Mutation of the β1-tubulin gene associated with congenital macrothrombocytopenia affecting microtubule assembly

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Congenital macrothrombocytopenia is a genetically heterogeneous group of rare disorders. We identified the first TUBB1 mutation, R318W, in a patient with congenital macrothrombocytopenia. The patient was heterozygous for Q43P, but this single-nucleotide polymorphism (SNP) did not relate to macrothrombocytopenia. Although no abnormal platelet β1-tubulin localization/marginal band organization was observed, the level of β1-tubulin was decreased by approximately 50% compared with healthy controls. Large and irregular bleb protrusions observed in megakaryocytes derived from the patient's peripheral blood CD34+ cells suggested impaired megakaryocyte fragmentation and release of large platelets. In vitro transfection experiments in Chinese hamster ovary (CHO) cells demonstrated no incorporation of mutant β1-tubulin into microtubules, but the formation of punctuated insoluble aggregates. These results suggested that mutant protein is prone to aggregation but is unstable within megakaryocytes/platelets. Alternatively, mutant β1-tubulin may not be transported from the megakaryocytes into platelets. W318 β1-tubulin may interfere with normal platelet production, resulting in macrothrombocytopenia. (Blood. 2009; 113:458-461)

Introduction

Congenital macrothrombocytopenia is a genetically heterogeneous group of rare disorders.1,3 The most frequent forms include MYH9 disorders, such as May-Hegglin anomaly, and Bernard-Soulier syndrome. In approximately half of the cases the pathogenesis remains unknown; thus, a definite diagnosis is not possible. The linkage between the membrane skeleton and cytoskeletal actin filaments as well as the marginal microtubule band maintains normal platelet morphology.4,5 Defects in these systems may result in macrothrombocytopenia. The microtubules are assembled from α- and β-tubulin heterodimers. β1-Tubulin expression is restricted in the megakaryocyte/platelet lineage.6 Tubb1 knockout mice show thrombocytopenia and spherical platelets.7 TUBB1 Q43P functional polymorphism has been reported. However, it may not be relevant to macrothrombocytopenia.8 We identified the first TUBB1 mutation affecting microtubule assembly in congenital macrothrombocytopenia.

Methods

Patient

The patient was a 7-year-old boy who was incidentally found to have thrombocytopenia (platelets, 40-60 × 10^9/L). He was diagnosed with immune thrombocytopenic purpura. Peripheral blood smears showed the prominent appearance of giant platelets. Electron microscopy showed no other abnormalities (Figure 1A,B). There were no leukocyte inclusion bodies, confirmed by myosin IIA localization.9 The platelets aggregated normally with adenosine diphosphate (ADP), collagen, and ristocetin. Flow cytometry showed normal expression of platelet GPⅠb/Ⅸ. An initial bone marrow examination revealed normal megakaryocyte number and morphology. The mother of the patient also had macrothrombocytopenia. Peripheral blood samples were obtained after the mother gave informed consent in accordance with the Declaration of Helsinki for the study, which was approved by the institutional review boards (IRBs) of Nagoya Medical Center and Hokkaido University. Platelet size and TUBB1 gene frequencies were determined in 108 healthy controls and 16 consecutive, unrelated patients with macrothrombocytopenia who were enrolled in an institutional review board–approved collaborative study on congenital thrombocytopenia.

Antibodies

We raised an anti-β1-tubulin antibody (NB2301) in rabbits against a synthetic peptide corresponding to C-terminal 425-451aa of human β1-tubulin (KAVLEEDEEVTIEAEEMPEDKGH). Antiserum was collected and affinity-purified. NB2301 specifically reacted with recombinant β1-tubulin among 7 known recombinant human β-tubulin isoforms on immunoblots, and only stained platelets on blood smears (Figure S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). Other antibodies used were anti-GPⅠb (SZ22, Immunotech, Marseille, France), anti-α-tubulin (DM1A and RB9281, LabVision, Fremont, CA), anti-β-tubulin (TUB2.1), and anti-β5-tubulin (SAP-4G5, Abcam, Cambridge, United Kingdom).

Mutational analysis

The entire coding sequence of exons and exon-intron boundaries of TUBB1 was amplified by PCR, and the products were subjected to DNA sequence analysis (Table S1).
TUBB1 cloning, mutagenesis, and transfection

Full-length TUBB1 sequences were amplified from the patient’s platelet cDNA (Table S1). TUBB1 cDNA in-frame with the C-terminal myc epitope tag was cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA). We prepared 3 mutant constructs (P43, W318, and P43/W318), and used them to transfect Chinese hamster ovary (CHO) cells. At 24 hours after transfection, the cells were replated on fibronectin-coated (10 μg/mL) chamber slides, and cultured for 48 hours. For microtubule-depolymerization experiments, cells were incubated at 4°C for 4 hours or 33 mM nocodazole at 37°C for 2 hours.

Analysis of cultured megakaryocytes

CD34+ cells isolated from 10 mL of peripheral blood were cultured for 14 days in serum-free medium supplemented with thrombopoietin (TPO), stem cell factor (SCF), and interleukin-3 (IL-3). Cytospin preparations of differentiated megakaryocytes were analyzed by immunofluorescence staining.

Results and discussion

We searched for TUBB1 mutations in the patient, and found a novel conserved R318W mutation (Figure 1C,D). Restriction analysis confirmed heterozygosity in the patient and mother. The mutation was not found in 108 healthy controls or in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/). In a tubulin 3D model, it is buried inside the molecule and is near the α and β intradimer interface (Figure 1E). The side chain is oriented toward the inside of the molecule and faces that of E27. An R318W mutation replacing the charged arginine with bulky aromatic tryptophan will disrupt the side chain interactions. Because D249N, located at the interface, has recently been reported to cause canine macrothrombocytopenia, mutations disrupting the structure of the intradimer interface may affect platelet morphology.

Two other nonsynonymous substitutions, Q43P and R307H, were identified. The former was recently described as a functional polymorphism. The prevalence of the P43 allele was higher in patients with congenital macrothrombocytopenia (0.12) than controls (0.06). We also found a similar gene frequency of 0.16 and 0.06 in our patients and controls, respectively (Table S2). Although R307 is conserved, this position contains basic histidine in other human β-tubulins, and the gene frequency was the same in patients and controls. Control individuals with Q43P or R307H even in their homozygous state had normal-sized platelets (Table S3). We therefore suggested that both substitutions are not related to macrothrombocytopenia.

The expression and localization of platelet β1-tubulin were investigated using the newly produced NB2301 antibody. β1-Tubulin was normally localized in the marginal microtubule band in resting patient platelets (Figure 2A). Immunoblotting showed the normal electrophoretic mobility. However, the expression level was decreased: the
1-tubulin/tubulin ratio was decreased by approximately 50% compared with controls (Figure 2B). Q43P is reportedly associated with the reduced platelet 1-tubulin. We found in a control P43 homozygote that the 1-tubulin/tubulin ratio was not decreased (Figure 2B). Thus, the decreased expression of 1-tubulin in the patient is likely to be due to R318W and not Q43P. We examined megakaryocytes cultured from CD34 peripheral blood cells. Cultured mature megakaryocytes frequently extend blebs, similar to demarcation membrane system-like structure. We observed large and irregular bleb protrusions in the patient (Figure 2C). This suggested impaired megakaryocyte fragmentation and release of large platelets. The plasma glycopcalicin concentration, a proteolytic fragment of GPIb (molecular marker of platelet production/destruction), was below the normal limit (1.02 g/mL; normal values, 1.40 ± 0.25 g/mL), indicating that thrombocytopenia is not due to underproduction of platelets but peripheral destruction.

To determine the functional and structural consequences of the R318W mutation on microtubule assembly, we monitored mutant 1-tubulins after their transfection into CHO cells. The wild-type
and P43 mutant myc-tagged β1-tubulin were localized as fine filamentous cytoplasmic networks, indicating normal incorporation of recombinant β1-tubulin into microtubules with endogenous α-tubulin. In contrast, W318 and P43/W318 mutants accumulated in punctuate structures in the cytoplasm (Figure 2D). Under conditions of microtubule depolymerization by low temperature (Figure 2E,F) or nocodazole treatment, aggregates of W318 mutants did not dissociate.

Our results suggested that the net effect of the TUBB1 R318W mutation in platelets is the instability of mutant protein. These data seem to contradict those obtained by in vitro expression experiments, whereby β1-tubulin W318 formed insoluble aggregates. One important difference is that the latter employs an expression vector with strong cytomegalovirus (CMV) promoter. Thus, the unstable but aggregate-prone W318 mutant was detected as insoluble aggregates. The coordinated production of α- and β-tubulins necessary for proper heterodimer assembly are regulated both transcriptionally and translationally and, when tubulin is overexpressed in cells, synthesis of the endogenous form is strongly inhibited.18-20 In Tubb1 knockout mice, up-regulation of β2- and β5-tubulins partially compensates for the null expression of β1-tubulin.2 Because heterozygous Tubb1 knockout mice do not exhibit giant platelets, simple haploinsufficiency alone cannot explain the pathogenesis of macrothrombocytopenia. Mutant β1-tubulin may dominantly affect microtubule assembly in some manner. Normal expression of mutant TUBB1 mRNA (not shown) and decreased β1-tubulin in platelets lead to an alternative hypothesis that mutant β1-tubulin is not transported from the megakaryocyte into platelets.

Eliciting the precise molecular mechanisms will not only advance our classification and diagnosis of congenital macrothrombocytopenias, but also our comprehensive understanding of megakaryopoiesis and platelet genesis.

Acknowledgments

We thank Ms Yoshimi Ito for her skillful technical assistance.

This work was supported by grants to S.K. from the Japan Society for the Promotion of Science (18591094 and 20591161), the Ministry of Health, Labor and Welfare (Grant for Child Health and Development 19C-2), Charitable Trust Laboratory Medicine Foundation of Japan, and National Hospital Organization (network research grant for congenital thrombocytopenia).

Authorship

Contributions: S.K. designed and performed research, analyzed data, and wrote the paper; R.K. contributed patient samples and clinical data; T.J.I. designed the tubulin experiments and wrote the paper; and M.H. and H.S. supervised the research.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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