeen misschien betekent dat de stolling van minder groot belang is voor het ontstaan van erythromelalgie, vormde het uitgangspunt voor de studies die in HOOFDSTUK 3 beschreven worden. Als belangrijkste bevindingen leverden deze studies op - naast dat erythromelalgie gepaard lijkt te gaan met aktivatie van bloedplaatjes en beschadiging van de vaatwand - dat de vorming van fibrine, het eindprodukt van de stolling (dit wordt geremd met bloedverdunners), inderdaad niet van groot belang lijkt te zijn voor het ontstaan van erythromelalgie. Uit weefselstukjes afgenomen uit de huid van patienten met erythromelalgie bleek dat de bloedstolsels bij erythromelalgie nauwelijks fibrine bevatten, hetgeen ondersteund dat erythromelalgie veroorzaakt wordt door het samenklonteren van bloedplaatjes en dat aktivatie van de bloedstolling van ondergeschikt belang lijkt te zijn.

In HOOFDSTUK 4 wordt de aktivatie van bloedplaatjes in relatie tot het ontstaan van erythromelalgie onderzocht. Bij het merendeel van de patienten met erythromelalgie in de voorgeschiedenis ontstaat binnen 10 dagen na het stoppen van aspirine opnieuw erythromelalgie. De klachten van erythromelalgie worden voorafgegaan door een toenemende mate van aktivatie van bloedplaatjes. Bij patienten die geen erythromelalgie ontwikkelen blijft de mate van aktivatie beperkt en gelijk. Met lage dosis aspirine (50 mg of 100 mg per dag), waarbij de stofwisseling van alleen het bloedplaatje geremd wordt, is het mogelijk om de aktivatie van het bloedplaatje bijna volledig te remmen, waarna de klachten van erythromelalgie verdwijnen. Deze bevindingen ondersteunen de gedachtengang dat erythromelalgie veroorzaakt wordt door het spontaan samenklonteren van bloedplaatjes in de kleinere slagaders van handen en voeten. De reden waarom bij sommige patienten de bloedplaatjes spontaan worden geaktiveerd en bij anderen niet, is onduidelijk. Dit zal in andere studies nog onderzocht moeten worden.

Patienten met ET hebben naast trombotische complicaties soms ook last van bloedingen. De bloedingsneiging (blauwe plekken, neusbloedingen, nabloedingen na een operatie en bloedverlies via de darmen) lijkt op die van patienten met de ziekte van von Willebrand. Sommige onderzoekers hebben gevonden dat bloedingen vaker dan trombose lijken voor te komen bij ET patienten als zij een hoger aantal bloedplaatjes hebben. In HOOFDSTUK 5 hebben wij dan ook gedurende een aantal jaren bij één ET patient de von Willebrandfaktor spiegels en aktiviteit in relatie tot klachten en het aantal bloedplaatjes vervolgd. Deze patient maakte twee episodes door van bloedingen. Beide keren bleken op dat moment het aantal bloedplaatjes hoog (> 2000 x 10^9/L) en de von Willebrand factor aktiviteit laag te zijn. Als met medicijnen, Hydrea het aantal bloedplaatjes omlaag werd gebracht, nam de aktiviteit van von Willebrand factor weer toe. Als alle waarden, die in een aantal jaren verzameld waren, met elkaar werden vergeleken bleek dat er een omgekeerde relatie bestond tussen het aantal bloedplaatjes en de activiteit van von Willebrand factor in plasma.

In HOOFDSTUK 6 wordt deze bevinding bij grotere groepen van zowel ET patienten als patienten met een reactieve trombocytose bevestigd. In HOOFDSTUK 7 wordt onderzocht waarom patienten met een hoog aantal bloedplaatjes een lagere aktiviteit van von Willebrand factor hebben. Het blijkt dat bij deze patienten de klaring van een bepaald deel van von Willebrand factor dat met name van belang is voor de aktiviteit (de zgn. hoog-moleculair-gewichts multimeren) sneller te zijn dan bij een laag aantal bloedplaatjes. Alhoewel dit nog niet bewezen is, lijkt het voor de hand te liggen dat de hoog-moleculair-gewichts multimeren sneller worden afgebroken door bloedplaatjes in geval van een hoger aantal circulerende bloedplaatjes. In HOOFDSTUK 8 wordt onderzocht of de lagere spiegel van de hoog-moleculair-gewichts multi-

meren van von Willebrand factor een verklaring vormt voor de onevenredige verlenging van de bloedingstijd die bij sommige ET patienten optreedt na inname van aspirine. Het blijkt dat een lage spiegel van de hoog-moleculair-gewichts multimeren van von Willebrand factor in plasma inderdaad een belangrijke factor is bij deze verlenging van de bloedingstijd na inname van aspirine aangezien deze verlenging niet optreedt indien de lage bloedspiegel van de hoog-moleculair-gewichts multimeren met medicijnen (DDAVP) wordt opgehoogd. Deze bevindingen veronderstellen dat met name de hoog-moleculair-gewichts multimeren van von Willebrand factor in het bloed van belang kunnen zijn voor het voorkomen van bloedingen. Dat een lagere spiegel van deze multimeren ook daadwerkelijk een hoger bloedingsrisico inhoudt zal uit toekomstige klinische studies moeten blijken.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 31 juli 1966 geboren te Spijkenisse. Hij volgde zijn middelbare schoolopleiding aan de 'Blaise Pascal' te Spijkenisse, alwaar hij in 1984 het VWO diploma behaalde. In dat zelfde jaar werd aangevangen met de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. In juli 1991 behaalde hij zijn arts-examen (met lof). Van september 1991 tot april 1992 verrichtte hij op de afdeling Hematologie van het Dijkzigt ziekenhuis (supervisie Dr. J. Stibbe) onderzoek op het gebied van hypercoagulabiliteit bij (niet-)hematologische maligniteiten. Na een aantal maanden van voorbereidend werk, werd in het kader van een AGIKO (assistent-geneeskundige in opleiding tot klinisch onderzoeker) constructie van 1 januari 1993 tot juli 1995 het onderzoek verricht (supervisie Dr. J.J. Michiels), dat uiteindelijk tot dit proefschrift geleid heeft. Sinds 1 juli 1995 is hij in het Academisch Ziekenhuis te Rotterdam in opleiding tot internist (opleider Prof. J.H.P. Wilson).

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