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The Bleeding Time Is Inversely Related to Megakaryocyte Nuclear DNA Content and Size in Man

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Key words

Bleeding time – Megakaryocytes – Platelets – Platelet volume

Summary

The relation between the bleeding time and the megakaryocyte nuclear DNA content and size was evaluated in eleven consecutive patients with normal steady state thrombopoiesis undergoing thoracotomy. A statistically significant inverse correlation was found between the bleeding time and both megakaryocyte DNA content ($r = -0.71$, $p < 0.05$), megakaryocyte total size ($r = -0.58$, $p < 0.05$), megakaryocyte cytoplasmic size ($r = -0.64$, $p < 0.05$) and megakaryocyte nuclear size ($r = -0.58$, $p < 0.05$). The megakaryocyte total size and the megakaryocyte cytoplasmic size were statistically significantly larger in men than women ($p < 0.02$ and $p < 0.03$ respectively). Changes in the megakaryocytes in the bone marrow are associated with changes in primary haemostasis in normal individuals.

Introduction

The cutaneous bleeding time is generally accepted as an indicator of in vivo platelet reactivity (1). The bleeding time is prolonged in patients with decreased platelet reactivity and in severe thrombocytopenia (1) and has recently been shown to be shortened in the acute phase of a myocardial infarction (2).

Platelets are produced from the cytoplasm of megakaryocytes. The bleeding time was first shown to be related to the platelet count (3) but this was later shown only to be true for platelet counts lower than 100×10^9/l (1). Studies using more accurate methods for measuring the bleeding time have indicated that in humans with normal steady state thrombopoiesis (4) and in patients with chest pain (2) the bleeding time is determined by the platelet mass (platelet count × mean platelet volume). Recent studies indicate that platelet volume does not change during the platelets lifespan in the circulation (5, 6). This suggests that the haemostatic potential of the platelet is determined at the time of thrombopoiesis and that the nature of the megakaryocyte is a determinant of the bleeding time.

In the present study the link between haemostasis and megakaryocyte DNA content and size in humans with normal thrombopoiesis was evaluated for the first time.

Patients, Materials and Methods

Eleven consecutive patients undergoing thoracotomy for repair of a hiatus hernia (10 patients) or resection of a condroma (1 patient) were included in the study. The patients gave informed consent and the study was approved by the Ethics committee. None of the patients had taken aspirin or other non steroidal antiinflammatory drugs for 2 weeks prior to the study. The patients were not allowed to smoke for 6 hours before the study. None of the patients were on anticoagulants and none of them had received blood transfusions prior to the operation. None had cancer or acute or chronic inflammatory disease. Smoking habits and medication were recorded.

The following variables were measured on the day before the operation: the bleeding time was measured after 15 minutes supine rest using the Simplate II device (General Diagnostics, New Jersey, U.S.A.). A blood pressure cuff was inflated to a pressure of 40 mm Hg and horizontal incisions were made on the lateral aspect of the forearm approximately 5 cm below the antecubital crease (7). All measurements were done by the same investigator. Blood samples were obtained from the cubital vein of the contralateral arm immediately after measurement of the bleeding time. Blood was anticoagulated with sodium citrate/prostaglandin E_1 and the platelet count and mean platelet volume were measured as described earlier (5). Briefly, platelet count was measured in whole blood using a Coulter ZB and the platelet volume distribution was measured by a resistive particle counter coupled to a computer via an analogue-to-digital converter in a representative population of platelets separated from whole blood by velocity sedimentation in continous non-linear Percoll gradients. The platelet recovery was calculated as the ratio between the platelet count in the platelets obtained from the gradient and the platelet count in whole blood. The platelet mass was calculated as the product of the platelet count and mean platelet volume.

During the operation a piece of rib approximately 3 cm in length was removed and dissected free of muscular and connective tissue. The rib was opened longitudinally. For measurement of the megakaryocyte DNA content approximately ⅔ of the bonemarrow was gently washed out into 18 ml of precooled phosphate-buffered saline (12° C) containing 3 mmol/l K_2-EDTA, 7.5 mmol/l d-glucose, 1 mmol/l Na_2SO_4, 5 mmol/l KCl, 100 U/ml DNA'ase (Sigma, St. Louis, U.S.A.) and 50 µg/l freshly prepared prostacyclin (Wellcome Research Laboratories, Beckenham, Kent, U.K.). Osmolarity of the medium was adjusted to 280 mOsm/l (pH = 7.4). The solution was syringed once through a 21G needle and 6 ml aliquots of the monodispersed cell suspension were layered on top 5 ml of gradients (12° C) consisting of 30% Percoll (Pharmacia, Sweden) and 70% of the medium described above (volume/volume). After centrifugation at 190 g for 20 minutes (12° C) the red cells and their precursors lay at the bottom of the gradient. The supernatant above the red cells was removed and centrifuged at 140 g (12° C) for 4 minutes. The supernatant was discarded and the pellet resuspended in medium giving a cell suspension containing approximately 10^6 cells/ml. These procedures were carried out within 40 minutes after resection of the rib. The cell suspension was stained with mithramycin (Sigma) and ethidium bromide (Sigma) (8). The DNA cell distribution was measured using flow cytometry on a FACS 420 (Beckton Dickinson) coupled to a Hewlett Packard computer. The 2N and 4N cells were gated off and the megakaryocyte DNA distribution was given as the relative frequency of 8N, 16N, 32N and 64N cells. The arithmetric mean of the DNA distribution was calculated (9).

For measurement of megakaryocyte size pieces of marrow were immediately placed in 10% phosphate-buffered-formaline and fixed for 24 hours. These were embedded in glycol methacrylate, sectioned at 3 µm and stained with haematoxylin-eosin. The mean nuclear and total megakaryocyte size were calculated from measurements of microscopic planimetric area of 50 randomly selected megakaryocytes with eosinophilic cytoplasm and at least 3 nuclear lobes [stage II, III and IV according to Levine (10)] using a Kontron MOP AM03 Videoplan with a distribution facility. Megakaryocyte cytoplasmic areas were calculated as total area minus nuclear area. All measurements were done blindly on coded samples by the same investigator. The coefficient of variation based on 6 double measurements was 6.8%.

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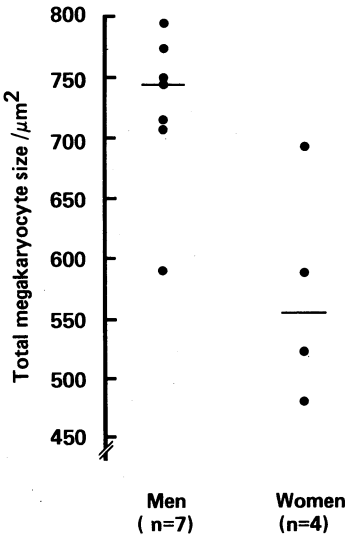

Fig. 1 The total megakaryocyte size given as the mean of the area of 50 megakaryocytes measured by microscopic computerized planimetry in bone marrow sections from humans with normal thrombopoiesis. The total megakaryocyte size was statistically significantly larger in men than in women ($p < 0.02$)

Statistics

All results are given as medians with range values in brackets. The variables were compared between men and women using the Mann-Whitney U test. Correlation studies performed between the measured variables was done using Spearman's rank correlation test. All probabilities were based on two tailed tests.

Results

Four of the 11 patients were women. The median age was 52 years (24–67 years). There was no statistically significant difference in age between men and women. Three of the patients were smokers (one woman, two men). The platelet count [224 × 10^9/l (140 × 10^9/l–345 × 10^9/l)] and the bleeding time [323s (216s–402s)] for all patients were within the normal range. The median platelet recovery was 90% (80–99%). The bone marrow when examined using light microscopy was normal in all patients.

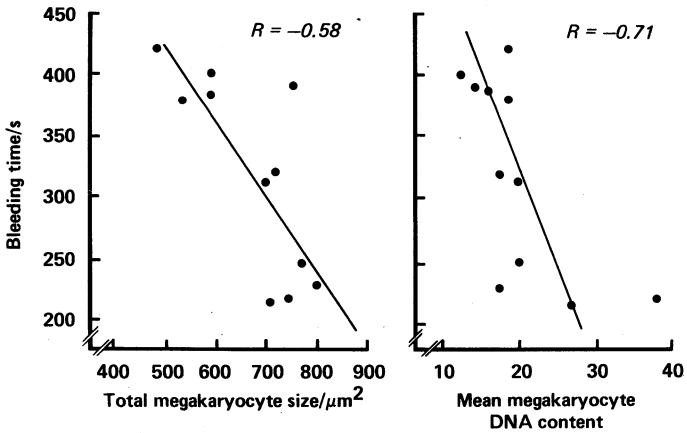

Fig. 2 Scattergrams illustrating the significant inverse relationship between the bleeding time and total megakaryocyte size ($p < 0.05$) and between the bleeding time and mean megakaryocyte DNA content ($p < 0.05$) in 11 humans with normal thrombopoiesis

The total megakaryocyte size was statistically significantly larger in men than in women [744 μm² (589 μm²–795 μm²) versus 558 μm² (481 μm²–691 μm²); $p < 0.02$ (Figure 1)] as were the megakaryocyte cytoplasmic volume (569 μm² (433 μm²–591 μm²) versus 422 μm² (374 μm²–495 μm²); $p < 0.03$). The bleeding time was shorter in men than in women [250 s (216s–402s) versus 383s (315s–420s)] but the difference was not statistically significant. No statistically significant difference in megakaryocyte nuclear size or DNA content or in any of the platelet variables could be demonstrated between men and women.

The bleeding time was found to be significantly inversely correlated to both total megakaryocyte size [$r = -0.58$, $p < 0.05$ (Figure 2)], megakaryocyte cytoplasmic size ($r = -0.64$, $p < 0.05$) and megakaryocyte nuclear size ($r = -0.58$, $p < 0.05$). There was also a significant inverse correlation between bleeding time and megakaryocyte DNA content [$r = -0.71$, $p < 0.05$ (Figure 2)]. No significant correlation between the bleeding time and platelet count, mean platelet volume or platelet mass could be demonstrated.

Megakaryocyte nuclear size was significantly inversely correlated to both platelet count ($r = -0.69$, $p < 0.05$) and platelet mass ($r = -0.79$, $p < 0.01$). Platelet count and platelet mass were both significantly inversely correlated to age ($r = -0.75$, $p < 0.05$ and $r = -0.87$, $p < 0.01$ respectively). An inverse relationship between mean platelet volume and platelet count was found ($r = -0.55$) as previously reported (11, 12) but this was not statistically significant. No relationship between mean platelet volume and any of the megakaryocyte variables could be demonstrated.

Discussion

This study shows for the first time that the bleeding time is inversely related to the size and DNA content of the megakaryocytes in individuals with normal thrombopoiesis. The previously described relationship between bleeding time and platelets (2, 3, 4) may now be referred to the progenitor cell. The present result is strengthened by the fact that the megakaryocyte size and DNA content were measured independently in whole marrow and in enriched cell suspensions obtained from the same individual.

Megakaryocytes are unique among mammalian cells in that they can redouble their DNA content without dividing (13). Animal studies indicate that when platelets are destroyed by injection of serum containing antiplatelet antibodies an increase in megakaryocyte DNA content and size in the bone marrow occur 1–4 days later (14, 15, 16). The platelets produced from these large megakaryocytes with high ploidy have been shown to be more reactive than platelets produced by megakaryocytes of normal size (16). The control mechanism is not understood but one or more hormones [thrombopoietin(s)] have been postulated to be involved (17). In normal steady state in humans the presence of high ploidy megakaryocytes is associated with a short bleeding time. It is thus likely that similar control mechanism(s) that can induce an increase in megakaryocyte ploidy in response to an increased haemostatic demand exist in man.

In the present study the megakaryocyte DNA distribution was measured by flow cytometry after DNA labelling with DNA fluorescent stain. This method is a modification of the technique used in a study on patients with various haematological disorders or coronary artery disease, where a significant inverse correlation between the arithmetic mean megakaryocyte DNA content and the mean platelet volume was found (9). We were unable to demonstrate an inverse correlation between the arithmetic mean megakaryocyte DNA content and the mean platelet volume in humans with a normal thrombopoiesis in steady state. It is difficult to obtain a sufficient amount of suitable bone marrow

from normal humans, and the lack of such correlation in our study could be due to the small number of subjects studied. Aggregates of diploid or tetraploid cells have been claimed to be erroneously included in the megakaryocyte DNA histogram. Using the present technique with prostacyclin and DNA'ase in the cell medium, we did not observe peaks of cells in the DNA histogram corresponding to a cellular DNA content of 6N, 10N, 12N etc. Also no cell aggregates were found when cell suspensions were examined by phase contrast microscopy.

Our present finding of an increased megakaryocyte size in men compared to women suggests that in individuals with thrombopoiesis in steady state men may produce platelets with a higher reactivity compared to women. This is supported by the report by O'Brien et al. who found a shorter bleeding time in normal men than in normal women (18). In the present study the bleeding time was also found to be shorter in men than in women but this did not reach statistical significance probably due to small number of subjects studied.

It has been shown that patients suffering from acute myocardial infarction or sudden cardiac death have larger megakaryocytes than age matched control groups (19). In another study the bleeding time was found to be shorter in patients suffering from acute myocardial infarction than in patients with chest pain but no definite infarction (2). The bleeding time has also been shown to be shorter in patients who had had a myocardial infarction 3 month to 5 years prior to investigation compared to age and sex matched controls (18). Our present results give an explanation for the concomitant occurrence of large megakaryocytes and shortened bleeding time in myocardial infarction. In myocardial infarction primary changes at the megakaryocyte level or a stimulation of the above described control mechanism(s) may be present and may in some patients be involved in the pathogenesis of the disease. Furthermore, bearing in mind the higher incidence of acute myocardial infarction in men, our present finding that the megakaryocytes are larger in men than in women highlights the necessity for further studies on megakaryocytes in cardiovascular disease.

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