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Erythroid Involvement in CD36 Deficiency.

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Abstract *Objective.* The CD36 molecule is expressed in platelets, monocytes, erythroblasts, and other different tissues. There are 2 types of platelet CD36 deficiency; types I and II, associated with the absence and presence of CD36 on monocytes, respectively. To clarify the involvement of the erythroid lineage in CD36 deficiency, we investigated the phenotype and RNA expression of CD36. *Materials and Methods.* CD36 expression was examined in 296 patients with several cardiovascular diseases in our outpatient clinic. Of them, there were 12 patients with type I and 16 with type II CD36 deficiency. A bone marrow sample was examined in 5 type I and 4 type II patients. The expression of CD36 mRNA was examined in BFU-e. The sequences of RT-PCR products of the CD36 mRNA from monocytes were examined. *Results.* CD36 was deficient in erythroblasts from all the 5 patients with type I as expected. CD36 was present in erythroblasts from 3 of the 4 with type II, suggesting that their abnormality is restricted to platelets (type IIa). CD36 was unexpectedly absent from erythroblasts of a single type II patient (type IIb). CD36-specific mRNA was identified in BFU-e from each of 2 normals, 6 type I and 6 type II including type IIb. The sequences of RT-PCR products of the CD36 mRNA in a patient with type IIa and another with type IIb showed homozygous wild alleles. *Conclusion.* The findings provide evidence for further heterogeneity among CD36 deficient individuals, and existence of basic principle mechanism of type II, such as glycosylation abnormality.

Key words: CD36 deficiency, haematopoiesis, erythroblast, BFU-e, lineage involvement

Introduction

CD36 is a heavily N-glycosylated glycoprotein which is also known as glycoprotein IV of platelets, the Nak[a]-antigen of post-transfusion purpura [1], a long-chain fatty acid binding protein (FAT) of adipocytes [2], and an integral membrane protein (PAS IV) of the mammary epithelial cells [3]. CD36 is a member of a superfamily that includes the receptor for the high-density lipoprotein SR-BI (CLA-1) [4], the lysosomal protein LIMP II, the Drosophila epithelial molecule Emp, and the human muscle fatty acid-binding protein M-FABP [5]. CD36 functions as a receptor of several different types of ligands: the cell adhesion molecule-type receptor of extracellular collagen type I [6, 7] and thrombospondin-1 [8]; the transporter-type receptor of long-chain fatty acids [9]; the receptor of the surface antigen expressed in malaria-parasitized erythrocytes, Plasmodium falciparum erythrocyte membrane protein-1 (PfEMP1) [10, 11]; and the scavenger receptor of oxidized low-density lipoprotein (OxLDL) [12, 13], anionic phospholipids [4] and photoreceptor outer segments [14]. CD36 also functions as a signal transducer of the Src family protein tyrosine kinases Fyn, Lyn and Yes [15]. The functions of CD36 expressed in macrophages as the scavenger receptor of OxLDL and anionic phospholipids are thought to have important roles in atherosclerosis and in the phagocytic clearance of apoptotic cells, respectively.

There are 2 types of platelet CD36 deficiency; types I and II, associated with the absence and presence of CD36 on monocytes, respectively [16]. The frequency of Nak[a]-negative blood donors, i.e., of CD36 deficiency, of whites in Europe is extremely low [17], while the frequency in Japanese subjects is very high at 4.0% (14 of 354),

including 2 individuals (0.56%) with type I deficiency [16].

The CD36 molecule is expressed in several types of cells, especially on the haematopoietic cells including megakaryocytes and platelets, monocytes and macrophages, and erythroblasts. Although nucleated erythrocytes in the bone marrow lose most of their CD36 molecules at their terminal maturation, mature human erythrocytes still have a low amount of CD36 which may have an important role as an adhesion molecule of adult red cells [18]. It has been reported that platelets from subjects with CD36 deficiency showed normal aggregations in response to collagen as well as ADP, epinephrine, arachidonic acid and thrombin [19]. Since there has been no report from a large number of CD36-deficient individuals, the hematopoiesis and especially the involvement of the erythroid lineage in CD36 deficiency are not fully understood. It is also known that CD36 deficiency may have a role in the etiology of cardiovascular diseases, including hypertrophic cardiomyopathy [20]; we therefore examined the CD36 expression in the blood cells of patients with cardiovascular diseases in our outpatient clinic. We report the haematopoietic status and erythroid involvement in the 28 individuals with CD36 deficiency examined.

Materials and methods

Patients.

Between April and November 1997, 296 patients seen in our outpatient clinic for cardiovascular diseases were examined for CD36 expression in the blood cells. Among these patients, there were 12 with type I and 16 with type II CD36 deficiency. The primary cardiovascular diseases of these CD36-deficient individuals were hypertrophic cardiomyopathy (2 cases), dilated cardiomyopathy (1), old myocardial infarction (2), angina pectoris (4), hypertension (2) and chronic heart failure (1) in the type I deficiency group, and hypertrophic cardiomyopathy (4), dilated cardiomyopathy (1), angina pectoris (7), old myocardial infarction (1), hypertension (1) and ischemic heart disease (2) in the type II group. The haemoglobin level, white cell count and platelet count of the patients was analyzed. Further examination was done in 5 of the 12 type I and 4 of the 16 type II patients using bone marrow aspirates to analyze the complication of haematopoiesis and erythroid involvement in CD36 deficiency, after informed consent was obtained. The smear specimens of bone marrow aspirates were examined after May-Gruenwald and Giemsa staining. The CD36 expression in the blood platelets, monocytes, and bone marrow nucleated red cells was examined by flow cytometry. A mixed-colony formation of circulating blood cells was also applied in 2 normal, 6 type I and 6 type II patients, and 10 erythroid colonies (BFU-e) were chosen and utilized for RT-PCR analysis of the CD36 mRNA. The RT-PCR products of the CD36 mRNA from the peripheral blood monocytes in 2 type II patients were analyzed to determine the sequences in all the translated regions of CD36.

Flow cytometric analysis of CD36 expression

Fluorescein isothiocyanate (FITC)-conjugated control IgG1, and phycoerythrin (PE)-conjugated anti-LeuM3 (CD14) and control IgG1 were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA, USA). FITC-conjugated anti-CD36 and PE-conjugated anti-glycophorin A (GPA) were purchased from Immunotech (Marseille, France). Peripheral blood samples in EDTA-tubes were obtained from several patients with heart disease after informed consent. Platelet-rich plasma (PRP) was separated after centrifugation at 1,000 rpm for 10 min. Low-density mononuclear cells (MNC) were also separated from the rest of the sample by density centrifugation, using a Lymphoprep (1.077, Nycomed Pharma AS, Oslo, Norway) at 1,500 rpm for 30 min. The PRP and MNC samples were used to analyze the CD36 expression in platelets and monocytes, respectively. Heparinized bone marrow aspirates were also obtained from some of the patients with CD36 deficiency after informed consent, and MNC were analyzed to detect the CD36 expression in the erythroblasts. Fifty microliters of PRP was incubated with anti-CD36-FITC for 30min on ice, washed with a working buffer (phosphate-buffered saline (PBS) supplemented with 4% citrate and 1% human serum albumin), resuspended in 1ml of the same buffer, then analyzed with the use of a flow cytometer and a gate on a light-scattering dot plotgram in the platelet area (Fig. 1). The peripheral blood MNC were incubated simultaneously with anti-CD14-PE and anti-CD36-FITC for 30 min on ice, and then washed with PBS. The CD14-positive cells were analyzed using a gate on an FL2(PE) vs. SSC dot plotgram. The bone marrow MNCs were simultaneously stained with anti-

GPA-PE and anti-CD36-FITC, and then the GPA-positive cells were analyzed using a gate on an FL2 vs. SSC dot plotgram, as well. The cell fluorescence was analyzed with a FACScan Flow Cytometer and Lysis II software (Becton Dickinson). Ten thousand cellular events were acquired.

Mixed colony assay of the peripheral blood

A mixed-colony formation (CFU-mix) was applied to analyze the expression of the CD36 mRNA in the erythroid lineage cells by means of RT-PCR after informed consent. A clonal cell culture was performed in 35mm petri dishes (Becton Diskinson). For analyzing the CFU-mix, 1 x 10^5 low-density cells from blood were seeded in 1 ml of complete methylcellulose medium, MethoCult GF H4435 (0.9% Iscove's methylcellulose, 30% FBS, 1% BSA, 3 U/ml erythropoietin, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, 50 ng/ml rh stem cell factor, 20 ng/ml rh GM-CSF, 20 ng/ml rh IL-3, 20 ng/ml rh IL-6, and 20 ng/ml rh G-CSF: StemCell Technologies, Vancouver, Canada), and incubated at 37^0C in a humidified atmosphere in air with 5% CO_2 for 14 days. Ten erythroid colonies (BFU-e) were chosen, and stored separately in Eppendorf tubes with 1ml of Trizol reagent (Gibco BRL, Grand Island, NY) and 2 μl of glycogen (Boehringer Mannheim, Germany), then utilized for RT-PCR.

Amplification and sequencing of the CD36 cDNA

Total RNA of the peripheral blood MNC was isolated by Trizol, and 5 μg was then utilized for the cDNA synthesis. The cDNA was synthesized from the total RNA of BFU-e or MNC with random primers and murine Moloney leukemia virus reverse transcriptase in a final volume of 20 μl. The CD36 cDNAs were amplified with AmpliTaq polymerase (TOYOBO, Osaka, Japan), each primer was used to determine all the sequences of the translated regions (Table 1) from 1 μl of the cDNA according to the following amplification profile: 35 cycles at 94℃ for 60 seconds, 58℃ for 90 seconds, and 73℃ for 120 seconds. The amplified products were separated on 3% agarose gels and stained with ethidium bromide. The PCR products were purified with microcon-100 (Millipore, Bedford, MA). The purified PCR products were sequenced with a TaqDye Deoxy terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA) and a DNA sequencer (model 310, PE Applied Biosystems).

Results

Haematopoiesis in patients with the CD36 deficiency

The haemoglobin levels, white cell counts and platelet counts of the patients with the CD36 deficiency were almost normal considering that the age distribution of the patients was comparatively high, as shown in Table 2. No evidence of haematopoietic abnormality, or at least minimal abnormality, was observed in the smear specimens of the bone marrow aspirates (Table 2). The bone marrow samples from all 5 type I patients and 1 of the 4 type II patients showed no CD36 expression in the nucleated red cells (NRCs), while 3 of the 4 type II patients showed CD36 expression in NRCs. Thus, there were 2 subtypes of type II CD36 deficiency, i.e., type IIa (CD36 was present in NRCs) and type IIb (absent in NRCs), as shown in Table 2 and Fig. 2.

CD36 gene involvement in the patients

The CFU-mix assay was performed in 2 normal, 6 type I and 6 type II patients including all 5 type I patients, 1 of the type IIa patients and the type IIb patient in Table 2. The CD36 mRNA was present in all 10 BFU-e examined from each patient with 2 normal, 6 type I and 6 type II CD36 deficiency, and the cDNA size was normal in each sample using the primers CD36 3-5' and 5-3' (exons 3 to 5, E3xE5 in Fig. 3) or the primers CD36 4-5' and 6-3' (exons 4 to 6, E4xE6, not shown) in Table 1. The RT-PCR products of the CD36 mRNA from the blood MNC in the 2 type II patients in Table 2 were then sequenced using each primer in Table 1 to clarify the mutations as markers to analyze the expression of the mRNA of CD36 in the erythroid lineage and to understand the mechanism of CD36 deficiency in the erythroid, such as the silent alleles in erythroid. The cDNA from the 2 patients showed only wild type bands, and their sequences in all the translated regions had no mutations, therefore, further analysis of the BFU-e was not performed. To confirm the technical reliability of the methods, the sequences of the CD36 alleles in 4 patients with type I reported in a previous paper (submitted) are also shown in Table 2.

Discussion

The CD36 gene is located on chromosome 7q11.2 [21], and is encoded by 15 exons consisting of 12 translated exons from III to XIV [22]. The CD36 cDNA consists of 1870 bp residues, and the resulting polypeptide possesses 471 residues with a predicted molecular weight of 53 kDa. The final molecular weight of the CD36 glycoprotein is 88 kDa, after N-linked glycosylation at 10 sites of the extracellular domain [23]. CD36 is thought to have 2 transmembrane regions (residues from 7 to 34 and 440 to 466), 1 membrane-associated domain (184 to 204), and 2 cytoplasmic domains at both the N- and C-termini (1 to 6 and 467 to 472) consisting of 4 cysteine residues at the 3, 7, 464 and 466 cytoplasmic domains [15, 24]. The structural, surface-exposed, immunogenic and functional domain of the human CD36 is an epitope of the 155 to 183 amino acid residues [25, 26].

Deficiency of an antigen may be caused by abnormalities in several steps of molecular events involved in the synthesis of the antigen, such as mutations of the encoding gene, failure of the transcription, dysfunction of the translation, or abnormality of post-translational maturation of the polypeptide. There are 2 types of abnormal CD36 alleles involved in the pathogenesis of CD36 deficiency: mutations have been found in the exon regions and in an intron of the CD36 gene. All 3 types of the abnormal alleles including a point mutation at C478 to T478 in exon IV, a dinucleotide deletion at nt 539 to 540 in exon IV, and a single nucleotide insertion at nt 1159 in exon X of the CD36 gene result in the absence of the CD36 glycoprotein [27-30]. The "platelet-specific silent allele" caused by an abnormal $(TG)_n$ repeat polymorphism in intron C of the CD36 gene results in a megakaryocyte-platelet-specific mRNA expression defect [31]. As a result, both homozygotes and heterozygotes of the exon-type abnormal alleles cause the type I CD36 deficiency, while compound heterozygotes of the exon-type abnormal allele and a platelet-specific silent allele cause the type II deficiency. Therefore, erythroblasts as well as endothelial cells are expected to have a deficiency of the CD36 glycoprotein in type I subjects, while the erythroblasts in subjects with the type II deficiency caused by the platelet-specific silent allele are expected to express the glycoprotein. In fact, all 5 type I CD36-deficient individuals showed CD36-deficient erythroblasts, while 3 of the 4 type II individuals showed CD36-positive erythroblasts (type IIa). We have previously reported on the CD36 expression in myocardial capillary endothelial cells obtained from normal, type I and type II CD36 deficient individuals [32, 33]. The endothelial cells in the endomyocardial biopsy specimens showed normal CD36 expression, absence of CD36 and a mosaic pattern (chimerism) formed by CD36-positive and negative cells in a normal individual, a type I patient and a type II patient, respectively. Interestingly, 1 of the 4 type II subjects showed CD36-negative erythroblasts (type IIb), which is not explained by the known mechanism of CD36 deficiency. Thus, there is another mechanism in the patients with type IIb CD36 deficiency.

A human CD36 isoform produced by the skipping of coding exons IV and V was identified in the human erythroleukaemia cell line, HEL [34]. The 57kDa isoform lacks amino acids 41-143 in the extracellular region. The presence of this variant was confirmed in RNA pools from human placental tissue. It is not known whether this type of alternative splicing of CD36 is present in human erythroid haematopoiesis. However, it is well known that the erythroid (E) and megakaryocytic (Meg) lineages have a common precursor [35, 36], and the regulations of these 2 CD36-positive E- and Meg-lineage specific genes share many features [37-39]. Hence, it is possible that 1 or some of these E- and Meg-specific regulators are involved in the pathogenesis of type IIb CD36 deficiency as an "erythromegakaryocytic-common silent allele". On the other hand, CD36 does not momentarily express in the common precursor stages for the E- and Meg-lineages, but is continuously produced during the proliferating/maturating stages of the nucleated red cells and megakaryocytes. The imaginary mechanism of an "erythromegakaryocytic-common silent allele", therefore, does not support or explain the observations in type IIb.

To study the molecular mechanism of the CD36 expression or deficiency in the erythroid lineage, we further examined the mutation of the CD36 gene using RT-PCR analysis of the monocytes and BFU-e. The mutant alleles of 478T or 970C are not expected to change the cDNA size synthesized using primers from exons 3 to 5 or 4 to 6. All the BFU-e examined in type I patients expressed the CD36 mRNA, therefore the exon-type mutant allele was translated in monocytes as well as in the erythroid lineage. In contrast, 2 patients with

type II examined for cDNA sequences from monocytes showed homozygous wild type alleles. Moreover, the CD36 alleles were not silent in the erythroid cells, because the CD36 mRNA was detected in all the BFU-e from both type IIa and type IIb patients examined. Thus, the mechanism of the CD36 deficiency in the erythroid lineage in type IIb may be explained in the stages after the translation of the CD36 allele, such as glycosylation of the polypeptide. A recently published paper has reported data calling into question the previous hypothesis that a platelet-specific silent allele existed near or at the CD36 gene [40]. Although they did not analyze the expression of the CD36 mRNA, the intensive study of the CD36 gene in 827 Japanese volunteers did not support the platelet-specific silent allele mechanism. There were, surprisingly, 22 individuals showing homozygous wild alleles of the CD36 gene in the 48 patients with the phenotype of type II CD36 deficiency.

Although the BFU-e in our study may have included contaminated monocytes derived from the CFU-GM, the CFU-mix assay was taken from $1x10^5$ MNC of the peripheral blood, and the numbers of both the BFU-e and CFU-GM were approximately 20 in each 35 mm dish. As no overlap of the CFU-GM on the BFU-e was observed, the numbers of contaminated monocytes in the BFU-e should be minimal. Moreover, all the 10 BFU-e made from each individual expressed the mRNA of CD36. The detected CD36 mRNA in the BFU-e was, therefore, not derived from possibly contaminated monocytes, but originated in the BFU-e. We did not analyze the mRNA expression in the platelets to verify whether an allele was silent for 2 reasons. To study this accurately, it would be necessary to obtain a pure platelet sample that does not contain monocytes or monocyte derived fragments. Even after such a complex procedure, RT-PCR is not sufficient to verify it, because the RT-PCR of the CD36 mRNA was so sensitive that the mRNA was detected from a small population of cells in the BFU-e, and minimal contamination of the monocytes in the purified platelet samples may still give false data. The other reason was because both the patients with types IIa and IIb showed homologous wild genotypes. Therefore, "platelet-specific silent allele" does not explain the events observed in the patients.

Platelets and nucleated red cells have common precursors in the bone marrow, and the 2 types of cells may utilize the same enzymes to modulate and mature the CD36 polypeptide after translation of the molecule at such stages as glycosylation. The possible mutations in such enzymes other than the CD36 gene itself may, therefore, rightly explain the mechanism of the type IIb deficiency. However, it seems difficult to identify the molecule responsible for the CD36 deficiency from all the related molecules with modification and maturation of the CD36 polypeptide, despite the successful study on the PIG-A gene mutation in patients with paroxysmal nocturnal haemoglobinuria [41, 42].

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Table 1 Primer sequence of CD36 for RT-PCR

Primer Name	Sequence
CD36 2-5'	aaaattcatgtcttgctgtt (exon 2)
CD36 3-5'	ctggctgtgtttggaggtattct (exon 3)
CD36 4-5'	agtgatgatgaacagcagcaaca (exon 4)
CD36 6-5'	gatcctcaattcacttattaacaa (exon 6)
CD36 8-5'	tattgggaaagtcactgcgac (exon 8)
CD36 13-5'	actatattgtgcctattctttgg (exon 13)
CD36 5-3'	agcgtcctgggttacattttcc (exon 5)
CD36 6-3'	agttctctcaaagttctgacttgg (exon 6)
CD36 9-3'	tacctggcttttctcaacaa (exon 9)
CD36 11-3'	acttgcatacagaaaatgaggaag (exon 11)
CD36 14-3'-1	aggaggtttatttttccagttac (exon 14)
CD36 14-3'-2	gcctaatatgtaacttctctttgat (exon 14)

Table 2 Distributions of age and blood counts (range and mean)

Type	Sex	n	Age	WBC	Neut	RBC	Hb	Plt
I	M	8	35 - 74 (63)	3.57 - 13.50 (5.07)	2.10 - 10.10 (3.46)	3.03 - 5.81 (4.52)	10.7 - 16.0 (13.9)	122 - 301 (191)
I	F	4	30 - 66 (57)	5.30 - 7.10 (5.80)	3.55 - 5.60 (3.68)	3.80 - 4.76 (4.24)	11.5 - 13.0 (12.2)	151 - 287 (271)
II	M	12	38 - 76 (68)	3.37 - 8.40 (6.09)	2.30 - 5.83 (3.90)	3.34 - 5.19 (4.71)	10.1 - 15.8 (14.3)	129 - 283 (211)
II	F	4	58 - 84 (72)	2.79 - 15.30 (4.52)	1.75 - 12.96 (2.16)	3.25 - 4.16 (3.51)	10.9 - 11.9 (11.1)	112 - 205 (175)

Table 3 Findings of the marrow smear preparations and the CD36 cDNA sequence of the blood monocytes in patients with the CD36 deficiency

Type	Age/sex	Primary	WBC	RBC	PLT	Cellularity	E (%)	M (%)	L (%)	M/E	cDNA sequence	BFU-e
I	70/M	OMI	4.31	4.54	207	normo	31.6	50.4	13.6	1.59	478T homo	tested
I	62/F	AP	5.90	3.87	264	normo	22.0	70.0	5.6	3.18	478T homo	tested
I	59/M	HCM	6.61	5.81	299	hypo	14.8	75.2	6.0	5.08	478T homo	tested
I	57/M	HCM	6.44	5.23	186	normo	34.4	51.2	10.4	1.49	970C homo	tested
I	71/M	AP	3.81	4.24	122	hypo	28.0	56.8	12.8	2.03	N.T.	tested
IIa	84/F	AP	4.16	3.43	168	normo	23.2	54.8	17.6	2.36	N.T.	N.T.
IIa	49/M	AP	7.09	5.19	206	normo	34.8	48.4	13.2	1.39	Wild homo	tested
IIa	60/M	AP	6.59	4.97	129	hypo	31.2	59.2	6.0	1.90	N.T.	N.T.
IIb	72/M	AP	5.83	4.65	133	hypo	26.4	61.2	10.4	2.32	Wild homo	tested

Primary disease: OMI, old myocardial infarction; AP, angina pectoris; HCM, hypertrophic cardiomyopathy. Blood tests: WBC and Neut, white cell count and neutrophil count, respectively (x103/ml); RBC, red cell count (x106/ml); Hb, hemoglobin (g/dl); PLT, platelet count (x103/ml). Bone marrow cellularity: normo, normocellular and hypo, slightly hypocellular. Hematopoietic cell proportion in bone marrow: E (%), erythroid; M (%), myeloid; L (%), lymphoid; M/E ratio. cDNA sequence: homo, homozygous; Wild, normal; N.T., not tested; 478T and 970C, point mutations at 478 (C → T) and 970 (T → C), respectively.

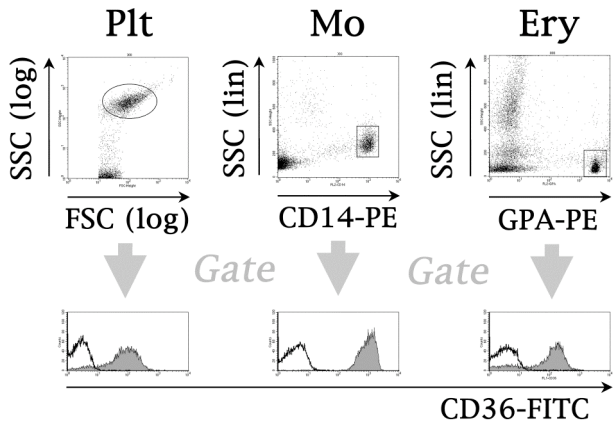

Figure 1. The acquisition gates to analyze the CD36 expression in the blood platelets (Plt), monocytes (Mo) and bone marrow nucleated red cells (Ery) obtained from a normal volunteer.

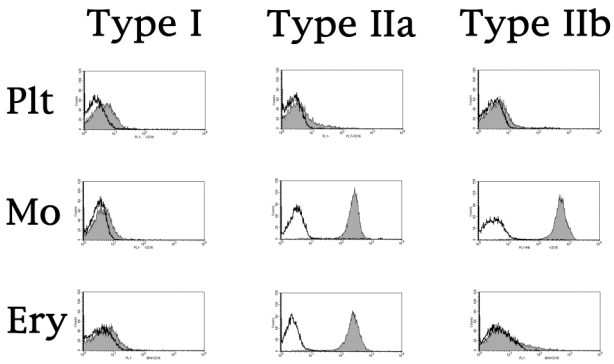

Figure 2. The CD36 expression in the peripheral blood platelets (Plt), monocytes (Mo) and bone marrow erythroblasts (Ebl) obtained from patients with type I, type IIa and type IIb CD36 deficiency. The gray and white peaks are histograms of the CD36 and isotype control fluorescence, respectively.

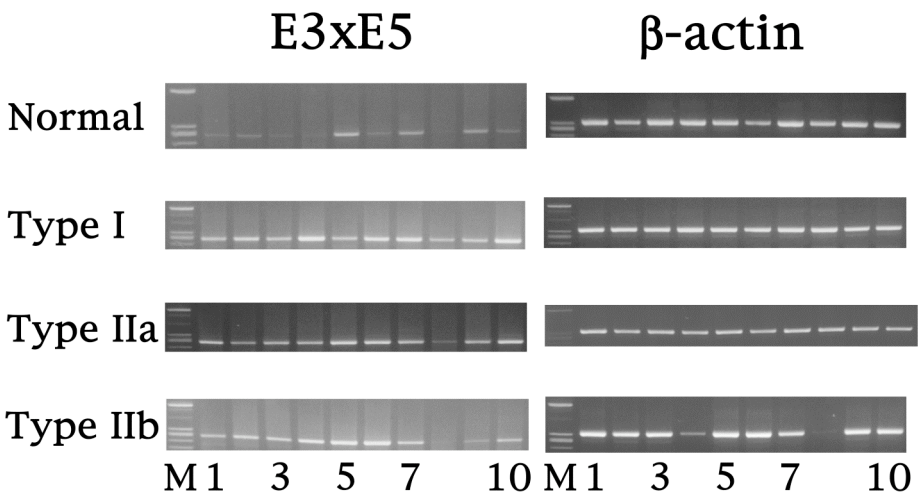

Figure 3.
RT-PCR for the CD36 mRNA extracted from 10 BFU-e of peripheral blood obtained from a normal volunteer, and types I, IIa and IIb CD36 deficient patients. Lanes M and 1 to 10, markers (603, 310, 281/271 and 234 bp) and 10 BFU-e. E3xE5, exons 3 to 5 using primers CD36 3-5' and 5-3' in Table 1.