CD47 is expressed at normal levels in patients with autoimmune haemolytic anaemia and/or immune thrombocytopenia

N. Ahrens, C. Pagenkopf, H. Kiesewetter and A. Salama
Institute for Transfusion Medicine, Charité–Universitätsmedizin Berlin, Berlin, Germany

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SUMMARY. CD47 deficiency results in lethal autoimmune haemolytic anaemia (AIHA) and mild spontaneous thrombocytopenia in non-obese diabetic mice. It is unknown whether CD47 has an impact on AIHA of the warm type or autoimmune thrombocytopenia (ITP) in humans.

Healthy blood donors (n = 11), patients with AIHA (n = 13), patients with ITP (n = 18) and one patient with Rh null phenotype were investigated. CD47 expression on red blood cells (RBC), platelets, granulocytes and lymphocytes and in plasma was determined by quantitative flow cytometry.

All types of blood cells studied were found to carry CD47. Although CD47 expression on Rh null RBCs was decreased, there was no significant difference between CD47 expression on RBCs of healthy blood donors and on those of patients with AIHA or ITP. Similarly, CD47 was detectable in the plasma of the studied subjects.

No evidence for a pathogenetic role of CD47 in autoimmune haemolysis or thrombocytopenia in humans could be demonstrated.

Key words: AIHA, CD47, ITP.

METHODS AND PATIENTS

For diagnosis of autoimmune cytopenias, detection of characteristic autoantibodies is still regarded as the ‘gold standard’. However, their prognostic value is limited and does not allow for a precise prediction of the severity of autoimmune haemolytic anaemia (AIHA) or autoimmune thrombocytopenia (ITP) (Salama et al., 2002).

CD47 (integrin-associated protein) is a ubiquitously expressed 50-kDa transmembrane glycoprotein that has been suggested to act as a ‘marker of self’, since murine red blood cells (RBCs) and platelets lacking this protein are rapidly eliminated by macrophages of wild-type mice (Oldenborg et al., 2000; Olsson et al., 2005). CD47 is not only expressed on all types of blood cells, including RBCs, platelets and leukocytes, epithelial and endothelial cells, fibroblasts and mesenchymal cells, but also on various tumour cell lines. It has been found to bind to signal regulatory protein (SIRP) α, SIRPβ2 and thrombospondin (Oldenborg, 2004; Piccio et al., 2005). Furthermore, it interacts with several integrins (Oldenborg, 2004), and is involved in cell migration and phagocytosis. In mice, the binding of RBC CD47 to SIRPα on macrophages contributes to the inhibition of phagocytosis, and CD47-deficient non-obese diabetic (NOD) mice develop severe AIHA (Oldenborg et al., 2002). On human RBCs, CD47 is part of the Rh complex (Cartron, 1999; Dahl et al., 2003), which is the target of warm-reactive autoantibodies (Salama et al., 2002). In addition, patients of the Rh null phenotype may exhibit mild compensated haemolytic anaemia. Haemolysis is also typical for protein 4-2-deficient human RBCs that express low levels of CD47, suggesting a causal relationship between haemolysis and CD47 expression (Bruce et al., 2002).

In this study, we measured CD47 expression in various blood cell types in patients with AIHA or ITP in comparison to healthy blood donors.
AIHA of warm type and/or ITP as well as from one patient with the Rhnull phenotype. All donors and patients gave their informed consent. The samples were processed immediately, and all incubation steps were performed at room temperature. Although some protocols (RBC, plasma) were available for all patients, platelets and leukocytes could only be tested during the second phase of this study.

Patients with serologically detectable RBC autoantibodies were regarded as having active disease if they required medical treatment, and as having AIHA in remission if they did not. None of the patients were transfused within the last 6 months prior to investigation.

Red blood cells

Blood samples were centrifuged at 1710 × gmax for 5 min. In order to reduce background staining by nonspecific antibody attachment, 10 μL of washed RBCs (suspended to 10 000 μL⁻¹) was pre-incubated with 20 μL human IgG (5% w/v Venimmun, Aventis Behring, Marburg, Germany) for 15 min and subsequently stained with 20 μL anti-CD47 phycoerythrin (PE) (Becton-Dickinson, Heidelberg, Germany) for 30 min. Before measuring CD47, 500 μL phosphate-buffered saline (PBS) was added.

Platelets

Blood samples were centrifuged at 120 × gmax for 25 min. The supernatant (platelet-rich plasma) was transferred to a second tube and centrifuged at 1710 × gmax for 5 min. The pellet was resuspended in Hanks’ solution to a concentration of 5000 platelets μL⁻¹, 50 μL was blocked with IgG as described for RBCs and stained with 10 μL anti-CD41 fluorescein isothiocyanate (FITC) (Becton-Dickinson) and 10 μL anti-CD47 PE. Following incubation for 30 min, 500 μL Hanks’ solution was added to each sample.

Leukocytes

Two hundred microlitres of whole blood was lysed by the addition of 200 μL OptiLyse (Immunotech, Marseille, France) for 10 min, and 4 mL distilled water for an additional 10 min, according to the manufacturer’s instructions. The leukocytes were washed once with 2 mL PBS (615 × gmax, 10 min). Fifty microlitres of resuspended cells (2000 leukocytes μL⁻¹) was blocked with IgG and stained with 10 μL anti-CD45 FITC (Becton-Dickinson) and 20 μL anti-CD47 PE. After a 30-min incubation, 500 μL PBS was added.

Plasma CD47

The following anti-CD47 clones were investigated pairwise in fluorescence quenching assays: BRIC126 (Serotec, Düsseldorf, Germany), miap301 that cross-reacts with human CD47 and B6H12 (Becton-Dickinson) and BRIC125 (IBGRL, Bristol, UK). Non-competitive binding was found for no pair of the clones. Pre-incubation of RBCs with any of the clones decreased the RBC fluorescence intensity of every other clone.

Therefore, the amounts of soluble CD47 in plasma were measured indirectly by a consumption assay. Ten microlitres of microfiltered plasma (0.45 μm, Mini-Sart, Sartorius, Göttingen, Germany) was pre-incubated with 20 μL anti-CD47 PE (12.5% in PBS) for 30 min, and unbound anti-CD47 PE was captured by a standard sample of healthy donor RBCs as described above. Following a 30-min incubation, 500 μL PBS was added. The remaining fluorescence intensity was subtracted from the fluorescence intensity of negative controls using PBS instead of plasma in order to acquire values that directly correlate with the quantity of soluble CD47.

Flow cytometry

All samples were analysed by FACScan flow cytometry (Becton-Dickinson) and CellQuest software. The flow cytometer settings and output for RBC, platelet and leukocyte were calibrated using isotype controls and standardized beads with four defined levels of PE molecules (Quantibrite, Becton-Dickinson). The calculated regression (Excel, Microsoft, Redmond, WA) for each setting was used to estimate the units of fluorescence intensity (U) from the cells’ geometric mean.

Cell types were identified according to forward and side light scatter qualities. Platelets and leukocytes were additionally stained with and gated for anti-CD41 and anti-CD45, respectively (Fig. 1), and 20 000 events were analysed for CD47. After the labelling, quantitative flow cytometry was done without further washing.

Statistical analysis

Data were processed using the statistical package for social sciences (SPSS version 11, Chicago, IL). The fluorescent intensity units (U) of the donor, AIHA and ITP groups are reported as medians, and normal distribution was calculated with the one-sample Kolmogorov–Smirnov test. Groups with equal variances (Levene’s P > 0.05) were compared with Tukey’s honestly significant difference test. In all other cases, Tamhane’s T2 test was performed.
RESULTS

Red blood cells

Unlike Rhnull RBCs that were observed to express very small quantities of CD47 (2017 U, Fig. 2a), RBCs from patients with AIHA were found to express CD47 with the same strong intensity as normal RBCs (25 069 and 23 493 U, respectively, $P = 0.55$). CD47 was detectable on RBCs independent of disease activity and did not change

Fig. 1. Flow cytometric CD47 quantification. RBCs (a), platelets (b), granulocytes (c + d) and lymphocytes (e + f) were gated as described in Methods. An appropriate percentage of the events was chosen to display the distribution.
significantly during follow-up in samples from 10 patients (Fig. 2b, \(P = 0.53\)).

As indicated by the interquartile distances in Fig. 2, CD47 expression on RBCs from patients with AIHA was variable. Though the RBCs of patients with AIHA were somewhat smaller than donor RBCs as indicated by the forward scatter (geometric mean fluorescence 219 and 242, respectively), a difference in size does not correlate with CD47 expression (Fig. 2c).

**Platelets**

CD47 expression on platelets was predominantly identical in healthy blood donors (1977 U) and ITP patients (2129 U), with no significant difference observed (Fig. 3a, \(P = 0.86\)).

**Lymphocytes**

CD47 was also detectable on lymphocytes of donors (38 916 U), patients with AIHA and patients with ITP (38 141 and 34 044 U, respectively; Fig. 3b, \(P = 1.0\)). However, the expression levels in ITP lymphocytes were observed to be slightly decreased (\(P = 0.065\)).

**Granulocytes**

There was considerable variation in CD47 expression on the cells in the granulocyte gate (Fig. 3c). Patients with AIHA and/or ITP had slightly higher levels of CD47 expression than healthy donors (17 629 and 16 785 U vs 13 687 U, respectively), although a statistically significant difference was not observed for AIHA (\(P = 0.066\)) and ITP patients (\(P = 0.58\)).
CD47 was detectable in the plasma of healthy donors (4517 U) as well as in that of AIHA patients (4550 U). Significantly higher levels of CD47 were observed in the plasma of patients with ITP (4946 U; Fig. 4, \( P = 0.0026 \)).

DISCUSSION

There is good evidence to suggest that CD47 may play a role in AIHA and ITP in mice (Oldenborg et al., 2002). In this study, we analysed the expression of CD47 on various blood cells of different patients with AIHA and/or ITP. A significant difference between the expression of CD47 on RBCs and platelets in these patients in comparison to healthy donors was not observed. This finding does not support the hypothesis that CD47 may be involved in AIHA and ITP but on the contrary, is in agreement with results from in vitro experiments demonstrating that Rhnull RBCs with reduced CD47 expression do not show increased interaction with peripheral blood monocytes (Arndt & Garratty, 2004). In addition, patients with weak CD47 expression may exhibit some haemolysis but, unlike NOD mice, do not develop AIHA, i.e. Rhnull phenotype and protein 4.2 deficiency (Avent et al., 1988; Rybicki et al., 1993; Cartron, 1999; Bruce et al., 2002; Dahl et al., 2003; Mouro-Chanteloup et al., 2003). It is possible that these patients have sufficient residual CD47 to prevent autoimmunization. A dosage effect, as seen with murine platelets (Olsson et al., 2005), has not been observed with RBCs (Oldenborg et al., 2000). Thus, a preventive contribution of CD47 to AIHA and ITP cannot be completely excluded. Furthermore, opsonized RBCs lacking CD47 are much more rapidly cleared by macrophages than opsonized normal RBCs (Oldenborg et al., 2001). The same phenomenon has been demonstrated using opsonized and non-opsonized platelets lacking CD47 (Olsson et al., 2005). A possible explanation for the apparent difference between the rapid elimination of CD47-negative murine red cells \textit{in vivo} (Oldenborg et al., 2001; Olsson et al., 2005) and survival of human CD47 weak RBCs (Arndt and Garratty, 2004) might lie in the origin of the involved antibodies and macrophages. The antibodies used for opsonization may have a higher avidity, thus not being comparable to human autoantibodies. In addition, the CD47–SIRP\(\alpha\) interaction has been shown to be both species and cell-type specific (Subramanian et al., 2006). Its affinity in humans may be low and therefore not as significant as in mice. The cell-type specificity is also demonstrated by the fact that only splenic macrophages have been shown to phagocytose CD47-deficient cells (Oldenborg et al., 2000). However, a contribution of CD47 in the development of AIHA in humans cannot be completely excluded. It is possible that functional alterations or isoforms of CD47 may exist.

CD47 inhibits not only antibody-dependent phagocytosis but also unspecific phagocytosis. Clearance of CD47-deficient RBCs in CD47-positive mice has been observed in strains that are C3 or antibody deficient (Oldenborg et al., 2000). Moreover, it has been shown that the phagocytosis of perfluorocarbon particles is markedly reduced by co-infusion of recombinant CD47 molecules (Hsu et al., 2003). Interestingly, this study has demonstrated CD47 expression not only on blood cells but also in plasma. It is unclear whether plasma CD47 stems from microparticles and/or from shedding or secretion. Microparticles are small membrane fragments from platelets and other cells that can be detected in all plasma samples and are known to be present at higher concentrations in patients with ITP (Jy et al., 1992; Morel et al., 2004). This is in agreement with our observation of higher CD47 levels in ITP patient samples. CD47 of cellular origin has also been observed in the supernatant of old RBC units (Anniss & Sparrow, 2002). Whether the loss of RBC-bound CD47 and the increase in microparticle CD47 has an impact on transfusions (Bessos & Seghatchian, 2005; Stewart et al., 2005), AIHA or ITP has yet to be elucidated.
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