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Chapter 6

Juvenile cyclic amegakaryocytic thrombocytopenia, a novel entity

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Summary

Cyclic thrombocytopenia is a rare disorder described in adults, characterized by periodic platelet count fluctuations of unknown etiology. We report on a male child with cyclic changes of platelet counts ranging from $2 \times 10^9/L$ to $224 \times 10^9/L$ with a periodicity of 25 days. Since birth, the patient had periods of bruising. Platelet counts, measured first at the age of eleven months, were periodically low during these periods of bruising. Thrombopoietin plasma levels oscillated inversely with the platelet count, whereas glycofalin levels oscillated in phase with the platelets. No oscillation was seen in neutrophil and reticulocyte numbers. The bone marrow showed periodic reduction in megakaryocyte counts. In an *in-vitro* megakaryocytopoiesis assay, the patients' CD34+ cells showed megakaryocyte formation, although to a lower level than controls. Addition of patients' plasma, collected during the rise in platelet numbers, to cultures with normal bone marrow-derived CD34+ cells caused an increase in the development of CD41+ megakaryoblasts.

Because the periods with bruising existed since birth, apparently this is a form of congenital cyclic thrombocytopenia. The underlying mechanism of the cyclic thrombocytopenia in this patient is not yet clear, and until now, no therapy has been found for this patient. However, platelet transfusions have resulted in cessation of bleedings during thrombocytopenic periods.

Introduction

Cyclic thrombocytopenia is a rare acquired disorder described for only a few adult patients and characterized by a regular periodic fluctuation in the platelet count, varying from severe thrombocytopenia to a normal or even increased platelet count. The fluctuations in platelet count can be secondary to a defective platelet production (amgakaryocytic type) or to an increased platelet destruction.^{1,2,3} Both forms have only been described in adult patients. In the described cases, male patients suffered from amgakaryocytic cyclic thrombocytopenia, whereas in most female patients cyclic destruction of platelets, sometimes in concordance with hormonal variations, seemed to be the origin of platelet variations.

We describe a male child with a cyclic bleeding tendency since birth, and proven cyclic thrombocytopenia since the first year of life. The possible pathogenesis of this disorder is discussed.

Materials and methods

Thrombopoietin (Tpo) and glycofalin (GC) measurements

For the measurement of Tpo and GC plasma levels, EDTA-anticoagulated blood was collected. Tpo levels were measured with a solid-phase sandwich ELISA as previously described.⁴ Normal Tpo levels range from 4 to 34 AU/ml. GC plasma levels were measured with an ELISA as described by Porcelijn et al.⁵ Normal GC values as determined in 95 healthy adult individuals were between 144-444 AU/ml. In a former study on different causes of congenital thrombocytopenia,⁶ Tpo and GC plasma levels were measured in an age-related control group composed of 56 children. The mean Tpo and GC plasma levels in this group were in the same range as found in healthy adults.

Megakaryocytopoiesis

Bone marrow samples were obtained five times throughout the platelet cycle. Quantitation of megakaryocytes in the bone marrow smears were performed on randomly numbered slides by three experienced hematologists.

Megakaryocyte expansion culture

Bone marrow cells for expansion cultures were taken from the patient at two time points. The first bone marrow sample was taken six days after the platelet count had reached its nadir (ascending leg), the second bone marrow sample was taken six days before the platelet count had reached its nadir (descending leg). After informed consent had been obtained, control bone marrow cells were derived from healthy children who served as stem-cell donor for a sibling. Bone marrow was anticoagulated with heparin. Mononuclear cells were isolated from bone marrow by density gradient centrifugation over Ficoll (1.077 g/cm³; Pharmacia Biotech, Uppsala, Sweden). Subsequently, CD34⁺ cells were isolated by magnetic cell sorting (VarioMACS system; Myltenyi Biotec, Gladbach, Germany) according to the manufacturer's instructions. This resulted in a purity of more than 95% CD34⁺ cells, as determined by FACS analysis. CD34⁺ cells were cryopreserved until needed. After thawing, cells were washed in IMDM (BioWhittaker, Europe) + 0.2% (w/v) human serum albumin (Sanquin) and subsequently cultured in Stemspan SF expansion medium (StemCell Technologies, Vancouver, BC, Canada). Two x 10⁵ CD34⁺ cells in a total volume of 1 ml were cultured in the presence of full-length rhTpo (10 ng/ml, a generous gift of Genentech, San Francisco, CA, USA) and Interleukin-1 β (IL-1 β ; 10 ng/ml, Strathmann Biotec AG, Hamburg, Germany) in a 12-well plate (Costar, Cambridge, MA, USA) as described by van den Oudenrijn et al.⁷ Indicated cultures were supplemented with 10% patient plasma (v/v) or AB plasma from a healthy donor. Cells were cultured for seven days at 37°C, 5% CO₂ without additional feeding of growth factors or medium.

Flow cytometry and monoclonal antibodies

Cells were harvested on day seven of culture. Directly after harvesting, the cells were fixed on ice with 1% (w/v) paraformaldehyde for 10 minutes. The cells were gently centrifuged for 10 minutes (180g) with the brake on half maximum and were resuspended in PBS containing 0.2% (w/v) bovine serum albumin (BSA). Subsequently, cells were incubated with the indicated antibodies.

The cells were incubated with fluorescein isothiocyanate- (FITC) or phycoerythrine- (PE) labeled monoclonal antibodies (moabs) for 30 minutes at 4°C. Isotype-matched mouse IgG subtypes served as controls. After 30 minutes of incubation, the cells were washed with PBS 0.2% (w/v) BSA and analyzed by FACScan (Becton Dickinson, San Jose, CA, USA).

The following FITC-conjugated moabs were used: IgG1 isotype control (CLB-203; Sanquin), CD41 (megakaryocytes/platelets; CLB-thromb/7, 6C9; CLB) and CD42b (megakaryocytes/platelets; CLB-704; Sanquin). Phycoerythrin (PE)-conjugated moabs were: IgG1 isotype control (X40; Becton Dickinson, San Jose, CA, USA), CD33 (myeloid cells; Leu-M9; Becton Dickinson, San Jose, CA, USA) and CD34 (stem cells; 581; Immunotech, Marseille, France).

Detection of platelet antibodies

Platelet immunoglobulin G antibodies against glycoprotein (GP) IIb/IIa and GPIb/IX were analyzed with the Platelet Immune Fluorescence Test (PIFT)⁸ and the Monoclonal Antibody-Based Immobilization of Platelet Antigens (MAIPA) as described before.⁹

Case report

The patient is a boy who suffered from mild bleeding tendency since birth. At the age of eleven months, thrombocytopenia was detected; platelet counts varied between $20 \times 10^9/L$ and $80 \times 10^9/L$. His pediatrician diagnosed chronic idiopathic thrombocytopenic purpura (ITP) without a need for treatment because there was no serious bleeding tendency. The boy was referred to our hospital for further investigations. Then, the boy was admitted with an acute serious bleeding from nose, stomach and bladder, resulting in a hypovolemic shock with a platelet count of $4 \times 10^9/L$ and hemoglobin level of 7 g/dl. Red cell and platelet transfusions were given. The platelet count after transfusion was $110 \times 10^9/L$ and decreased gradually over the next week. Thus, the diagnosis of immune-mediated platelet destruction or ITP could be rejected. In the next months, platelet counts varied between $2 \text{--} 224 \times 10^9/L$ with serious bleeding tendency when platelet counts decreased below $20 \times 10^9/L$. Figure 1 shows the course of the platelet numbers during this time. Platelet transfusions were given in case of bleeding.

The family history was blank. Patient was the fifth child of consanguineous parents (first cousins). Parents and sibs had normal platelet counts. Physical examination was unremarkable and the patient had no dysmorphic signs. Blood counts were in the normal range for hemoglobin, reticulocytes, leukocytes and differentiation. Table 1 shows the results of the diagnostic investigations in this period. Beside a slightly shortened in vivo platelet-survival time no abnormalities were found. Bone marrow aspirates were taken twice at different times. In the first BM smear erythropoiesis and myelopoiesis were normal. The number of megakaryocytes was normal, but the megakaryocytes were small, and no release of platelets was seen. The second BM smear was similar, concerning cellularity and differentiation of erythropoiesis and myelopoiesis. However, there were only a few, small megakaryocytes without platelet release. No classifying diagnosis could be made.

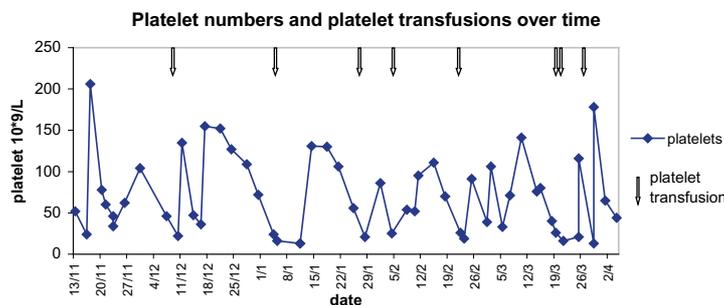


Figure 1: Platelet counts over a 5-month period during which eight platelet transfusions were given, with only a temporary rise in the platelet count.

Parameter	Value	Reference Range
Mean platelet volume	6.4-12.3 fl	6.5-11 fl
Thrombopoietin	5-80 AU/mL	2-34 AU/mL
Glycocalicin	67-234 AU/mL	144-444 AU/mL
Activated partial thromboplastin time (sec)	35	34-41
Prothrombin time (sec)	12.2	11-13
Platelet functional analysis	Normal	
Platelet survival (hr)*	45.3	60-110
Glycoprotein profile platelets [∇]	Normal	
Chromosome pattern (blood and bone marrow)	XY	
Chromosome breakage analysis (mitomycin)	Normal	
WASP gene analysis	Normal	
Platelet autoantibodies (GPIIb/IIIa, GPIb/IX)	Negative	

*Radiolabeled autologous platelets were used.

[∇]Expression of glycoproteins GPIa, GPIb, GPIIb, GPIIIa, GPV, and GPIIX was tested in the PIFT.

Table 1: Selected laboratory test results

Treatment

Intravenous gammaglobulin (0.8 gram/kg) and plasma infusions (10 ml/kg) were not of any benefit. Subsequently, the patient was treated with corticosteroids but no beneficial effect on platelet count and bleeding tendency was observed (fig 2).

We then decided to start an observation period of twelve weeks, in which no interventions like platelet transfusions or any medication were made. Platelet counts, Tpo, GC, and platelet auto- antibodies were measured weekly. From the parents and sibs, samples for blood counts were taken weekly during six weeks as well. Fig 3 shows the results of the blood platelet counts, GC and Tpo levels during this observation period. Platelet antibodies were not detected.

The platelet counts were found to follow a cyclic pattern with a periodicity of about 25 days; the GC plasma levels, which reflect the total platelet mass in the body, oscillated with the same pattern. Tpo levels mirrored this pattern, which is in

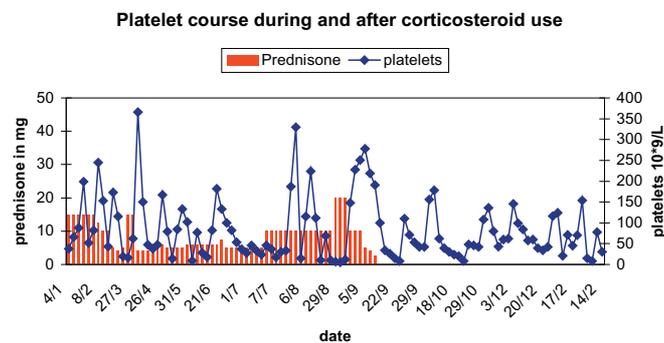


Figure 2: Platelet counts during and after corticosteroid use. Prednisone treatment did not influence the cycling pattern of the platelets.

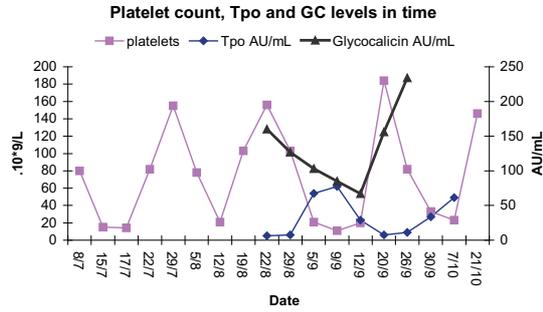


Figure 3: Platelet counts (left *y*-axis, platelet count in $10 \times 10^9/L$) and thrombopoietin (Tpo) and glycocalicin (GC) levels (right *y*-axis, values in AU).

concordance with the role of platelets in Tpo plasma level regulation. After recognizing the periodically cycling of platelet counts, a bone marrow aspirate was taken in the ascending leg of the platelet count cycle. This bone marrow was normocellular and showed an increased number of small megakaryocytes. In contrast, the bone marrow sample taken at the descending leg was also normocellular, but only very few small megakaryocytes were seen.

Bone marrow cultures

Outgrowth of the patients' CD34⁺ cells with Tpo and IL-1 was compared with outgrowth of control childrens' CD 34⁺ cells. The patients' cultures show a diminished percentage of CD34⁺/CD41⁺ cells and CD42b⁺ cells (fig 4) pointing to both low formation of megakaryocyte progenitors and to a low level of maturation of the formed megakaryocytes. To evaluate whether a plasma-derived factor resulted in impaired megakaryocytopoiesis, in vitro studies were performed with donor CD34⁺ cells with patients' plasma. Figure 4 shows a low percentage of differentiation towards CD41-positive cells upon addition of AB plasma or patient's plasma derived at the time of descending platelet counts (11%). Addition of patient's plasma taken at the time of ascending platelet counts induced a rise in CD 41-positive cells up to 25%. This suggests a stimulating effect on the differentiation of CD34⁺ cells towards mature megakaryocytes by plasma taken at the ascending leg of the cycle. In Table 2 the results of CD34⁺ cultures with two different patient BM samples and a control BM sample are listed; BM from the patient was taken during the ascending leg of the platelet cycle and during the descending leg of the platelet cycle. The table shows that CD34⁺ cultures performed in presence of plasma from the descending (dP) leg or AB plasma (AB) resulted in the formation of 3-11% CD41⁺ positive cells (megakaryocytes), whereas cultures with addition of plasma from the ascending (aP) leg resulted in a higher yield of megakaryocytes (27-37%). So it seems again that there is a stimulating factor present in the plasma of the patient taken at the time of the ascending leg of the platelet cycle.

Discussion

Cyclic thrombocytopenia is a rare acquired disorder described in adult patients. The mechanism of fluctuations in platelet counts is unknown. At least two different

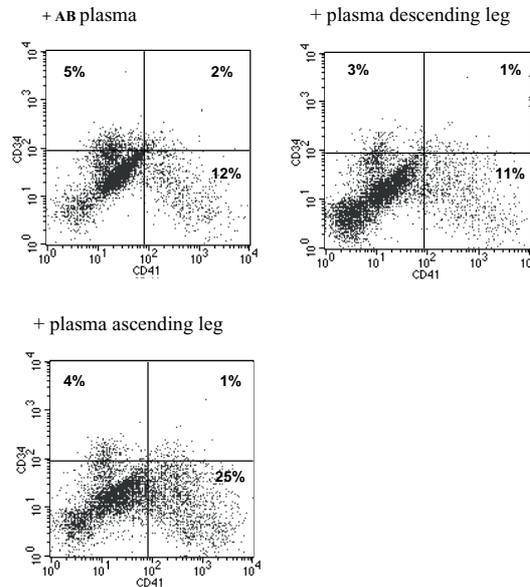


Figure 4: Donor CD34⁺ cells were cultured with Tpo, IL-1, and AB plasma, with the patient's plasma collected during the descending leg, or with the patient's plasma collected during the ascending leg of the platelet cycle.

pathways have been proposed in the literature. The first is periodic destruction of platelets due to platelet autoantibodies.¹⁰ The second is a periodic change in megakaryopoiesis, idiopathic or mediated by cyclic variations in growth factors², GM-CSF-blocking IgG¹¹ clonal T cells¹² or mononuclear cells¹³. The mechanisms responsible for the onset of periodic oscillations are still unclear. The patient we present suffers from a bleeding tendency since birth and a cyclic thrombocytopenia, with a 25-day periodicity. Immune modulation did not alter either the cyclic pattern of the thrombocytopenia or the amplitude. The variation in megakaryocyte numbers in bone marrow smears and in the outcome of the megakaryocyte cultures, as well as the oscillation in Tpo and GC levels supports the diagnosis Cyclic Amegakaryocytic

		% CD41	%CD34/CD41	%CD34	%CD42b
BMa	TPO+IL-1	5.5	0.8	12.9	1.1
	TPO+IL-1+aP	32.1	0.8	12.4	0.4
	TPO+IL-1+dP	7.6	1.2	19.9	1.1
	TPO+IL-1+AB	3.5	0.8	16	0.7
BMd	TPO+IL-1	11.3	0.9	9.2	1.6
	TPO+IL-1+aP	37.0	1.3	12.4	5.5
	TPO+IL-1+dP	7.5	1.7	10.0	1.5
	TPO+IL-1+AB	3.1	0.4	17.2	0.8
ChBM	TPO+IL-1	8.6	5.2	5.2	1.4
	TPO+IL-1+aP	27	6.8	6.8	2.1
	TPO+IL-1+dP	10.9	4.8	6.0	1.6
	TPO+IL-1+AB	2.9	2.2	8.7	4.3

Table 2: BMa, patient's bone marrow taken at the ascending leg of the platelet cycle; BMd, patient's bone marrow taken at the descending leg of the platelet cycle; ChBM, control donor bone marrow; aP, plasma derived during the ascending leg; dP, plasma derived during the descending leg of the platelet cycle.

Thrombocytopenia (CAT).

The oscillation in clinical picture and platelet count in our patient has similarities with the pattern of the cyclic neutropenia (CN). However, there is an important difference. The oscillation in the levels of our patient's blood cells is restricted to the platelets, whereas in CN oscillation occurs not only in numbers of neutrophils and monocytes but also in those of the reticulocytes and platelets.¹⁴ In their review on cyclical neutropenia and other periodic hematological disorders, Haurie et al.¹⁵ distinguish two major categories of mechanism for the origin of oscillation in hematopoiesis. The first concerns the loss of stability in peripheral control loops of colony-forming units, mediated by lineage-specific growth factors such as Tpo, Epo or G-CSF. The second group concerns oscillations in stem cell population as a result of loss of control in the local regulatory loop within the totipotent human stem cell population. In the cyclic amegakaryocytic thrombocytopenia in our patient, the origin of the problem may be found both in an intrinsic defect in megakaryocytopoiesis and in a disturbed feedback between CFU-Meg and the platelet mass. Until now we did not detect specific factors influencing this feedback loop. No evidence was found for autoantibodies directed against platelets or the Mpl receptor. We only found an extra megakaryocytopoiesis stimulating effect on CD34⁺ cells, both from the patient and from a healthy donor, when culturing these cells in the presence of patient's plasma taken at the time of increasing platelet counts. Kimura et al.¹⁶ discussed the role of various growth factors in their patient with cyclic thrombocytopenia. No definitive conclusions can be drawn from his observations because their patient showed periodic thrombocytosis, which might influence the levels of growth factors. Until now we can conclude that our patient suffers from juvenile, possible congenital, cyclic amegakaryocytosis of unknown origin. This condition has not yet been described before in children.

Acknowledgement

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