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N.B.: When citing this work, cite the original article.

Original Publication:

Ann-Charlotte B. Svensson Holm, Torbjörn Bengtsson, Magnus Grenegård and Eva G. Lindström, Platelet membranes induce airway smooth muscle cell proliferation, 2011, Platelets, (22), 1, 45-55.

<http://dx.doi.org/10.3109/09537104.2010.515696>

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Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-61615>

Platelet membranes induce airway smooth muscle cell proliferation

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Running title: Platelet membranes induce ASMC proliferation

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Abstract

The role of platelets in airway disease is poorly understood although they have been suggested to influence on proliferation of airway smooth muscle cells (ASMC). Platelets have been found localised in the airways in autopsy material from asthmatic patients and have been implicated in airway remodeling. The aim of the present study was to investigate the effects of various platelet fractions on proliferation of ASMC obtained from guinea pigs (GP-ASMC) and humans (H-ASMC). Proliferation of ASMC was measured by the MTS-assay and the results were confirmed by measurements of the DNA content. A key observation was that the platelet membrane preparations induced a significant increase in the proliferation of both GP-ASMC ($129.9 \pm 3.0 \%$) and H-ASMC (144.8 ± 12.2). However, neither supernatants obtained from lysed nor filtrate from thrombin stimulated platelets did induce ASMC proliferation to the same extent as the membrane preparation. We have previously shown the platelet-induced proliferation is dependent on the 5-lipoxygenase (5-LOX) and reactive oxygen species (ROS) pathways. In the present work we established that platelet membrane-induced ASMC proliferation was reduced in the presence of the NADPH oxidase inhibitor DPI and the 5-LOX inhibitor AA-861. In conclusion, our results showed that platelet membranes significantly induced ASMC proliferation, demonstrating that the mitogenic effect of platelets and platelet membranes on ASMC is mainly due to membrane-associated factors. The effects of platelet membranes were evident on both GP-ASMC and H-ASMC and involved 5-LOX and ROS. These new findings are of importance in understanding the mechanisms contributing to airway remodeling and may contribute to the development of new pharmacological tools in the treatment of inflammatory airway diseases.

Keywords: platelets; platelet membranes; airway smooth muscle cell; 5-lipoxygenase; reactive oxygen species; airway remodeling

Introduction

Airway remodeling is a contributory cause to structural changes observed in inflammatory airway diseases [1]. These structural changes include both increased smooth muscle cell mass and proliferation [1]. Furthermore, different animal models are used within respiratory research to study airway remodelling, e.g. guinea pig [2, 3], murine [4, 5] and human models [6]. Platelets possess both inflammatory and proliferative qualities [7-10] and are suggested to play a role in airway inflammation [11, 12]. Platelets release different growth factors upon activation e.g. the mitogen platelet-derived growth factor (PDGF) and we have recently found that functional platelets have the capacity to induce airway smooth muscle smooth muscle cell (ASMC) proliferation [13]. We also found that ASMC proliferation induced by functional platelets, as well as platelet-mediated proliferation of fibroblasts, is dependent on phospholipase A₂ (PLA₂), 5-lipoxygenase (5-LOX) and reactive oxygen species (ROS) production [13-15]. Activation of PLA₂ leads to the formation of AA, which is further metabolized by e.g. 5-LOX to leukotrienes. Leukotrienes play a central role in airway inflammation and have also been shown to affect cell proliferation [16]. The mitogenic effect of leukotrienes seems to be cell type specific, e.g. it was recently shown that leukotrienes are involved in proliferation of glioma cell lines [17] while they had no effect on endothelial cell proliferation [18].

ROS are generated by several sources, e.g. the plasma membrane NADPH oxidase and the mitochondria, and are believed to be important mediators of cell proliferation [15, 19, 20] as well as of cell differentiation [20] and cell adhesion [21, 22]. However, ROS might also be produced by xanthine oxidase, mitochondria and metabolism of AA through cyclooxygenase (COX) and LOX [23]. In addition, AA have also been shown to have a direct effect on NADPH-oxidase resulting in ROS formation [24, 25].

When platelets become activated, e.g. by thrombin or in response to high stress e.g. low temperature, they release two different types of microparticles, either budded from the plasma membrane or released from the α -granule [26]. Platelet microparticles (PMP) vary in size and the largest microparticles are the ones budded from the plasma membrane and they can be between 0.1 and 1 μm , i.e. almost the same size as resting platelets. The smaller microparticles, the exosomes, are stored within α -granules and are between 40-100 nm in size [26]. Furthermore, PMP express some of the glycoproteins that intact platelets do, e.g. GpIb (CD42b), CD31, CD62P and GpIIb-IIIa [27]. PMP have been shown to be very important for the coagulation cascade [28], and also for adhesion of platelets to the subendothelium matrix [29]. Interestingly, PMP have also been shown to induce proliferation of e.g. hematopoietic cells [30] and vascular smooth muscle cells [31, 32]. In addition, PMP contain bioactive lipids such as arachidonic acid (AA) that PMP deliver to different cells, e.g. platelets and endothelial cells resulting, in a metabolism of AA to other more active lipid metabolites, e.g. TXA_2 [33]. It has also been shown that PMP are able to metabolize AA delivered from endothelial cells to TXB_2 , the stable metabolite of TXA_2 , indicating that PMP also possess enzymatic activity [34].

Platelets have been shown to induce proliferation in different cell types, e.g. endothelial cells [35], hepatocyte [36] and fibroblasts [15]. However, the findings that cell proliferation induced by both platelet membranes and platelet microparticles is PDGF-independent also suggest a role for membrane-associated factors [31, 37]. Furthermore, unstimulated platelets stored either in room temperature or $-80\text{ }^\circ\text{C}$ have been shown to possess greater wound healing properties on diabetic wounds than plasma [38]. In addition, both freeze dried

platelets [39] and freeze dried platelet-rich plasma [40] have wound healing properties suggesting that released growth factors do not solely explain platelet-induced proliferation.

In this study, we wanted to further characterize the role of platelets in airway smooth muscle cell (ASMC) proliferation by clarifying the effects of platelet membranes and other fractions of platelets on ASMC proliferation and also evaluate the involvement of 5-LOX and ROS. Furthermore, our study aimed to compare platelet and platelet membrane-induced proliferation of ASMC obtained from guinea pigs (GP-ASMC) with humans (H-ASMC). Increased knowledge of the mechanism of platelet interaction with cells located in the airways may lead to the development of new pharmacological strategies in the treatment of patients with inflammatory disorders.

Methods

Chemicals

The chemicals used were as follows: Dulbecco's Modified Eagle Medium, non-essential amino acids, sodium pyruvate, penicillin and streptomycin (PEST), foetal bovine serum (FBS) and Trypsin-EDTA (Gibco, Paisley, Scotland); CellTiter 96[®] Aqueous One solution cell proliferation assay (Promega, Madison, WI, USA); AA-861 (Biomolecular Research Laboratories Inc., Plymouth Meeting, PA, USA); arachidonyl trifluoromethyl ketone (ATK) (Cayman Chemical, Ann Arbor, MI, USA); bodipy phalloidin and Alexa Fluor[®]594-phalloidin (Molecular Probes, Eugene, OR, USA); lysophosphatidylcholine (LPC), thrombin, platelet-derived growth factor (PDGF), anti-PDGF (Sigma Chemical Co., St. Louis, MO, USA); diphenyleneiodonium chloride (DPI) (Merck, Darmstadt, Germany); paraformaldehyde (PFA) (Labkemi, Stockholm, Sweden); phycoerythrin conjugated mouse antibodies against GpIb and GpIIb (Dako, Glostrup, Denmark); imatinib (kindly provided by Novartis Pharma AG, Basel, Switzerland).

Buffers and media

The following buffers and media were used in the experiments: phosphate-buffered saline pH 7.3 (PBS; 137 mM NaCl, 27 mM KCl, 6.74 mM Na₂HPO₄·2H₂O, 1.47 mM KH₂PO₄ and 0.5% BSA); PBS pH 7.3 (1.2 % BSA); Krebs-Ringer phosphate buffer pH 7.3 (KRG; 120 mM NaCl, 4.9 mM KCl, 1.2 mM MgSO₄, 1.7 mM KH₂PO₄, 8.3 mM Na₂HPO₄, 1 mM CaCl₂ and 10 mM glucose); KRG without CaCl₂; acid citrate/dextrose solution (ACD; 85 mM C₆H₅Na₃O₇, 71 mM H₃C₆H₅O₇ and 111 mM D-glucose);); lysis buffer for membrane preparation (7.5 mM NaCl, 2.5 mM TRis-HCl, 1 mM EDTA, 5 mM EGTA, 100 µM Phenylmethylsulphonyl fluoride); starvation medium (DMEM, 1mM sodium pyruvate, 1%

non-essential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin); complete medium (starvation medium with 10 % foetal bovine serum).

Cell culture

Airway smooth muscle cells (ASMC) obtained from guinea pigs (GP-ASMC) was isolated using explant technique, approved in advance by the ethical review committee on animal experiments (Linköping, Sweden, Dnr 41-03). Identification of ASMC where done as previously described [13]. ASMC obtained from humans (H-ASMC) where bought from Promocell (Heidelberg, Germany). ASMC were cultured in complete medium in a humidified atmosphere at 37°C and 5% CO₂. ASMC displayed all the reported characteristics of viable smooth muscle cells in culture when examined by light microscopy [41]. ASMC in passages 5 to 20 were used in the following experiments.

Preparation of platelets, platelet membrane, cell lysate and supernatant

Fresh blood from healthy donors was obtained from the blood bank at Linköping University Hospital, Linköping, Sweden and isolated as previously described [13]. In short, five parts of the blood were mixed with one part of ACD solution and centrifuged for 20 minutes at 220 x g. The platelet-rich plasma obtained in the upper layer were removed and centrifuged for 20 minutes at 480 x g. The platelet pellet was gently washed and resuspended in KRG without calcium, and the platelets were counted in a Bürkner chamber. This platelet suspension was used to prepare lysate, supernatant from platelet lysate and platelet membranes.

Lysate of platelets where prepared using a Branson sonifier cell disrupter B15 (Branson sonic Power company, Danbury, CT, USA) for 3 x 15 seconds. The disrupted platelets where then put in the -70 °C freezer followed by two cycles of thawing, vortexing and freezing. The cell

lysate where stored in the -70° freezer until use. The supernatant was prepared by centrifugation of the cell lysate at $200\,000 \times g$ for 30 minutes and the supernatant was thereafter removed and stored in the -70° freezer until use.

Platelet membranes was performed according Regan and Matsui 1990 [42]. Platelet suspension was centrifuged for 30 minutes at $2,800 \times g$ at $+4^{\circ}\text{C}$. The pellet was resuspended in lysis buffer and homogenized, and then centrifuged for 45 minutes at $29,500 \times g$ at $+4^{\circ}\text{C}$. The lysis-homogenization procedure was repeated three times, and the resulting platelet membrane fraction was resuspended in calcium-free KRG to the original volume of the platelet suspension. Consequently, the amount of platelet membranes used in the experiments corresponded to the original platelet concentration.

The effect of thrombin-activated platelets on ASMC proliferation was studied by exposing 0.5 ml of platelets (3×10^8 platelets/ml) to 0.5 U/ml thrombin. Platelet activation was monitored by analysing platelet aggregation, a method that measures changes in light transmission using a Chronolog Dual Channel lumi-aggregometer (Model 560, Chrono-Log, Haverston, PA). Some samples were filtrated through a sterile filter with a pore size of $0.2 \mu\text{m}$ that allows different soluble agents such as growth factors to pass according to the manufacturer (Sarstedt, Sweden). ASMC were thereafter incubated with unstimulated platelets, thrombin-activated platelets or filtrate from thrombin-activated platelets for 24 hours followed by proliferation measurements as described in *ASMC proliferation*.

ASMC proliferation

ASMC proliferation was measured as previously described [13]. Briefly, ASMC (3000/well) were seeded in 96-well plates, after 24 hours the medium was changed to starvation medium

and incubated for 24 hours. Thereafter, cells were incubated for further 24 hours in medium supplemented with 0.1% FBS, in the absence (controls) or presence of platelets (ASMC/platelet ratio of 1/1,000), platelet membranes, cell lysate or supernatant (corresponding to ASMC/platelets ratio of 1/1,000). ASMC were also incubated with platelet membranes with or without (AA-861, ATK, DPI, imatinib and anti-PDGF).

ASMC proliferation was analysed using the CellTiter96[®] Aqueous One Solution Cell Proliferation Assay (MTS-assay). Briefly, after incubation with the stimulus, new medium supplemented with 0.1% FBS was added together with the CellTiter 96[®] Aqueous One Solution Reagent, and the amounts of viable cells were measured spectrophotometrically at 490 nm using a microplate reader (Spectra MAX 340, Molecular Devices, Sunnyvale, CA) [43]. The MTS-assay was verified by measuring DNA using a NanoDrop ND-100 UV-visible light spectrophotometer (Saveen Werner AB, Malm; Sweden). All drug and solvents used were tested for interference with the assay.

Microscopic examination of the platelet/platelet membrane-ASMC interaction

The interaction between ASMC and platelets or platelet membranes was studied morphologically by fluorescent staining of F-actin using Bodipy phalloidin for GP-ASMC and Alexa Fluor[®] 594-phalloidin for H-ASMC followed by fluorescence microscopy. Briefly, 10,000 ASMC/well in an 8-well chamber slide (Nunc, Rochester, NY, USA) were incubated in DMEM supplemented with 0.1% FBS, in the absence (controls) or presence of platelets (ASMC/platelets ratio of 1/1,000) or platelet membranes (corresponding to ASMC/platelets ratio of 1/1,000). After 24 hours, the samples were permeabilized and stained in a mixture of lysophosphatidylcholine (100 µg/ml) and Bodipy phalloidin (0.6 µg/ml) or Alexa Fluor[®]594-phalloidin (8 µg/ml) in PBS pH 7.3. Thereafter the chamber slides were mounted with

coverslips using fluorescent mounting medium and put in the refrigerator prior fluorescence microscopy analysis (Carl Zeiss, Oberkochen, Germany).

Flow cytometry

The platelet membranes were characterized upon size, density and expression of the platelet specific structures GpIb (CD42b) and GpIIb (CD41) using flow cytometry (Coulter Epics XL-MCL, Beckman Coulter, Miami, FL, USA). 10 μ l of platelet membranes (concentration corresponding to 2.1×10^9 platelets/ml) were mixed with 10 μ l of either the phycoerythrin-conjugated mouse GpIb (diluted 1:5) or the phycoerythrin-conjugated mouse GpIIb antibody (diluted 1:5) and HEPES buffer to a final volume of 100 μ l followed by incubation for 10 min in room temperature. The samples were thereafter diluted 1:10 in HEPES buffer before analysis on the flow cytometer. The platelet membrane population was identified using forward scatter, side scatter and fluorescence (GpIb or GpIIb-phycoerythrin).

Statistical analysis

The results are normalized in relation to unstimulated ASMC and are expressed as mean values (% of control) $\pm$ standard error of the mean (S.E.M). Student t-test was used to evaluate differences where the number of observations differed substantially between the groups to be compared. One-way ANOVA followed by Dunnet's multiple comparison test was used in the remaining analyses. The statistical analyses performed are indicated in the figure legends. A p-value < 0.05 was considered to be significant, and significance is denoted * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). Data were analysed using GraphPad PrismTM (GraphPad Software, San Diego, CA).

Results

Effects of various platelet preparations on ASMC proliferation

We previously found that intact platelets have the capacity to induce proliferation of airway smooth muscle cells obtained from guinea pigs [13]. In the present study, we used the MTS-assay to investigate the effect of intact platelets, platelet membranes, platelet cell lysate and supernatant from platelet lysate on guinea pig airway smooth muscle cells (GP-ASMC) proliferation after 24 hours of coincubation at an ASMC/platelet ratio of 1/1000 or ASMC/platelet fraction corresponding to 1/1000. As shown in Figure 1, both platelets membranes and platelet cell lysate induce a significant increase in GP-ASMC proliferation. The increase in proliferation was equivalent with that provoked by viable platelets (Figure 1). Platelet-derived supernatant also caused an increase in proliferation although not as pronounced as membranes and platelet cell lysate (Figure 1). Measurement of DNA content confirmed that GP-ASMC stimulated with platelets membranes at a ratio corresponding to ASMC/platelet ratio of 1/1000 increased the DNA content to $158.6 \pm 9 \%$ compared to control ($n = 3$). We also clarified if platelet membranes and intact platelets at an affected proliferation of human airway smooth muscle cells (H-ASMC) in the same way as GP-ASMC. The results showed that platelet membranes significantly stimulated H-ASMC proliferation but the effect was less pronounced compared to viable platelets (Figure 1, inset).

Platelets stimulated with thrombin (0.5 U/ml) induced both GP-ASMC and H-ASMC proliferation to the same extent as unstimulated platelets. Filtrate from thrombin stimulated platelets induced a small increase in both GP-ASMC and H-ASMC proliferation (Figure 2 and Figure 2 inset). In the absence of platelets, thrombin induced a small but non significant increase in both H-ASMC and GP-ASMC proliferation (H-ASMC stimulated with 0.5 U/ml thrombin: $117 \pm 12\%$ compared to unstimulated H-ASMC, $n = 4$; GP-ASMC stimulated with

0.5 U/ml thrombin: $111 \pm 9\%$ compared to unstimulated GP-ASMC, $n=5$). Morphological analyses based on staining of F-actin with either bodipy phalloidin or Alexa[®]594-phalloidin was done to investigate the interaction between ASMC and platelets or platelet membranes and showed that platelets bind to both GP-ASMC and H-ASMC in accordance with our previous study [13]. Importantly no sign of intact platelets was when ASMC were coincubated with platelet membranes, which confirms the absence of intact platelets in these samples (data not shown).

Figure 1. Effects of platelets and different fractions of platelets on ASMC proliferation. The MTS assay was used to analyze changes in airway smooth muscle cell proliferation after incubating the cells for 24 hours with unstimulated platelets (1/1000 ASMC/platelet ratio) or fractions of platelets (corresponding to ASMC/platelet ratio of 1/1,000). ASMC obtained from guinea pigs (GP-ASMC) where stimulated with either intact platelets, platelet membranes, cell lysate or supernatant obtained from platelet cell lysate. ASMC obtained from humans (H-ASMC) where stimulated with intact platelets or platelet membranes (inset). Both intact platelets and fractions of platelets increased ASMC proliferation. Data are expressed as means $\pm$ SEM from 4-16 separate cell passages and blood donors (GP-ASMC: platelets $n=10$, platelet membranes $n=14$, cell lysate $n=10$, supernatant $n=4$; H-ASMC: platelets $n=10$, platelet membranes $n=16$). Student *t*-test was used for statistical analysis where stimulated ASMC were compared to unstimulated ASMC.

Figure 2. Effects of thrombin-stimulated platelets on ASMC proliferation.

The MTS-assay was used to analyze changes in ASMC growth after incubating the cells for 24 hours with thrombin-stimulated platelets or filtrate from thrombin-stimulated platelets at an ASMC/platelet ratio of 1/1,000 for 24 hours. Both unstimulated platelets and thrombin-stimulated induced proliferation, whereas filtrate from thrombin-stimulated platelets affected ASMC proliferation poorly. Data is expressed as means $\pm$ SEM from 3-4 separate cell passages. One-way ANOVA followed by Dunnet's multiple comparison tests were used for statistical analysis where stimulated ASMC were compared to unstimulated ASMC.

Flow cytometry was used to characterize the platelet membrane population with regard to size, density and expression of the platelet specific structures GpIb (CD42b) and GpIIb (CD41). We found that the platelet membrane preparation was in the same size or smaller than intact platelets (Figure 3A). The platelet membrane population was also positive for both GpIb and GpIIb (Figure 3 B and C).

Figure 3. Characterization of the platelet membrane preparation. The platelet membranes were characterized by size and expression of platelet specific structures using flow cytometry. Both GPIb and GPIIb was labelled using specific phycoerythrin-conjugated mouse antibodies. Panel A show the size and density of the membrane particles, panel B show the expression of GPIb and panel C show the expression of GPIIb in the membrane preparation.

The role of platelet derived growth factor in platelet membrane-induced ASMC proliferation

The tyrosin kinase inhibitor imatinib and an antibody directed against platelet derived growth factor (PDGF) was used to analyse the involvement of PDGF in platelet membrane-induced GP-ASMC proliferation. The antibody directed against PDGF did not influence platelet-membrane-induced GP-ASMC proliferation (Figure 4A), while 0.1 μ M imatinib significantly inhibited the cell proliferation induced by platelet membranes (Figure 4B). The concentrations of anti-PDGF and imatinib did not affect basal ASMC proliferation (data not shown).

Figure 4. Effects of imatinib and anti-PDGF on ASMC proliferation induced by platelet membranes. The MTS-assay was used to analyze changes in GP-ASMC growth after incubating the cells for 24 hours with platelet membranes corresponding to an ASMC/platelet ratio of 1/1,000 for 24 hours in the presence or absence of the tyrosin kinase inhibitor imatinib or anti-PDGF. The platelet membrane-mediated increase in GP-ASMC proliferation was reduced by imatinib but not affected by anti-PDGF. Data are expressed as means $\pm$ SEM from 2-9 separate cell passages. Student *t*-test was used for statistical analysis where inhibited ASMC and platelets were compared to unstimulated ASMC.

The role of eicosanoids in platelet membrane-induced ASMC proliferation

We have previously shown that ASMC obtained from guinea pigs express 5-LOX, whereas platelets do not, and that the increase in GP-ASMC proliferation induced by intact platelets is 5-LOX dependent [13]. To elucidate the role of eicosanoids in platelet membrane-induced ASMC proliferation we used the phospholipase A₂ (PLA₂) inhibitor ATK (0.1-10 μ M) and the 5-LOX inhibitor AA-861 (0.1-10 μ M). We found that platelet membrane-induced proliferation of both GP-ASMC and H-ASMC was significantly inhibited by 10 μ M AA-861 (Figure 5A and 5A inset) and the mitogenic effects of platelet membranes on GP-ASMC and H-ASMC proliferation were also reduced by ATK (Figure 5B and 5B inset). The concentrations used of both AA-861 and ATK did not affect basal ASMC growth.

The role of reactive oxygen species in platelet membranes-induced ASMC proliferation

To investigate the role of reactive oxygen species (ROS) in platelet membrane-induced GP-ASMC and H-ASMC proliferation the NADPH-oxidase inhibitor DPI was used. We found that 0.1 μ M DPI significantly inhibited the mitogenic effect of platelet membranes on both GP-ASMC proliferation (Figure 6) and H-ASMC proliferation (Figure 6, inset) measured by using the MTS assay. These concentrations of DPI did not affect basal cell growth (data not shown).

A

B

Figure 5. Effects of eicosanoid inhibitors on ASMC proliferation induced by platelet membranes. The MTS-assay was used to analyze changes in GP-ASMC and H-ASMC growth after incubating the cells for 24 hours with platelet membranes corresponding to an ASMC/platelet ratio of 1/1,000 for 24 hours in the presence or absence of eicosanoid inhibitors. The platelet membrane-mediated increase in GP-ASMC and H-ASMC proliferation was impeded by the 5-LOX inhibitor AA-861 (A) and also reduced by the PLA₂-inhibitor ATK (B). Data are expressed as means $\pm$ SEM from 4-6 separate cell passages. One-way ANOVA followed by Dunnet's multiple comparison tests were used for statistical analysis where inhibited ASMC and platelet membranes were compared to stimulated ASMC.

Figure 6. The effect of the NADPH oxidase inhibitor DPI on platelet membrane-induced ASMC proliferation. GP-ASMC and H-ASMC were incubated with platelet membranes in the absence or presence of the NADPH-oxidase inhibitor DPI. Thereafter, ASMC proliferation was analysed using the MTS-assay, which indicated that both platelet membrane induced GP-ASMC and H-ASMC proliferation was inhibited by DPI. Data are expressed as means $\pm$ SEM from 3-9 separate cell passages. Student t-test was used for statistical analysis where inhibited ASMC and platelet membranes were compared to stimulated ASMC.

Discussion

Platelets have been implicated in proliferative activities of airway smooth muscle cells and on that account in the remodelling process observed during airway inflammation. However the relative role played by the large number of platelet derived mediators is unclear.

Different models are used to study airway remodelling, e.g. guinea pig e.g. guinea pig [2, 3], murine [4, 5] and human models [6]. We have previously found that platelets have the ability to induce proliferation of airway smooth muscle cells (ASMC) obtained from guinea pigs

(GP-ASMC) [13]. The present study focused on the possible proliferative effect of platelets with respect to soluble mediators and membrane components. Furthermore, our study aimed to clarify if platelets and platelet membranes modulate the proliferation of GP-ASMC and ASMC obtained from humans (H-ASMC). We have used several fractions of platelets and a key observation was that platelet membrane preparations induced a significant increase in the proliferation of both GP-ASMC and H-ASMC. Platelets store well characterised growth factors in their alpha granules, but interestingly we found that the platelet lysate, but not the supernatants obtained from lysed platelets, induced GP-ASMC proliferation to the same extent as the membrane preparation. In accordance, it has previously been shown that platelet membranes stimulate proliferation of coronary artery smooth muscle cells coronary artery smooth muscle cells [37], human dental pulp-derived cells [44] and bone cells [45]. However, Matsuo et al. [36] found that platelet membranes do not induce growth of hepatocytes. The reason why the platelet membrane has different effect on cell proliferation could be due to differences in the platelet membrane isolation methods. Furthermore, the mitogenic effect of platelet membranes could be cell type specific.

We also studied the ability of thrombin-activated platelets and filtrate from thrombin-activated platelets to induce ASMC proliferation. When we used intact platelets it was found that preactivation with thrombin did not further influence on ASMC proliferation. Hence, we propose that preactivation of the platelets is not a prerequisite for the observed effects. It should be noted that filtrate from thrombin-stimulated platelets, in which membrane components have been separated from soluble mediators, only induce a modest increase in ASMC proliferation. In line with our finding it was recently shown that unstimulated platelet-rich plasma (PRP) induced bone cell proliferation while the supernatant from thrombin-

activated PRP did not have any effect [46]. Filtrate and supernatant from thrombin-stimulated platelets have been shown to increase proliferation of human pulp-derived cells, bone cells and endothelial cells [44, 45, 47], respectively. However, the experimental setup in these studies differs substantial from our study. The difference in incubation time and cell systems may explain the discrepancy between our results and the other studies.

Studies have shown that ASMC from asthmatic airways react more excessively to soluble factors compared with ASMC from healthy individuals [48-50]. The role of soluble mediators released from activated platelets on ASMC proliferation *in vivo* cannot be estimated by the present *in vitro* study on non-asthmatic ASMC.

Morphological experiments using fluorescence microscopy were performed to visualise the presence of platelets and platelet membranes after coincubation with ASMC. In accordance with our previous study [13], we observed that platelets bind to the ASMC after 24 hour of coincubation. In samples with ASMC coincubated with platelet membranes we did not reveal any intact platelets. In addition, flow cytometry analyses revealed that membrane fraction expressed the platelet-specific structures GpIb and GpIIb showing that it originates from platelets and not contaminating blood cells. The results also revealed that platelet membranes are in the same size span as platelet-derived microparticles (PMP) [26].

To exclude the role of PDGF in platelet-induced GP-ASMC proliferation an antibody directed against platelet-derived growth factor (PDGF) and the tyrosine kinase inhibitor imatinib were used. We found that the antibody did not affect the proliferation while high concentration of imatinib had an inhibitory effect. Imatinib is a potent inhibitor of the PDGF tyrosin kinase receptor with an IC_{50} value of 0.1 μM [51], but the drug can also influence other kinases e.g.

ERK, Akt and MAPK [52, 53]. It is thus possible that the inhibitory effect of imatinib is unrelated to the PDGF receptor. The interpretation of the results is that PDGF does not contribute in an extensive way to the membrane induced proliferation. In accordance, other studies have shown that proliferative effect of platelet membranes and platelet derived microparticles on coronary artery smooth muscle cells are independent of [31, 37]. Consequently, our results show that the platelet membrane fraction resembles platelet-derived microparticles in size and that PDGF is not trapped within the membrane compartment.

Many studies *in vitro* have shown that arachidonic acid metabolites influence on cell proliferation although the effects seems to be cell-type specific [54-58]. Cysteinyl leukotrienes, products of the 5-LOX pathway, have been implicated in various stages of airway inflammation. In this study we found that the mitogenic effect of platelet membranes on ASMC proliferation could be inhibited by the 5-LOX inhibitor AA-861 and reduced, albeit not significantly, by the PLA₂ inhibitor ATK. These results demonstrate that platelet membranes activate 5-LOX located in ASMC and that this activation results in enhanced proliferation. The PLA₂ inhibitor ATK did not inhibit the platelet membrane-induced ASMC proliferation, indicating that PLA₂ is not the major enzyme for releasing arachidonic acid from the plasma membrane. The mechanism by which the membrane fraction brings about the 5-LOX activation is not clarified but one possible explanation is that platelet membranes serve as a lipid reservoir for biosynthesis of mitogenic lipids. PMP contain bioactive lipids such as arachidonic acid (AA) that PMP deliver to different cells, e.g. platelets and endothelial cells resulting, in a metabolization of AA to other more active lipid metabolites, e.g. TXA₂ [33]. The membrane fraction could also interact with surface structures on the ASMC and trigger intracellular events that activate 5-LOX.

Reactive oxygen species (ROS) have been described as important mediators in cell growth. We recently found that ROS is involved in platelet-induced proliferation of both fibroblasts [15] and ASMCs [13]. Huo et al. 2009 showed that ROS are involved in growth of corneal epithelial cells induced by epidermal growth factor [59]. In the present study we investigated the role of ROS in platelet membrane-induced H-ASMC proliferation using the NADPH-oxidase inhibitor DPI. We found that DPI inhibited the mitogenic effect of platelet membranes on GP-ASMC and H-ASMC growth indicating a proliferative role of ROS.

Increased proliferation of smooth muscle cells plays a central role in the remodeling process observed during airway inflammation. The role of platelets in inflammatory airway diseases is not fully investigated even though platelets have been found localised to locations of bronchial smooth muscle, underneath the epithelium and in areas of eosinophil infiltration [60]. In addition, depletion of platelets in a murine model reduced the remodeling process [5], indicating a role for platelets in inflammatory airway diseases. Our results showed that platelet membranes effectively induced airway smooth muscle cell proliferation. The effects of platelet membranes were evident on both GP-ASMC and H-ASMC and involved 5-LOX and ROS. This action of platelet membranes represent a novel mechanism that may be of importance for airway remodeling. The results may also have an impact in the development of new pharmacological strategies in the treatment of patients with inflammatory airway disorders.

Acknowledgements

The author would like to thank Sofia Ramström for all the help with the flow cytometry analyses. This work was supported by grants from the strategic areas Cardiovascular Inflammation Research Centre (CIRC) and Material in Medicine, the Heart- and Lung foundation, Östergötlands Läns Landsting and the Swedish Scientific Council.

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