

Residual platelet ADP reactivity after clopidogrel treatment is dependent on activation of both the unblocked P2Y₁ and the P2Y₁₂ receptor and is correlated with protein expression of P2Y₁₂

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Abstract Two ADP receptors have been identified on human platelets: P2Y₁ and P2Y₁₂. The P2Y₁₂ receptor blocker clopidogrel is widely used to reduce the risks in acute coronary syndromes, but, currently, there is no P2Y₁ blocker in clinical use. Evidence for variable responses to clopidogrel has been described in several reports. The mechanistic explanation for this phenomenon is not fully understood. The aim of this study was to examine mechanisms responsible for variability of 2MeS-ADP, a stable ADP analogue, induced platelet reactivity in clopidogrel-treated patients. Platelet reactivity was assessed by flow cytometry measurements of P-selectin (CD62P) and activated GpIIb/IIIa complex (PAC-1). Residual 2MeS-ADP activation via the P2Y₁₂ and P2Y₁ receptors was determined by co-incubation with the selective antagonists AR-C69931 and MRS2179 in vitro. P2Y₁ and P2Y₁₂ receptor expression on both RNA and protein level were determined, as well as the P2Y₁₂ H1 or H2 haplotypes. Our data suggest that the residual platelet activation of 2MeS-ADP after clopidogrel treatment is partly due to an inadequate antagonistic effect of clopidogrel on the P2Y₁₂ receptor and partly due to activation of the P2Y₁ receptor, which is unaffected by clopidogrel. Moreover, a correlation between increased P2Y₁₂ protein expression on platelets and decreased response to clopidogrel was noticed, $r^2=0.43$ ($P<0.05$). No correlation was found between P2Y₁₂ mRNA

levels and clopidogrel resistance, indicating post-transcriptional mechanisms. To achieve additional ADP inhibition in platelets, antagonists directed at the P2Y₁ receptor could be more promising than the development of more potent P2Y₁₂ receptor antagonists.

Key words AR-C 69931 · clopidogrel · MRS 2179 · P2Y₁ · P2Y₁₂ · platelets

Introduction

ADP receptors play an important role in the activation of platelets by acting as a positive feedback mechanism. There is evidence of two different ADP receptors expressed on human platelets: the Gq-coupled P2Y₁ and the Gi-coupled P2Y₁₂ [1, 2]. The P2Y₁₂ antagonist clopidogrel has been shown to be an important platelet inhibitor. The Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events (CAPRIE) and Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) studies demonstrate that inhibition of the P2Y₁₂ receptor is beneficial, both alone and as a complement to standard aspirin treatment [3, 4]. At present, the standard regimen after a non-ST elevation myocardial infarction or coronary stenting is a combined treatment with both clopidogrel and aspirin.

However, evidence for a variable response to clopidogrel has recently been studied [5–7], and clopidogrel resistance has been associated with an increase in cardiovascular events [8]. The mechanisms underlying this phenomenon have not been fully elucidated. An association of clopidogrel resistance with the activity of the hepatic cytochrome

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CYP3A4 has been suggested [9]. Medication with atorvastatin has also been proposed to influence the metabolism of clopidogrel, by acting as a competitive substrate for CYP3A4 [10]. However, later studies have not been able to confirm this relationship [11–13]. Other potential explanations may be variations in receptor expression and function or variation in intracellular signaling pathways. A single nucleotide polymorphism (SNP) in the P2Y₁₂ receptor associated with increased function of the receptor and increased risk for peripheral arterial disease (PAD) has been described and characterized [14, 15]. Again, a more recent study has not been able to confirm this association [16]. It is also plausible that the second ADP receptor expressed on human platelets, P2Y₁, may be involved in the insufficient inhibition of ADP-mediated platelet activation after clopidogrel treatment.

We recently developed a real-time quantitative polymerase chain reaction (PCR) method for studies of transcriptional regulation of specific mRNA transcript in human platelets [1]. It has been useful in clinical studies demonstrating a down-regulation of P2Y₁₂ receptor mRNA in patients with systemic lupus erythematosus (SLE) compared with healthy controls [17].

The aim of the present study was to investigate whether residual 2MeS-ADP, a stable ADP analogue, induced platelet reactivity after clopidogrel treatment due to unblocked P2Y₁ or to insufficient inhibition of P2Y₁₂ receptor activity. We were also interested in evaluating if variations in residual platelet reactivity after clopidogrel treatment could be correlated with receptor properties: P2Y₁₂ expression, on mRNA or protein level, or to the P2Y₁₂ haplotype.

Material and methods

Selection of patients

Our study group consisted of 19 patients (six women and 13 men) recently treated by percutaneous coronary intervention (PCI). Blood samples were collected during a visit to an out patient clinic, at least 2 weeks after the intervention. All patients were on both aspirin and clopidogrel and had a median age of 66 years (range 49–84 years). Eight had a history of previous acute myocardial infarction, and four had documented diabetes mellitus. There were 17 on statins and 14 on either angiotensin-converting enzyme (ACE) inhibitors or angiotensin II receptor blockers.

The Ethics Committee of Lund University approved the project. All patients had given written consent prior to participation in the study.

Blood collection and handling

For flow cytometry, blood was collected in vacutainer 4.5 ml citrate tubes. All experimental procedures were carried out as fast as possible and always within 10 min of blood collection. For RNA and protein analysis, blood was drawn in six vacuette 6 ml EDTA tubes. Platelet rich plasma (PRP) was then prepared by centrifugation at 160 *g* for 20 min at 4°C. PRP was carefully collected without disturbance of the buffy coat and was centrifuged again at 160 *g* for 10 min at 4°C to remove contaminating leukocytes. The PRP was then transferred to a new tube, and the platelets were collected by centrifugation at 1000 *g* for 10 min at 4°C. The platelet pellet was immediately dissolved in 1 ml TRIzol and frozen at –80°C.

Flow cytometric assessment of platelet activation

Flow cytometric studies were performed on a Becton and Dickinson FacScan. The following antibodies were used: fluorescein isothiocyanate (FITC)-conjugated anti-GPIIb/IIIa (PAC-1) antibody, phycoerythrin (PE)-conjugated anti-P-selectin (CD62P) and peridinin chlorophyll-a protein (PerCP)-conjugated anti-CD61. 2MeS-ADP (1 μM) was used as a stable P2Y agonist. We used the same concentration, 10 μM, of both antagonists, AR-C 69931 and MRS 2179, based on a dose-finding study made prior to the clinical study. AR-C 69931 is more potent (IC₅₀ around 0.4 nM) than MRS 2179 (reported IC₅₀ in human platelets 147 nM), but the fact that the P2Y₁₂ receptor in the clopidogrel-treated patient is already partly blocked changes this relationship [18, 19]. To achieve roughly the same amount of inhibition a more potent P2Y₁₂ antagonist is needed. Five microliters of whole blood was incubated in phosphate buffered saline (PBS) solution, pH 7.4, containing agonist, appropriate antagonist and saturating concentrations of antibodies in a total volume of 50 μl for 20 min in the dark at room temperature. The reaction was then stopped by the addition of 3 ml 1% paraformaldehyde solution at 4°C followed by 20 min incubation at 4°C in the dark. Three-color flow cytometry was then performed. Forward scatter and side scatter were used to gate the platelet population. A total of 10,000 gated events was collected. To further exclude other cell types, we included only CD61 positive cells in the analysis. P-selectin positive platelets were quantified by determining the percentage of cells with fluorescence greater than mouse IgG control. For anti-GPIIb/IIIa antibody, PAC-1, the mean fluorescence intensities (MFIs) were measured. Inhibition of platelet activation was expressed as the relative inhibition of platelet activation after addition of drugs compared with stimulated platelets.

RNA and protein extraction

Total cellular RNA and protein was extracted using TRIzol reagent (Gibco BRL, Life Technology) according to the supplier's instructions. The RNA concentration was determined spectrophotometrically, with a ratio of optical density (OD)_{260:280} > 1.6 being considered as pure. Samples were stored at -70°C until required. The quality of RNA and protein was fully satisfactory in only 14 subjects. Protein pellets were vacuum dried and dissolved in 1% sodium dodecyl sulfate solution. DC protein assay (BioRad Laboratories, USA) was used to determine protein concentrations. Protein samples were stored at -20°C until required.

Quantitative analysis of P2 receptors by real-time reverse transcription polymerase chain reaction

Total RNA was reverse-transcribed using Multiscribe RT Kit (Qiagen, CA, USA) following the manufacturer's instructions. Real-time PCR on platelet cDNA was performed using the following primers: P2Y₁₂ 5'-CTATCGATCGCTACCA GAAGAC-3', 5'-TCATGCCAGACTAGACCGAA-3', GAPDH 5'-GTCCACTGGCGTCTTCACCA-3', 5'-GTGGCAGTGATGGCATGGAC-3'. Relative gene expression levels were determined using LightCycler (Roche, Basle, Switzerland). As a template, 1 μl platelet cDNA was used in a total volume of 10 μl [3 mM MgCl₂, 0.5 μM of each primer (described below) and 1 \times LightCycler DNA Master SYBR Green I mix (Roche)]. The following PCR amplification protocol was used: initial denaturation at 95°C for 600 s, followed by 45 PCR cycles at 95°C for 1 s, 55°C (P2Y₁₂) or 58°C (GAPDH) respectively for 6 s, and 74°C for 23 s. Target gene amplification levels were quantified using a second-derivative maximum method available in the Light-Cycler software. P2Y₁₂ receptor expression was determined using the housekeeping gene GAPDH as a reference. To validate specificity of amplification reactions, the Light-Cycler PCR products were sequenced using BigDye v.3.1 (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as described elsewhere [20]. Briefly, 20 μg protein per lane was separated using 10% SDS-polyacrylamide gels and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). Sequential detection of P2Y₁ and P2Y₁₂ (at 1:200 dilution) and GAPDH (at 1:500 dilution) was

performed according to the following procedure in the above-mentioned order. Membranes were incubated for 1 h at room temperature with the appropriate primary antibody, washed three times with TBS 0.1% Tween, incubated for 1 h with HRP-conjugated secondary antibodies, washed three times and visualized using the ECL western blotting system and Hyperfilm. After each round of immunodetection, the membranes were incubated for 30 min at 50°C in stripping buffer (100 mM 2-mercapto-ethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) to remove previously bound antibodies, washed twice in TBS 0.1 % Tween, blocked with 5% fat-free milk in TBS 0.1% Tween and used again for the next set of antibodies.

The signal intensity (integral volume) of the appropriately sized bands was scanned and analyzed using Quantity One software (BioRad). The signal intensities of the different P2 receptors were normalized to GAPDH to account for variations in the amounts of total protein present in each lane.

Determination of P2Y₁₂ haplotype

DNA was isolated from whole blood using the TRIzol reagent according to the manufacturer's instructions. A PCR using HotStarTaq (Qiagen) and the primers 5'-GAGCACTCAAGACTTTACTGACG-3' and 5'-GAGAA GATCAGAAATGACTGTGTTC-3' was used to amplify the entire second and parts of the third exon of the of the P2Y₁₂ gene. The 2233 bp amplification product, containing two *Tai*I restriction sites, [at polymorphism i-T744C (1008 bp from the 5' end of the amplification product)] and at a control *Tai*I site (at 1588 bp), was diluted three times with water, digested for 1 h with 2U *Tai*I at 65°C in a total volume of 30 μl and visualized by agarose gel electrophoresis. The P2Y₁₂ H1 allele was identified by the presence of two bands (1588 bp- and 645 bp-long restriction fragments), and the H2 allele by three bands (1008 bp-, 580 bp- and 465 bp-long restriction fragments) [13].

Reagents, antibodies and drugs

Unless otherwise stated all reagents and drugs were purchased from Sigma Chemical Corp., St. Louis, MI, USA. PCR and RT-PCR consumables were purchased from Perkin-Elmer Applied Biosystems Inc., Foster City, CA, USA. PCR primers were from MWG Biotech AG, Ebersberg, Germany and TRIzol from Invitrogen, Carlsbad, CA, USA. Western blot reagents were from Amersham Pharmacia Biotech, UK, or Bio-Rad Laboratories, USA. Anti-P2Y₁ and anti- P2Y₁₂ antibodies were purchased from Alomone Labs, Israel, and the anti-GAPDH was from Chemicon, USA. Anti-CD61, anti-CD62P and anti-PAC1 antibodies

were from BD, USA. MRS 2179 was from Tocris, and AR-C 69931 was a kind gift from Astra-Zeneca, Sweden. All drugs were dissolved in 0.9% saline solution.

Statistics and analysis

Calculations and statistics were performed using GraphPad Prism 4.00 software. Repeated-measures analysis of variance (ANOVA) with Bonferroni's post-test was used to compare flow cytometry data. Values are presented as mean \pm SEM. Comparison of quantitative real-time data was performed using the method described by Pfaffl [21]. The amplification efficiencies of each primer pair were tested prior to analysis.

Results

Flow cytometry assessment of platelet activation

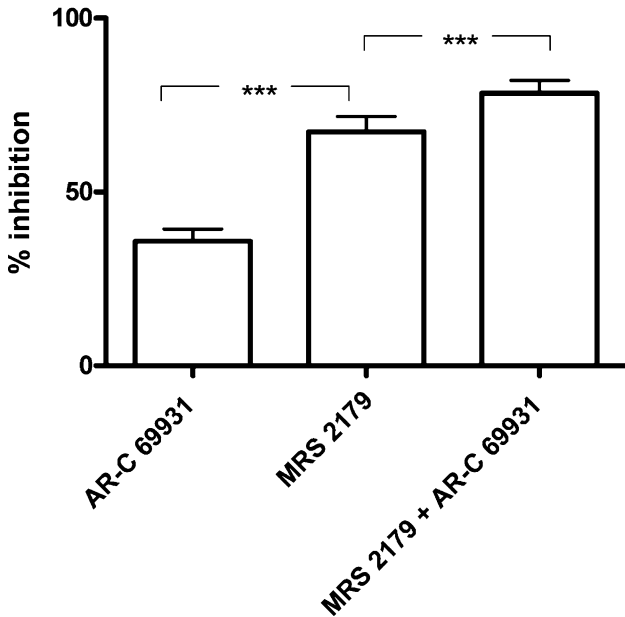
After stimulation of platelets with 1 μ M 2MeS-ADP, additional antagonism of the P2Y₁₂ receptor with AR-C 69931 (10 μ M), to platelets from patients already treated with

clopidogrel and aspirin, inhibited the residual percentage of P-selectin positive platelets by 35.8 \pm 3.5% (mean \pm SEM) ($n=19$) and the expression of activated GPIIb/IIIa by 43.5 \pm 3.4%. The blocking of the P2Y₁ receptor with MRS 2179 (10 μ M) resulted in a significantly greater inhibition of the percentage of P-selectin positive platelets by 67.3 \pm 4.4% and of activated GPIIb/IIIa to 54.2 \pm 2.6%. Combining the antagonists yielded an even greater significant inhibition: 78.4 \pm 3.8% for P-selectin and 72.2 \pm 2.5% for GPIIb/IIIa. Data are presented in Figure 1.

Correlation of function with protein and RNA expression

Proteins were run on western blot, and bands from the P2Y₁₂ receptor and GAPDH were detected. Signal intensities were quantified, and each P2Y₁₂ reading was normalized to the GAPDH expression in the same sample to correlate with different loading amounts. The relative normalized expression of P2Y₁₂ protein correlated with the percentage of cells expressing P-selectin after stimulation by 2MeS-ADP, with a linear regression of $r^2=0.43$ ($P<0.05$) ($n=14$). No correlations were found between P2Y₁ protein levels and P-selectin positive platelets ($r^2=0.002$, $P=0.88$, $n=14$). Data are presented in Figure 2.

a Inhibition of P-selectin positive platelets



b Inhibition of activated GPIIb/IIIa

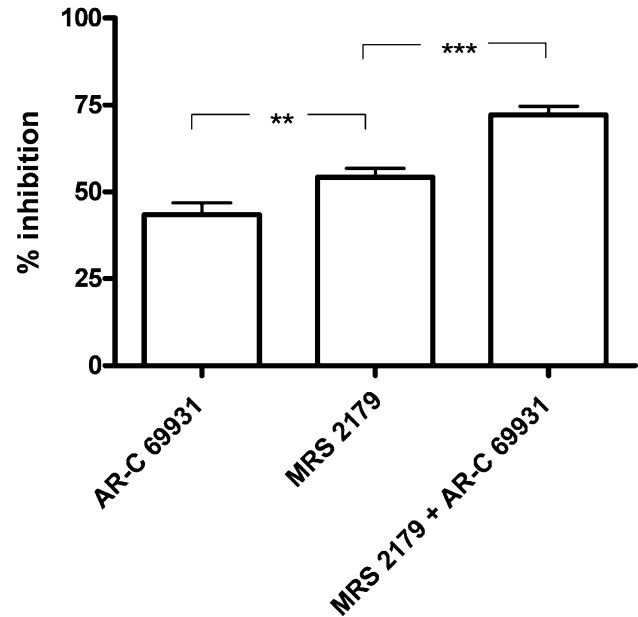


Figure 1 Graph showing the increased inhibition of residual ADP-induced P-selectin positive platelets (a) and expression of activated GPIIb/IIIa (b) after the further blocking of the P2Y₁₂ receptor with AR-C 69931 (10 μ M) and the P2Y₁ receptor with MRS 2179 (10 μ M) and the blocking of both receptors with a combination of both antagonists. These effects are seen in platelets already blocked by clopidogrel and aspirin. Blocking the P2Y₁₂ receptor further with AR-C 69931 (10 μ M) significantly inhibited the percentage of P-selectin positive platelets by 35.8 \pm 3.5% (mean \pm SEM) ($n=19$) and the expression of activated GPIIb/

IIIa by 43.5 \pm 3.4%. A significantly greater inhibition was seen when the P2Y₁ antagonist MRS 2179 (10 μ M) was used, where the inhibition of P-selectin increased to 67.3 \pm 4.4% and the inhibition of activated GPIIb/IIIa increased to 54.2 \pm 2.6%. Combining the antagonists yielded an even greater significant inhibition: 78.4 \pm 3.8% for P-selectin and 72.2 \pm 2.5% for activated GPIIb/IIIa. There were significance differences between the groups, $P<0.01$, in all cases ($n=19$). Repeated-measures ANOVA with Bonferroni's post-test were used to compare data

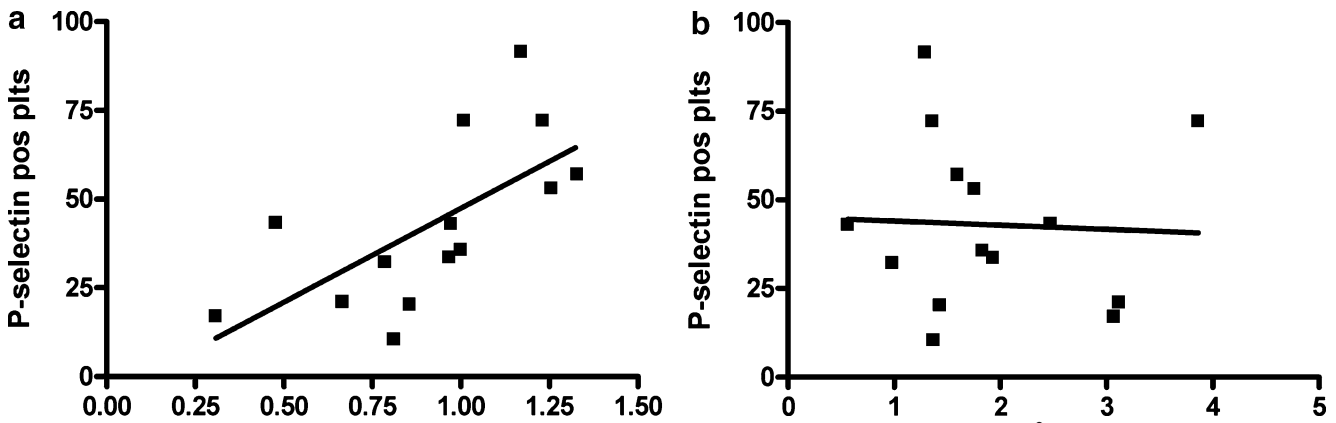


Figure 2 The relative expression and GAPDH-normalized expression of P2Y₁₂ (a) and P2Y₁ (b) protein correlated with the percentage of P-selectin positive platelets after stimulation with 1 μ M of 2MeS-ADP. In (a) there is a significant correlation of P-selectin positive platelets

with P2Y₁₂ protein expression, with $r^2=0.43$ ($P<0.05$) ($n=14$). In (b) there is no significant correlation of P-selectin positive platelets and P2Y₁ protein expression ($r^2=0.002$, $P=0.88$, $n=14$) (*pos plts* positive platelets)

P2Y₁₂ and GAPDH mRNA expressions were quantified, and no correlation was found between the relative expression levels of the P2Y₁₂ mRNA and the percentage of cells expressing P-selectin or activated GPIIb/IIIa after stimulation with 2MeS-ADP ($r^2=0.30$, $P=0.20$ for P-selectin; $r^2=0.04$, $P=0.19$ for GPIIb/IIIa) (data not shown).

P2Y₁₂ haplotype and function

The haplotype was determined by PCR sequencing and restriction digestion. Thirteen patients were homozygous for the H1 haplotype (H1/H1) and six patients were heterozygous (H1/H2), whereas none of the patients was homozygous for the H2 haplotype. There was no difference in the percentage of platelets expressing P-selectin after 2MeS-ADP (1 μ M) in the homozygous (H1/H1) group compared with the heterozygous (H1/H2) group ($45.2\pm 6.4\%$ for H1/H1 vs $37.0\pm 9.0\%$ for H1/H2, $P=0.47$) (unpaired *t*-test).

Discussion

This study suggests that the residual 2MeS-ADP platelet reactivity after clopidogrel treatment is in part due to an inadequate antagonistic effect of clopidogrel on the P2Y₁₂ receptor and partly due to the P2Y₁ receptor, which is unaffected by clopidogrel. Moreover, a correlation between increased P2Y₁₂ protein expression on platelets and decreased response to clopidogrel was noticed. No correlation was seen between platelet activation and P2Y₁₂ mRNA expression or the P2Y₁₂ H1/H2 haplotype.

Our cohort of patients was chosen to reflect the real clinical situation as much as possible, resulting in a heterogeneous group having other illnesses and taking several other medications apart from clopidogrel and aspirin. The *cohort* was too small for us to be able to do any

subgroup analyses in terms of other medication, previous diseases or gender. Since all of the patients were on aspirin, the impact of reduced prostaglandin synthesis was *present throughout*. Despite the inhibition by both clopidogrel and aspirin, we were able to activate their platelets further with 2MeS-ADP. The remaining 2MeS-ADP activation was dependent on both insufficient P2Y₁₂ blockade by clopidogrel and the unaffected ADP receptor P2Y₁. Most likely, many different factors contribute to produce the observed clinical phenotype. One of the most important factors is still the second ADP receptor, P2Y₁, that remains totally unaffected by clopidogrel. To achieve full ADP inhibition in the future, increasing the effect of clopidogrel or other P2Y₁₂ blockers will not suffice. Thus, the clinical benefit of a P2Y₁ blocker will be an interesting alternative.

Despite the use of both AR-C 69931 and MRS 2179, platelets were not completely inhibited after 2MeS-ADP stimulation. This was probably due to the very high concentration of agonist used to stimulate the system; 1 μ M of 2MeS-ADP corresponds to a significantly higher dose of unmodified ADP. The platelet stimulation was also conducted in whole blood, where leukocytes and erythrocytes may also influence the degree of platelet stimulation.

Resistance to the COX-1 inhibitor acetylsalicylic acid (aspirin) has been studied and debated for many years. Small observational studies suggest that non-responders to aspirin are at greater risk of cardiovascular events than are responders [22–25]. Recently, resistance to clopidogrel has emerged as an interesting occurrence with a clear clinical relevance [6–8]. The fact that clopidogrel resistance has an impact on the cardiovascular outcome in patients with coronary artery disease merits further studies to elucidate the underlying mechanisms [8]. Theoretically, many factors may influence the clopidogrel response: bioavailability, genetic or transcriptional variations in the metabolizing enzymes or in the target receptor and interactions with other

compounds. One early study demonstrated a correlation between clopidogrel resistance and atorvastatin medication, but this hypothesis was later dismissed by several other studies [11–13]. Currently, two studies have investigated possible mechanisms underlying clopidogrel resistance. One study was focused on the hepatic metabolism of the drug, and the other was focused on the bioavailability of clopidogrel [9, 26]. The bioavailability and the hepatic metabolism of the pro-drug clopidogrel do seem to be important for clopidogrel resistance [9, 26]. For the first time we find that increased platelet P2Y₁₂ receptor protein expression is a component of clopidogrel resistance. Increased abundance of a receptor protein may thereby result in less inhibition by its antagonist. However, whether these patients are constituent high P2Y₁₂ expressers that increase their risk of myocardial infarction is not known. Animal studies have shown a correlation between function and overexpression of P2Y₁ protein, but we could not find this association in our clinical material [27].

Platelets are anuclear cells and are therefore unable to produce new nuclear-derived mRNA transcripts. However, it has been shown that platelets contain megakaryocyte-derived mRNA that can be activated upon platelet stimulation to produce, for example, IL1- β and PAI-1 [28, 29]. Thus, translational regulation can account for the variation in protein expression. Interestingly, there was no correlation between P2Y₁₂ mRNA levels and variation of clopidogrel inhibition. These results suggest that post-transcriptional regulation of the P2Y₁₂ receptor expression is responsible for the correlation between P2Y₁₂ protein expression and the clopidogrel effect. Also, mRNA concentrations in platelets are low, and any mRNA correlation that might have existed in the megakaryocyte may have been lost in circulating platelets.

Haplotype studies have suggested that the P2Y₁₂ H2 haplotype may have a higher mRNA expression than the H1 haplotype, and that this would explain the functional differences between the different genotypes [14]. In our small sample group we could not find any such correlation, and our results are in concordance with a larger P2Y₁₂ haplotype study by Hetherington et al. [16]. It should be emphasized that our sample is small and it is therefore hard to draw any reliable conclusions between polymorphisms and function.

There are several P2Y₁₂ antagonistic compounds in early and late clinical trials at the moment: prasugrel, cangrelor, AZD6140. It will be very interesting to see if these will encounter the same response variation problems as clopidogrel has. It might also be that different drugs will be better suited for different clinical phenotypes. With many different P2Y₁₂ antagonists available in the clinic, a simple clinical test evaluating patient phenotype would be useful in choosing the optimal platelet inhibitor. This study also

shows the relevance of designing drugs targeting the P2Y₁ receptor.

Conclusion

Variation of clopidogrel inhibition is partly due to the unblocked P2Y₁ receptor and partly due to a variable antagonistic effect of clopidogrel on the P2Y₁₂ receptor. A correlation between increased P2Y₁₂ protein expression on platelets and decreased response to clopidogrel was seen, indicating a novel explanation to the clopidogrel resistance phenomenon.

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