First Report of Elevated Monocyte-Platelet Aggregates in Healthy Children

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Abstract
Platelets are subcellular fragments which circulate in blood and have well established roles in thrombosis and haemostasis in adults. Upon activation, platelets undergo granule exocytosis and express P-Selectin on the cell membrane which binds a ligand on monocytes, leading to monocyte-platelet aggregation. Elevated circulating monocyte-platelet aggregates in adults are linked to atherothrombosis, but have not been investigated in children where thrombosis is less common. This study aimed to measure monocyte-platelet aggregate formation in children using whole blood flow cytometry. Monocyte-platelet aggregates as well as activation and granule exocytosis of platelets were measured in healthy adults (n = 15, median age 28 years) and healthy children (n = 28, median age 7 years). Monocyte-platelet aggregates in healthy children were elevated compared to healthy adults (37.8±4.4% vs. 15.5±1.9% respectively, p<0.01). However, this was not accompanied by any difference in platelet activation (PAC-1 binding 6.8±1.5% vs. 6.3±2.0% respectively, p = ns) or granule exocytosis (P-selectin expression 4.4±0.5% vs. 3.1±0.5% respectively, p = ns). Despite comparable numbers of platelets bound per monocyte (GPIb MFI 117.3±13.7 vs 130.9±28.6 respectively, p = ns), surface P-selectin expression per platelet-bound monocyte was lower in children compared to adults. We therefore provide the first data of elevated monocyte-platelet aggregates in healthy children.

Introduction
Platelets are small cell fragments of large importance in medicine. Their role in haemostasis and the late stage thrombotic complications of cardiovascular disease are well characterised [1]. However, platelets also play an important and central role in inflammation [2], with recently discovered antigen presenting capacity [3] and ability to influence the phenotype of other blood and vascular cells through cell-cell signalling [4,5]. Monocyte-platelet aggregate (MPA) formation is a sensitive marker of platelet activation in adults and is an early marker of acute atherothrombotic events [6,7]. The mechanism by which MPAs form in adults has been well characterised, where activated platelets which have undergone exocytosis express α-granule P-selectin (CD62P) on the cell surface. The platelet P-selectin then interacts with P-selectin glycoprotein ligand-1 (PSGL-1), which is constitutively expressed on the surface of circulating monocytes [8]. Following this initial tethering, the β2 integrin Mac-1 (CD11b/18), and to a lesser extent LFA-1 on the monocyte stabilise the adhesion [9]. However, these interactions do not develop if PSGL-1 is blocked, or CD62P is not expressed on the platelet [10,11].

In addition to acting as a marker of platelet activation, the heterotypic cellular association between monocytes and platelets triggers an adhesive and pro-inflammatory monocyte phenotype [5,12]. Although incompletely characterised, this is thought to arise both through outside-in signalling of the adhesion receptors interacting with the platelet surface, and through in situ delivery of pro-inflammatory platelet granule contents to the monocyte [5,12–19]. Monocyte-platelet aggregates promote a pro-thrombotic milieu at the site of platelet activation, and are suggested to contribute to atherogenesis and progression of coronary artery disease (CAD) [5,20,21]. A potential role for sub-clinical platelet activation as a contributor to cardiovascular risk is emerging [12,14].

Important age-related quantitative changes have been reported in haemostatic factors [22,23] platelet count [24,25] and reactivity [26,27] among children, including decreasing soluble P-selectin in serum with age [28]. These age-related changes in haemostasis have important implications in the clinical management of children [23]. Measurement of monocyte-platelet aggregates is used as a surrogate marker of early platelet activation in paediatric research for many thromboinflammatory diseases, including acute myocardial infarction, cystic fibrosis and thrombocytopenia [14,29,30]. However, the formation of MPAs in children and their circulating levels has not been systematically investigated in healthy children. We therefore sought to measure formation of MPAs in healthy children, and compare them with adults.

Results
Circulating MPAs were increased in children compared to adults as shown in Figure 1A (37.8±4.4% vs. 15.5±1.9%...
respectively, \( p < 0.01 \). However, when circulating platelets were examined for surface markers of activation with no addition of agonists in \( ex \) \( vivo \), there was no corresponding increase in activation of the GPIIb/IIIa receptor as measured by PAC-1 binding (6.8 ± 1.5% vs. 6.3 ± 2.0% respectively, \( p = \text{ns} \)) (Figure 1B); or platelet granule exocytosis, as measured by P-selectin expression (4.4 ± 0.5% vs. 3.1 ± 0.3% respectively, \( p = \text{ns} \)) (Figure 1C).

In order to determine whether circulating monocyte-platelet aggregates in children formed as a result of the P-Selectin/PSGL-1 adhesion mechanism known to be responsible for this process in adults, the relative MFI of P-selectin on platelet-bound and – unbound monocyte events was examined. P-selectin expression of platelets bound to circulating monocytes in blood from children have significantly lower P-selectin expression compared to platelets bound to monocytes in circulating blood from adults (17.8 ± 3.5 vs. 59.5 ± 13.0 MFI respectively, \( p < 0.05 \)) (Figure 2). However, \( \alpha \) \( vivo \) chemical stimulation of whole blood with 50 \( \mu \)M TRAP-6, a specific agonist of the platelet protease activated receptor, resulted in equal expression of P-selectin on monocyte-platelet aggregates between adults and children.

In order to compare the relative number of platelets bound per monocyte without \( \alpha \) \( vivo \) chemical stimulation, the expression of platelet-specific GPIX (CD42a) was compared. Mean fluorescent intensity of CD42a from children (117.3 ± 13.7 MFI) was not different to adults (130.9 ± 28.6 MFI, \( p = \text{ns} \)), indicating similar number of platelets bound per monocyte.

Discussion

We provide the first evidence of elevated MPAs without \( ex \) \( vivo \) chemical stimulation in children. Platelets bound to monocytes in children did not show the elevation of P-selectin expression associated with MPA formation in adults. These results suggest that circulating MPAs in children are not a result of increased platelet activation and granule exocytosis, and that a P-selectin independent mechanism of MPA formation may be more important in MPA formation in children. These findings are very different to what has been described in adults. This is the first report of such age-specific differences in monocytes platelet interaction and it is consistent with our previous findings of age-dependent changes in other haemostatic and platelet parameters.

The lower P-selectin expression on circulating MPAs in children was not associated with a corresponding decrease in CD42a expression, indicating similar numbers of platelets bound per monocyte in MPAs from children and adults. Further testing by stimulation with a supra-maximal concentration of the chemical platelet agonist TRAP-6 demonstrated that platelets bound to monocytes from both children and adult blood could be induced to a similar maximal expression of P-selectin. Therefore, while the majority of circulating MPAs form in the absence of platelet activation in children, there is the capacity for both platelet activation-dependent and -independent formation of monocyte-platelet aggregates. This also confirms that the binding of anti-CD62P monoclonal antibodies to platelets that are already bound through P-Selectin/PSGL-1 mechanisms is not impaired in our assay.

Age related differences in relative abundance of monocytes and platelets \([24,25,31]\) might potentially contribute to the observed differences in the level of MPAs in blood from children. In order to test this hypothesis, we collected and analysed full blood counts and MPAs on a subset of samples from 5 adults and 6 children (data not shown). No correlation (Spearman’s rank test) of MPAs with either absolute platelet count, monocyte count or the ratio of platelet to monocyte count was observed.
Pre-analytical variables, especially with relation to blood collection, are known to affect parameters of platelet function, including MPAs. We performed preliminary experiments using different sample collection methods using direct venipuncture compared with blood drawn through indwelling catheter. This confirmed the need for blood collection procedures to be consistent, and so blood from healthy adult volunteers was drawn through an indwelling catheter to match routine paediatrics collection in this setting. Bloods were collected using the same peripheral cannula type and size into the same type of anticoagulant tubes. With this stringent standardization, difference in MPAs formation we reported here is unlikely attributed by the pre-analytical variables.

While not routinely used as a diagnostic assay in clinical medicine, whole blood flow cytometric measurement of monocyte-platelet aggregates is considered an early and sensitive marker of in vivo platelet activation and atherothrombosis in adults [6,9], and proposed as a potential diagnostic tool in the assessment of acute coronary syndromes [9]. Monocyte-platelet aggregates have also been used as a sensitive marker of platelet activation in the context of thromboinflammatory disease state of relevance to paediatric populations, such as cystic fibrosis [29]. However, in the current study circulating monocyte-platelet aggregates observed in healthy populations, such as cystic fibrosis [29]. However, in the current study circulating monocyte-platelet aggregates observed in healthy children were not associated with platelet activation, and the adherent platelets had not undergone exocytosis. Therefore care must be taken in the use of MPAs as a marker of platelet activation in children.

While MPAs in adults are thought to contribute to atherogenesis through promotion of an inflammatory phenotype and by localizing monocytes to the endothelium, circulating MPAs in children are unlikely to play a role in this process in the absence of P-selectin and platelet activation. Further research should be directed at understanding the different mechanisms and physiological consequences of monocytes-platelets interaction in children. The potential role for this physiological adhesion between unactivated platelets and monocytes in the protection of children from a number of clinically significant platelet mediated diseases such as heparin induced thrombocytopenia [32] deserves further exploration.

It is well established in adult blood that following initial tethering to activated platelets by P-selectin/PSGL-1 interaction, firm adhesion via bridging fibrinogen bound to the activated GPIIb-IIIa integrin [33] and via direct interaction with GPIbα [34]. Our finding of elevated monocyte-platelet tethering in children without elevated P-Selectin expression therefore warrants further investigation into the role and relative importance of other adhesion molecules, such as Mac-1, LFA-1 and ICAM-2, which may have a role in the in the observed formation of circulating monocyte-platelet aggregates in the absence of P-selectin.

Following granule exocytosis and exposure of P-selectin on activated platelets, P-selectin is rapidly cleaved while activated platelets continue to circulate and function [35]. It is therefore possible that the elevated circulating MPAs in children represent platelets that are initially bound to monocytes in vivo through P-selectin expression and that subsequently, but prior to blood collection, P-selectin is cleaved off with the platelet remaining tethered to the monocyte through other adhesion molecules.

In summary, this is the first observation of increased monocyte-platelet aggregation occurs in healthy children compared to healthy adults. This interaction occurs without the increased expression of P-selectin seen with MPA formation in adults, suggesting a different physiological monocyte-platelet interaction in this young population. This interaction could contribute to the thromboprotective mechanism observed in children. Further investigation of the phenotypic consequences of platelet adhesion to monocytes in the absence of P-selectin is warranted.

Materials and Methods

Participants and Blood Collection

Institutional human research ethics approval (RMIT University Human Research Ethics Committee reference 55/11 and Royal Children’s Hospital Melbourne Human Research Ethics Committee reference 20031) was obtained for the research and the procedure of gaining consent. For adults, written informed consent was obtained, while for children both verbal assent from the child and written consent from a parent were obtained. Blood from healthy volunteer adults (n = 15, age 20–43 years old, median age 28 years old) and healthy children of either gender (n = 28, age 1–14 years old, median age 7 years old) scheduled for minor day surgery (e.g. trigger thumb release) was collected. Subjects did not receive any antiplatelet medication and had no family history of haematological disorders. Whole blood was collected from peripheral cannula into S-Monovette tubes (Sarstedt, Australia), containing 1 volume of citrate per 9 volumes of blood according to protocols previously described [22]. In order to minimise pre-analytical variables, blood collection procedures and blood handling for adults and children were identical.

Assays

Flow cytometer setup and calibration. All analyses were performed on a FACS Calibur flow cytometer (Becton Dickinson, Australia). Fluorophores were excited by a 30 mW 488 nm argon sapphire laser. Fluorescein isothiocyanate (FITC) emission was collected in the wavelength range 515–545 nm (530/30 band pass), phycoerythrin (PE) emission was collected in the wavelength range 564–606 nm (585/42 band pass) and tandem phycoerythrin-cy5 (PE-Cy5) emission was collected in the wavelength >670 nm (670 long pass). Voltages to photomultiplier tubes (PMTs) collecting each wavelength range were established to ensure emission peaks for unstained and positively stained cells fell within the linear range of the instrument (i.e. between first and third decades of MFI). Voltages were individually calibrated for platelet and monocyte-platelet parameters against hard-dyed broad spectrum 3.0–3.4 μm 8 peak calibration particles of known concentration of molecules of equivalent soluble fluorochrome (MESF) (Spherotech, USA) as previously described [36]. Briefly, for each day in which samples were analysed, 8 peak rainbow beads were first analysed and PMT voltages adjusted such that each peak of fluorochrome labelled beads of known MESF appear in the appropriate channel for each bandwidth range. Compensation for spectral overlap between all fluorophores was determined using platelets and monocytes with high and low expression individually single stained with antibody. A mixed population of high and low expressing cells was created by partial activation of platelets using 2 μM TRAP-6. Effective compensation was verified by antibody titration on high expressing cells (Figure 3).

Measurement of platelet activation and exocytosis. Platelet activation was measured by whole blood flow cytometric determination of PAC-1 binding, a monoclonal antibody which recognizes integrin αIIbβ3 in its high affinity activated conformation, indicating activation dependent inside-out signalling has occurred. Granule exocytosis was measured by expression of P-selectin, an α-granule component not normally expressed on the surface of resting platelets but expressed following fusion of granule and cell membranes. Briefly, within 30 min of collection whole blood was diluted 1:5 with HEPES saline (10 mM HEPES, 0.15 M NaCl, pH 7.3–7.4) and incubated with an
antibody coating containing 1.0 µg·mL⁻¹ PC5 conjugated anti-CD42b monoclonal antibody (clone HIP1, Becton Dickinson Pharmingen, USA), 6.25 µg·mL⁻¹ FITC conjugated PAC-1 (Becton Dickinson, Australia) with or without blockade by 5 µg·mL⁻¹ epilipitide (Schering-Plough, Australia), and PE conjugated anti-CD62P monoclonal antibody (clone AK4, Becton Dickinson, Australia) or 1 µg·mL⁻¹ PE conjugated mouse IgG1k isotopic control (clone MOPC-21, Becton Dickinson, Australia). After 15 min of incubation the reaction was stopped with addition of 800 µL of 1% formaldehyde (Sigma Aldrich, USA) in HEPES-Saline. Platelets activated with 50 µM of thrombin receptor activating peptide, TRAP-6 (Sigma Aldrich, Australia) were analysed with each sample as a positive control. Platelets were identified by characteristic forward and side laser scatter and expression of platelet-specific GPIIX (CD42b). The percentage of activated platelets was quantified by addition of CD62P-PE (clone RMO52, Beckman Coulter Immunotech, Australia) or 0.5 µg·mL⁻¹ FITC conjugated mouse IgG1k isotopic control. In a subset of samples, P-selectin expression of monocyte-monocyte events. Gates were determined by appropriate isotypic control. In a subset of samples, P-selectin expression of monocyte-bound platelets was quantified by addition of CD62P-PE (clone AK-4) to the antibody cocktail, with sequential gating and fluorochrome compensation as previously described [5].

Statistical Analysis

Data shown are mean ± SEM. Results for children and adults were compared using the non-parametric Wilcoxon rank sum test.

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Author Contributions

Conceived and designed the experiments: ML VI CY PM. Performed the experiments: CY CA. Analyzed the data: CY VI CM. Contributed reagents/materials/analysis tools: ML VI PM. Wrote the paper: ML VI CY CA.

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