Brief report

Megakaryocyte impairment by eptifibatide-induced antibodies causes prolonged thrombocytopenia

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Glycoprotein (GP) IIbIIIa inhibitors are used in the treatment of acute coronary syndromes. Transient immune-mediated acute thrombocytopenia is a recognized side effect of GPIIbIIIa inhibitors. We provide evidence that GPIIbIIIa inhibitor-induced antibodies can affect megakaryocytes in the presence of eptifibatide. In a patient with acute coronary syndrome, acute thrombocytopenia occurred after a second exposure to eptifibatide 20 days after the initial treatment. Despite the short half-life of eptifibatide ($t_{1/2}$ = 2 hours), thrombocytopenia less than 5×10^9/L and gastrointestinal and skin hemorrhage persisted for 4 days. Glycoprotein-specific enzyme-linked immunosorbent assay showed eptifibatide-dependent, GPIIbIIIa-specific antibodies. Bone marrow examination showed predominance of early megakaryocyte stages, and platelet transfusion resulted in an abrupt platelet count increase. Viability of cultured cord blood–derived megakaryocytes was reduced in the presence of eptifibatide and patient IgG fraction. These findings can be explained by impaired megakaryocytopoiesis complicating anti-GPIIbIIIa antibody-mediated immune thrombocytopenia. This mechanism may also apply to some patients with autoimmune thrombocytopenia. (Blood. 2009;114:1250-1253)

Introduction

Glycoprotein (GP) IIbIIIa antagonists are a heterogeneous group of integrin inhibitors used for treatment in acute coronary syndromes. A relatively frequent adverse effect of GPIIbIIIa inhibitors is immune-mediated thrombocytopenia. The risk of thrombocytopenia from immune and nonimmune causes differs between the drugs: abciximab carries a risk of 0.3%-1.6%; tirofiban, 0.2%-0.4%[1]; and eptifibatide, 0%-0.2%.[2,3] GPIIbIIIa inhibitor-immune thrombocytopenia typically manifests within the first 24 hours of treatment, which indicates that these patients already have circulating antibodies in their plasma.[4] The risk for thrombocytopenia is higher during second exposure compared with first-time treatment.[5] In addition, severe thrombocytopenia ($< 20 \times 10^9$/L) was more frequent after a second exposure with abciximab.[6] Usually, platelet counts decrease to values less than 50×10^9/L, often less than 20×10^9/L. Patients have an increased risk for bleeding, but, especially with tirofiban and eptifibatide, platelet counts normalize after cessation of the GPIIbIIIa inhibitor within 2 to 3 days. However, some patients have an unusually prolonged thrombocytopenia,[7] which is inconsistent with the relatively short half-lives of tirofiban and eptifibatide.

Methods

Case report

A 67-year-old male patient was treated with eptifibatide (20 mg intravenous bolus) during coronary stent implantation (Figure 1) and reexposed (20 mg intravenously) during a second coronary intervention 20 days later. Within 1 hour, diffuse bleeding occurred and the platelet count decreased to less than 5×10^9/L (Figure 1). Despite transfusion of 2 platelet concentrates, pericardial hemorrhage occurred, platelet counts remained less than 5×10^9/L for 4 days, and gastrointestinal bleeding, tracheal bleeding, and skin hemorrhage increased. Although acute thrombocytopenia was probably caused by clearing of drug-dependent antibody-coated platelets resulting from the short half-life of eptifibatide ($t_{1/2}$, 2 hours), prolonged thrombocytopenia could not be explained solely by phagocytosis of platelets resulting from antibodies specific for a complex of GPIIbIIIa and eptifibatide. On day 4, bone marrow showed impairment of platelet production; and, in contrast to the platelet transfusions at day 1 (when eptifibatide was still present in the circulation), platelet count abruptly increased after platelet transfusion, together with bleeding cessation.

Pseudothrombocytopenia was excluded by microscopy. Serum of the patient was obtained approximately 24 hours after the second exposure to eptifibatide.

Platelet antibody tests

Patient serum was incubated with platelets of normal donors in the presence and absence of eptifibatide and binding of IgG antibodies assessed by whole-platelet enzyme-linked immunosorbent assay (ELISA)[8] and by flow cytometry using fluorescein isothiocyanate (FITC)–labeled rabbit anti–human-IgG antibodies (DakoCytomation). Glycoprotein specificity was assessed by glycoprotein-specific ELISA monoclonal antibody immobilization of platelet antigens (MAIPA)[9] as described.[10]

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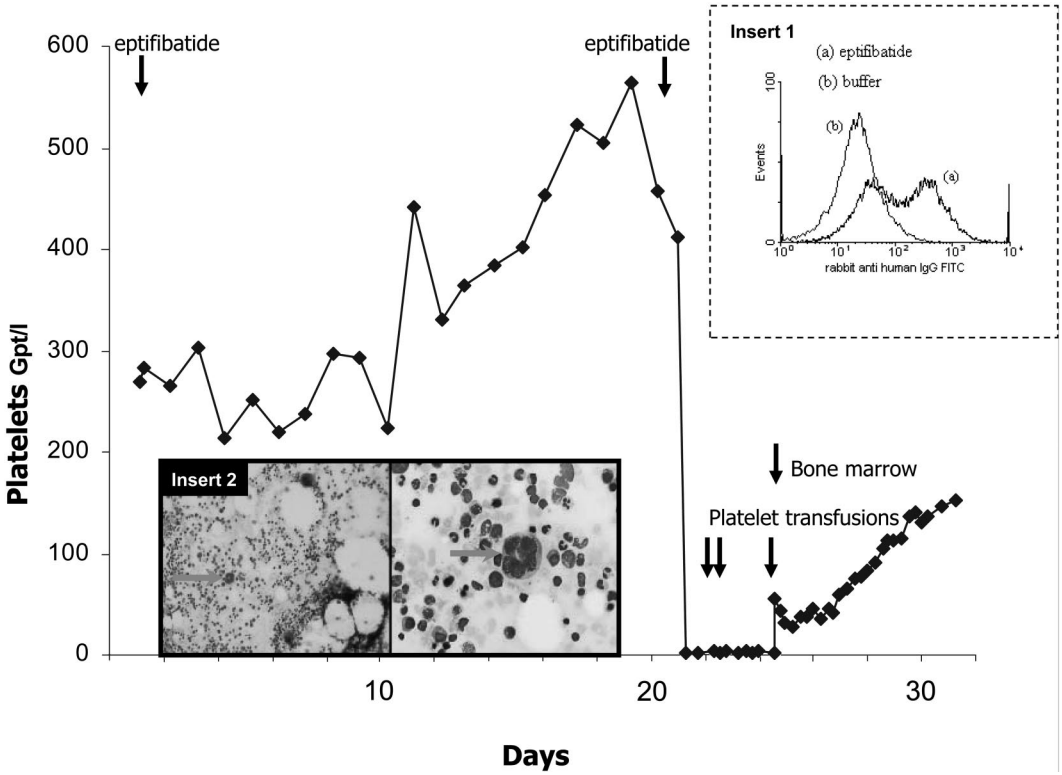

Figure 1. Clinical platelet count, antibody-binding characteristics, and bone marrow samples. The graph shows the time course of platelet counts in relation to 2 exposures to eptifibatide in a 67-year-old male patient with acute coronary syndrome (body weight 80 kg; compensated renal impairment). Inset 1 represents the increased binding of IgG, when normal platelets were incubated with patient serum in the presence of eptifibatide (1 μg/mL) but not in the presence of patient serum and buffer, as assessed by flow cytometry. Inset 2 represents a representative example of the bone marrow obtained on day 4 after start of thrombocytopenia. The left panel represents, in an overview, the reduced megakaryocyte count at a 10-fold magnification. Higher magnification (right panel, 60-fold magnification) reveals the young phenotype of the megakaryocyte marked with an arrow from overview microscopy at the left panel. Smears were stained by May-Gruenwald and Giemsa stain (Merck) following standard protocol (microscope: Olympus BX50F; objectives: UPlan FL 10×/0.30; PlanApo 60×/1.40 with oil; camera: ColorView IIIu; software: AnalySIS Five; all Olympus Europe).

Patient IgG was purified from the serum by protein G column according to standard and tested by flow cytometry and MAIPA for its reactivity with platelets and GPIIbIIIa, respectively, in the presence of eptifibatide.

Differentiation and treatment of CD34+ hematopoietic stem cells

Umbilical cord blood samples were harvested from normal full-term newborns as described.[11] Cells were resuspended in 2.5 mL of X Vivo-20 culture medium (Lonza Walkersville) containing 50 ng/mL thrombopoietin (Chemicon), 40 ng/mL human stem cell factor (BioSource), and (from days 0 to 5) 10 ng/mL IL-3 (BioSource). The cell suspension was transferred to bacterial tissue culture plates to prevent adherence (37°C; 5% CO_2) and replenished with fresh growth factors and culture medium every other day. At day 14, the cell cultures were divided and subjected to the different treatment regimens (Figure 2A). The cells were stained with trypan blue (Sigma-Aldrich) for determination of cell viability.

Immunocytochemistry of cultured cord blood stem cells

Immunocytochemistry was performed as previously described.[12] In brief, suspended cells were fixed (2% paraformaldehyde; 20 minutes at room temperature), spun onto vectabond-treated coverglass (Vector Laboratories), blocked with the appropriate serum (10% in phosphate-buffered saline); incubated (overnight; 4°C) with a mouse monoclonal anti-GPIIbIIIa antibody (clone VM16a; Abcam) or a rabbit polyclonal anti-CD41 antibody (Abcam), washed (phosphate-buffered saline) and incubated with a labeled isotype matching secondary antibody (Alexa Fluor 546; Invitrogen, 1 hour at room temperature), washed and counterstained with Alexa Fluor 488 phalloidin (Invitrogen), an Alexa Fluor 633 (Invitrogen) conjugate of wheat germ agglutinin, and 4,6-diamidino-2-phenylindole (Invitrogen; 30 minutes at room temperature), and assessed by fluorescence microscopy (60×/1.42 NA oil objective; Olympus IX81, FV1000 (Olympus).

Statistical analysis

The mean plus or minus SEM was determined for each experimental variable displayed in Figure 2B. Analysis of variance was conducted to identify differences that existed among multiple experimental groups. If significant differences were found, a Student-Newman-Keuls post hoc procedure was used to determine the location of the difference. For all of the analyses, *P* values less than .05 were considered statistically significant.

Approval to report these studies was obtained from the Ethics Board of the Ernst-Moritz-Arndt-University, Greifswald, and patient informed consent was obtained in accordance with the Declaration of Helsinki.

Results and discussion

Platelet antibody tests showed strong binding of IgG to platelets in the presence of eptifibatide (optical density [OD] 1.784 units) but not in the presence of buffer (OD 0.315 units) by ELISA and by flow cytometry (Figure 1). By MAIPA, IgG binding was shown to GPIIbIIIa (OD 0.276 units) but not to GPIbIX (OD 0.056 units) or GPIaIIa (OD 0.046 units), respectively. The bone marrow showed young megakaryocytes only (Figure 1), consistent with marked destruction of megakaryocytes several days earlier. Nevertheless, it is probable that megakaryocytes might have been absent in a bone marrow sample taken earlier in the episode.

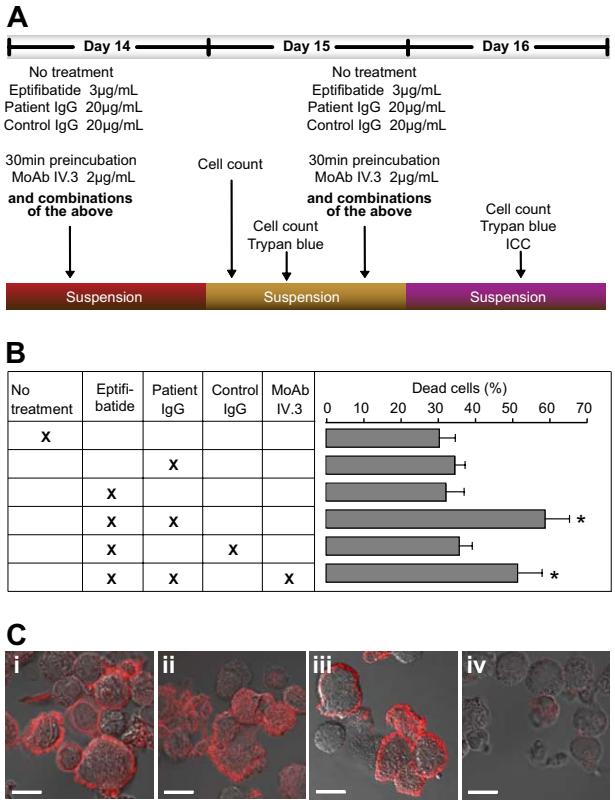

Figure 2. Treatment, proliferation, and viability of cultured cord blood-derived megakaryocytes. (A) Treatment plan for CD34$^+$-derived human megakaryocytes. The different interventions at different days are indicated by arrows. At days 15 and 16, cells were counted and trypan blue staining was performed to assess viability of the differentiated cells. ICC indicates immunocytochemistry; MoAb IV.3, FcRIIa-blocking monoclonal antibody. (B) The percentage of dead cells on day 16 in the different treatment groups (mean ± SEM; n = 4). Megakaryocytes treated with eptifibatide and patient IgG showed a significantly increased rate of cell death compared with the control experiments ($P = .01$). This effect was not inhibited by preincubation with the Fc-RIIa-blocking MoAb IV.3 ($P = .458$). * $P < .05$. (C) ICC images of cultured megakaryocytes that were stained for GPIIbIIIa (indicated in red) as described in "Immunocytochemistry of cultured cord blood stem cells." The buffer-treated cells (Ci), the eptifibatide-treated cells (Cii), as well as the cells treated with patient IgG only (Ciii) show intense staining for GPIIbIIIa, whereas eptifibatide + patient IgG (Civ) treated cells show minimal GPIIbIIIa expression. Scale bars represent 10 μm. This figure is representative of 4 independent experiments.

Cultured megakaryocytes show increased cell death resulting from patient IgG interaction. To recapitulate the in vivo finding suggesting antibody-induced megakaryocyte destruction, we used CD34$^+$ cord blood-derived hematopoietic stem cells differentiated into megakaryocytes as characterized in detail before.[11] Treatment of the differentiated megakaryocytes with eptifibatide in combination with the IgG isolated from the patient resulted in a significant decrease in cell viability compared with controls (Figure 2B). This effect was not the result of nonspecific effects of the IgG fraction or of eptifibatide, as control IgG and eptifibatide alone had no significant effect on cell viability. In addition, interactions of the patient IgG with the Fc-receptor probably did not cause megakaryocytotoxicity because an FcRIIa-blocking monoclonal antibody IV.3 did not reduce cell death substantially ($P = .458$). In addition, the complement system did not seem to be a major contributor because incubation with fresh human serum or heat-inactivated serum did not change the outcome of the experiment (data not shown). The immunostaining experiments using nonpermeabilized cells (Figure 2C) showed a significant reduction in cells expressing GPIIbIIIa on their surface (stained cells) in the eptifibatide + patient IgG group (Figure 2Civ) compared with no treatment (Figure 2Ci), eptifibatide only (Figure 2Cii), or patient IgG only (Figure 2Ciii). Under the culture conditions used, megakaryocytes in various stages of development will all be present in the culture, leading to a heterogeneous cell population with cells expressing different levels of GPIIbIIIa on their surface. GPIIbIIIa, which is expressed by nearly all cells intracellularly, is not accessible to the antibodies. Thus, some cells of megakaryocyte lineage did not expose the eptifibatide/GPIIbIIIa-dependent antigen(s) and remained unaffected by the antibody (Figure 2Civ). This provides further support for cytotoxicity being mediated through GPIIbIIIa/eptifibatide specificity of the antibodies. In addition, a polyclonal anti-GPIIbIIIa antibody showed the same staining pattern as a monoclonal GPIIbIIIa antibody, excluding a relevant competition between the patient antibody and the staining antibodies.

In conclusion, this study provides evidence that prolonged thrombocytopenia with severe hemorrhage after treatment with the short half-life GPIIbIIIa inhibitor, eptifibatide, can be caused by antibody-mediated impairment of megakaryocytes. Potentially, cytotoxicity mediated by GPIIbIIIa autoantibodies is also an explanation for reduced platelet production in some patients with amegakaryocytic thrombocytopenia[13,14] and in autoimmune thrombocytopenia.[15]

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Authorship

Contribution: A.G. designed the study and wrote the manuscript; B.F. performed the platelet antibody studies; H.Z. and B.M. are the intensive care unit physicians who treated the patient during the aplastic phase and obtained the patient material; W.K. performed the bone marrow study during aplasia; W.M. was the cardiologist who performed percutaneous coronary intervention (PCI) and recognized thrombocytopenia; and H.S. wrote parts of the manuscript and performed the megakaryocyte culture studies, together with N.M. All authors have read and approved the manuscript.

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