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Platelets stimulate airway smooth muscle cell proliferation through mechanisms involving 5-lipoxygenase and reactive oxygen species.

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Running title:

Platelet-induced ASMC proliferation

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Abstract

Continuous recruitment and inappropriate activity of platelets in the airways may contribute to airway remodeling, a characteristic feature of inflammatory airway diseases that includes increased proliferation of the smooth muscle.

The aim of the present investigation was to examine the effect of platelets on proliferation of airway smooth muscle cells (ASMC) in culture and to determine the possible role of 5-lipoxygenase (5-LOX) and reactive oxygen species (ROS) in this context.

ASMC obtained from guinea pigs were cultured and co-incubated with washed platelets for 24 hours. Thereafter, the proliferation was measured with the MTS-assay, the results were also verified by using thymidine incorporation, DNA measurements and manual counting.

The interaction between platelets and ASMC was visualised with fluorescence microscopy.

We found that platelets bind to the ASMC and the presence of platelets caused a significant dose-dependent increase in ASMC proliferation. Co-incubation of ASMC with platelets also increased ROS-production, detected by the fluorescent probe DCFDA. Furthermore, the platelet-induced proliferation was reduced in the presence of the NADPH-oxidase inhibitors DPI and apocynin.

A possible role of 5-LOX in platelet-induced proliferation and ROS-generation was evaluated by using the 5-LOX inhibitor AA-861 and the PLA₂-inhibitor ATK. The results showed that inhibition of these enzymes significantly reduced the platelet-induced proliferation. Moreover, Western blot analysis revealed that the ASMC but not the platelets express 5-LOX.

In addition, our experiments revealed that the presence of AA-861 and ATK significantly inhibited the ROS-production generated upon coincubation of platelets and ASMC.

In conclusion, we show that platelets have a marked capacity to induce ASMC proliferation.

Furthermore, our study indicates that the interaction between platelets and ASMC leads to activation of 5-LOX in the ASMC followed by an increased ROS-production, events resulting

in enhanced ASMC proliferation. The new findings are of importance in understanding possible mechanisms contributing to airway remodeling.

Keywords: platelet-induced proliferation; airway smooth muscle; 5-lipoxygenase; reactive oxygen species; airway remodeling

Introduction

Increased proliferation has been shown to play an important role in asthma, a chronic inflammatory disease in which repeated cycles of damage and repair change the structure of the airways. These structural alterations, referred to as airway remodeling, is a process that includes increased smooth muscle mass [1]. Several cell types and inflammatory mediators have been suggested to contribute to airway remodeling. A growing amount of evidence support a role of platelets as important players in inflammatory airway diseases [2, 3]. Platelets may play a role in that context, since they possess both inflammatory and proliferative properties. Platelets generate, express and release a variety of inflammatory mediators e.g. P-selectin and RANTES [4-6]. Furthermore, platelets express chemotactic and phagocytic receptors [7, 8] and are therefore able to undergo chemotaxis [9] and phagocytosis [10]. Studies have investigated the role of platelets in asthma and indicate that platelets from asthmatics express a different behaviour for example regarding aggregation response, cytosolic calcium mobilization and arachidonic acid metabolism [10, 11]. In addition, depletion of platelets in a murine model of chronic allergic inflammation markedly reduced airway remodeling [12].

Leukotrienes are formed through the action of the enzyme 5-lipoxygenase (5-LOX) and are potent mediators of inflammation and allergic reactions [13]. A number of studies have also shown that 5-LOX metabolites influence on cell proliferation although the effects are cell-type specific [14-17].

Reactive oxygen species (ROS) have recently been identified as important mediators of cell proliferation, differentiation [18] and adhesion [19, 20]. Exposure to low concentrations of hydrogen peroxide and/or superoxide anion stimulates growth of a variety of cell types, among them smooth muscle cells, fibroblasts, prostate cancer cells, and aortic endothelial cells [18, 21]. Ligand-receptor interactions induce production of ROS from several sources,

including mitochondria, xanthine oxidase, and NADPH oxidase. NADPH/NADH oxidase activity are found in a variety of non-phagocytic cell types, such as smooth muscle cells, epithelial cells, endothelial cells, and platelets [18].

Several studies have revealed interplay between ROS and eicosanoids in platelets, fibroblasts and smooth muscle cells from different species [22-24]. For example it has been shown that inhibition of 5-LOX blocks the rac-1-dependent ROS-generation [25], furthermore a new report suggest that 5-LOX dependent ROS-generation is crucial for fibroblast adhesion [20].

The aim of the present study was to evaluate a possible proliferative effect of platelets on airway smooth muscle cells (ASMC) in culture, and to assess the role of 5-LOX and ROS generation in this context. Increased knowledge of the mechanisms behind platelet-induced ASMC proliferation is important for understanding the mechanisms underlying airway remodeling and may provide new targets for treatment of inflammatory hyperproliferative disorders like asthma.

Methods

Chemicals

The chemicals used were as follows: Alexa Fluor 488 goat anti-mouse IgG, bodipy-phalloidin and 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (Molecular Probes, Eugene, OR, USA); 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); anti- α -actin, diphenyleneiodonium chloride (DPI), apocynin, xanthine oxidase, hypoxanthine, luminol, RIPA buffer, phenylmethanesulfonyl fluoride, leupeptin, pepstatin, acetylsalicylic acid (ASA), apyrase, PGE₁ (Prostaglandin E₁) and lysophosphatidylcholine (LPC) (Sigma Chemical Co., St. Louis, MO, USA); AA-861 (Biomolecular Research Laboratories Inc., Plymouth Meeting, PA, USA); arachidonyl trifluoromethyl ketone (ATK), LTB₄ and LTC₄ (Cayman Chemical, Ann Arbor, MI, USA); CellTiter 96[®] Aqueous One solution cell proliferation assay (Promega, Madison, WI, USA); paraformaldehyde (PFA) (Labkemi, Stockholm, Sweden); [³methyl-³H]-thymidine (Amersham Bioscience, Buckinghamshire, UK); mouse anti-5-lipoxygenase monoclonal IgG₁ antibody (BD Bioscience, San Jose, CA, USA); anti-CD42b antibody and goat anti-mouse horseradish peroxidase-linked IgG (DAKO; Glostrup, Denmark); Captopril[®] (kindly provided by Bristol-Mayers Squibb, Princeton, MA, USA); Western Lightning[™] Chemiluminescence Regent Plus, Optiphase SuperMix liquid scintillation cocktail (PerkinElmer, Boston, MA, USA); horseradish peroxidase (HRP) (Roche Diagnostics, Mannheim, Germany); GEA 3175 (Pharmaceutical Co., Copenhagen, Denmark).

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 $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, 1.47 mM KH_2PO_4 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 MgSO_4 , 1.7 mM KH_2PO_4 , 8.3 mM Na_2HPO_4 , 1 mM CaCl_2 and 10 mM glucose); KRG without CaCl_2 ; acid citrate/dextrose solution (ACD; 85 mM $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$, 71 mM $\text{H}_3\text{C}_6\text{H}_5\text{O}_7$ and 111 mM D-glucose); starvation medium (DMEM, 1mM sodium pyruvate, 1% non-essential amino acids, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin); complete medium (starvation medium with 10 % foetal bovine serum).

Cell culture

To exclude intra-human variations and host-donor reactions between platelets and ASMC we used ASMC from Dunkin-Hartley guinea pig trachea (Sahlins, Malmö, Sweden). The cells were obtained by explant technique, in accordance with procedures approved in advance by the ethical review committee on animal experiments (Linköping, Sweden, Dnr 41-03). ASMC identity was confirmed by using a primary smooth muscle-specific mouse anti- α actin antibody and a FITC-conjugated secondary antibody, visualized in a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). The culture was found to consist mainly of ASMC, more than 80% of the cells stained positively for α -actin and displayed all the reported characteristics of viable smooth muscle cells in culture when examined by light microscopy [26].

To achieve an optimal number of ASMC in the proliferation experiments 1,000-6,000 cells/well were seeded in 96-well microtiter plates, and they were subsequently incubated for 24 hours in a humidified atmosphere at 37°C and 5% CO_2 and analysed for cell growth with the MTS assay. Thereafter, we choose to use 3,000 cells per well, because that number

enabled registration of both increases and decreases in proliferation (absorbance value: 0.57 ± 0.03 , mean values $\pm$ S.E.M., $n = 36$).

ASMC in passages 7 to 20 were used in the following experiments.

Platelet preparation

Fresh blood from healthy donors was obtained from the blood bank at Linköping University Hospital, Linköping, Sweden. Only plastic utensils were used in the preparation procedure and all work was performed at room temperature to avoid activation of the platelets. In short, five parts of the blood were mixed with one part of ACD solution and centrifuged for 20 minutes at $220 \times g$. The platelet-rich plasma obtained in the upper layer were removed and centrifuged for 20 minutes at $480 \times g$. The platelet pellet was gently washed and resuspended in KRG without calcium, and the platelets were counted in a Bürker chamber. The isolated platelets were disc shaped, unaggregated and functional. The functionality of the platelets was confirmed by measuring aggregation and mobilization of intracellular calcium [27]. Each preparation of platelets was microscopically examined and no contamination of other blood cells was observed.

ASMC proliferation

ASMC (3000/well) were seeded in medium containing 10% FBS, after 24 hours incubation growth was arrested by changing to starvation medium 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 and drugs (AA-861, ATK, Captopril[®], LTC₄, LTB₄, PDGF, Apocynin, DPI).

In a set of experiments platelets were treated with a mixture of platelet-inhibitors (ASA 100 μ M, apyrase 0.5 U/ml, PGE₁ and GEA 3175 1 μ M).

ASMC proliferation was analysed using the CellTiter96[®] Aqueous One Solution Cell Proliferation Assay (MTS-assay) or by [³methyl-³H]-thymidine incorporation. Briefly, after incubation with the stimulus, new medium 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) [28]. Proliferation was also analysed using the thymidine incorporation technique. Briefly, 2 µCi/ml [³methyl-³H]-thymidine was added to DMEM supplemented with 0.1 % FBS during the 24-hour incubation with the stimulus and the cells were subsequently washed twice with PBS pH 7.3 and then exposed to 5% ice-cold trichloroacetic acid (TCA) for 30 minutes. Next, liquid scintillation cocktail was added to the 96-wellplate and after 1 hour the amount of thymidine was determined using a scintillation counter (1450 Microbeta Trilux Wallac, Eg & G[®] Lifescience, Turku, Finland). All drugs and solvents used were tested for interference with the assays. Proliferation was also measured by manual counting in a Bürker chamber and by measuring DNA using a NanoDrop ND-1000 UV-visible light spectrophotometer (Saveen Werner AB, Malmö, Sweden).

Reactive oxygen species

The intracellular redox state was registered using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) [29]. The ASMC were seeded and stimulated as described above (*ASMC proliferation*). Cells were incubated with the stimulus for 3 hours, and thereafter was 5 µM DCFDA added followed by 45 minutes incubation. The incubation time is based on the study by Berg et al. [30]. ROS production was evaluated by measuring the fluorescence in a FL-600 Microplate Fluorescence Reader (Bio-Tek instruments Inc,

Winooski, VT). The excitation and emission filters were set at 485 ± 10 nm and 530 ± 12.5 nm, respectively.

Moreover, ROS was generated in a cell free suspension and the drugs were tested for scavenger effects. ROS generation; 0.02 U/ml xanthine oxidase and 0.03 mM hypoxanthine or 1.5 mM CuSO_4 and 0.3% H_2O_2 . ROS was measured using horseradish peroxidase (HRP)-enhanced luminescence (4 U/ml HRP, 112 μM luminol).

Microscopic examination of the platelet-ASMC interaction

The interaction between ASMC and platelets was studied morphologically by fluorescent staining of the F-actin cytoskeleton in combination with fluorescence microscopy. ASMC were seeded on coverslips at a density of 10,000 cells/well. The seeded cells were incubated in complete medium for 24 hours in a humidified atmosphere at 37°C and 5% CO_2 . Thereafter, the medium was changed to starvation medium and incubation was continued for approximately 24 hours. The ASMC were then incubated in DMEM supplemented with 0.1% FBS, in the absence (controls) or presence of platelets (ASMC/platelets ratio 1/1,000). After 3 and 24 hours, the samples were washed twice in PBS pH 7.3 and fixed for 30 minutes in 4% paraformaldehyde at room temperature. The samples were subsequently permeabilized and stained in a mixture of lysophosphatidylcholine (100 $\mu\text{g}/\text{ml}$) and bodipy-phalloidin (0.6 $\mu\text{g}/\text{ml}$) in PBS pH 7.3. The coverslips were incubated for 30 minutes in dark at room temperature and then mounted in Dako fluorescent mounting medium on glass slides and put in the refrigerator prior fluorescence microscopy analysis (Carl Zeiss, Oberkochen, Germany).

Western blot analysis of 5-lipoxygenase

700,000 ASMC were seeded in petri dishes (ø 60 mm) in complete medium and incubated for 24 hours in a humidified atmosphere at 37 °C and 5% CO₂. Growth was arrested using serum-free DMEM and after 24 hours of serum deprivation DMEM with 0.1% FBS was added and the cells were incubated for 2 hours at 37 °C and 5% CO₂. Platelets were isolated as described above (*platelet preparation*).

Cells (both ASMC and platelet) were thereafter washed three times with ice-cold PBS and lysed by incubation in RIPA buffer supplemented with 1 mM phenylmethanesulfonyl fluoride, 20 µM leupeptin, 1 µM pepstatin for 5 min on ice. Cellular extracts were thereafter heated to 90 °C for 10 min. The amount of DNA was determined by NanoDrop ND-1000 UV-visible light spectrophotometer and homogenates light spectrophotometer and ASMC homogenates containing equal amounts of DNA and homogenates of 2×10^7 platelets were thereafter separated on 7.5%-SDS-PAGE using a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad, Hercules, CA, USA). The proteins were transferred to a nitrocellulose membrane using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). The membranes were blocked for 1 hour in room temperature with 5% (w/v) dry milk and 0.1% Tween 20 in PBS pH 7.4 to minimise unspecific binding and thereafter rinsed 3 times for 5 min in 0.1% (v/v) Tween-20 in PBS. The membranes were thereafter incubated for 1 hour at room temperature with a primary mouse anti-5-lipoxygenase monoclonal IgG₁ antibody (diluted 1:200 in PBS supplemented with 1% BSA (w/v), 0.1% (v/v) Tween 20 and 0.02% (w/v) sodium acid). The membrane was subsequently washed 3 times for 5 minutes in 0.1% (v/v) Tween-20 in PBS and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (diluted 1:10,000 in 0.1% (v/v) Tween 20 in PBS) for 1 hour. After that treatment, the cells were washed for 3 times for 5 minutes in 0.1% (v/v) Tween-20

in PBS. HRP antibody-conjugated proteins were visualized by chemiluminescence using Western Lightning™ Chemiluminescence Regent Plus reagents.

Statistical analysis

Results are expressed as mean values $\pm$ 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 platelets on ASMC proliferation

The MTS assay was used to study the effects of platelets on ASMC growth after 24 hours of co-incubation at an ASMC/platelet ratio of 1/100, 1/500, 1/1,000 or 1/2,000. The results show that the presence of platelets causes a marked dose-dependent increase in the proliferation of ASMC after only 24 hours (Figure 1). Incorporation of [³methyl-³H]-thymidine (Figure 1A, inset), DNA measurements (ASMC stimulated with platelets at an ASMC/platelet ratio of 1/1,000 increased DNA content to 228 ± 42 % compared to control, n = 8) and manual counting of ASMC (presence of platelet ratio of 1/1,000 increased ASMC proliferation to about 160% compared to control) was performed and the findings confirmed the mitogenic/growth promoting effect of platelets on ASMC. An ASMC/platelet ratio of 1/1,000 was chosen in subsequent experiments. To exclude the possibility that the platelet-induced proliferation was due to an immunological reaction, we investigated the impact of platelet preparations from guinea pigs (three individuals) on ASMC proliferation. The results showed that these platelets stimulated ASMC proliferation to the same extent as human platelets (Figure 1B).

Experiments performed in the presence of a mixture of platelet-inhibitors (the COX inhibitor ASA 100 μ M, apyrase 0.5 U/ml, the cAMP increasing drug PGE₁ and the NO donor GEA 3175 1 μ M) revealed that the drug treatment did not affect platelet-induced proliferation (176.6 ± 9.9 % and 176.9 ± 12.3 % respectively, n=4).

Stimulation with platelet-derived growth factor-BB (PDGF-BB) (50 and 150 ng/ml) resulted in increased growth of ASMC with 142 ± 14.8 % (n = 3) and 154 ± 4.0 % (n = 9) compared to control, respectively.

Figure 1. Effects of platelets on ASMC proliferation. The MTS assay was used to analyse changes in ASMC growth after incubating the cells for 24 hours with different numbers of platelets. The thymidine incorporation technique (inset in A) was used to confirm the results when using human platelets (results from 9 separate cell passages and blood donors). The growth-inducing effect of both human (A) and guinea pig (B) platelets was ratio-dependent and equally potent.

Data in experiments are expressed as means $\pm$ SEM and Student t-test was used for statistical analysis. Data obtained from 2-34 separate cell passages and blood donors at different ASMC/platelet ratios (1/100, $n = 6$; 1/500, $n = 4$; 1/1,000, $n = 34$; 1/2,000, $n = 2$).

The interaction between platelets and ASMC was visualized by staining F-actin with bodipy-phalloidin. Figure 2 shows that the platelets actually preferred to bind to the ASMC and not to the surface between the cells, and notably, a large number of platelets were still adhering to ASMC after 24 hours. We also performed morphological analyses based on staining of F-actin with bodipy-phalloidin and immunoreaction with phycoerythrin conjugated anti-CD42b antibodies to distinguish platelet structures from the ASMC (not shown).

A **B**

Figure 2. Interactions between platelets and ASMC visualised by fluorescent staining of the actin cytoskeleton. ASMC were incubated on glass coverslips in the presence of platelets (ASMC/platelet ratio 1/1,000) and then stained for F-actin with bodipy-phalloidin and studied by fluorescence microscopy (Carl Zeiss, Oberkochen, Germany). Incubations with platelets were carried out for 3 (A) or 24 (B) hours.

The role of eicosanoids in platelet-induced ASMC proliferation

Several studies have indicated that eicosanoids, especially 5-LOX metabolites, act as second messengers in cell proliferation [15, 31]. In the present study the Western blot analysis showed that the ASMC expressed 5-LOX, whereas the platelets did not (Figure 3).

Figure 3. Expression of 5-LOX in ASMC and platelets. Western blot analyses showed that ASMC, but not platelets, expressed 5-LOX. Expression of 5-LOX was analysed using a primary mouse anti-5-LOX monoclonal IgG₁ antibody and a secondary horseradish peroxidase conjugated goat anti-mouse IgG antibody. Prior to Western blotting, the amount of DNA in each sample of ASMC was determined. The blot shown is representative of 8 independent experiments.

To ascertain whether the phospholipase A₂ (PLA₂) and the 5-LOX pathways are involved in platelet-mediated ASMC growth the PLA₂ inhibitor ATK and the 5-LOX inhibitor AA-861 was used. In the initial dose-response experiments, we exposed merely ASMC to AA-861 (0.1-100 μ M) and ATK (0.1-100 μ M) for 24 hours and analysed proliferation. The results revealed that the highest concentration used significantly reduced basal ASMC growth (Figure 4A, 4B, insets). Consequently, the concentrations utilized in the subsequent experiments had no significant impact on basal ASMC growth, but both ATK (10 μ M) and AA-861 (0.1 and 10 μ M) were found to significantly antagonise platelet-induced ASMC proliferation (Figure 4A, 4B).

Inasmuch as our results indicated that the 5-LOX pathway of arachidonic acid metabolism is essential for the ability of platelets to induce growth of ASMC, we examined whether some 5-

LOX metabolites could mimic that effect. Neither LTC₄ nor LTB₄ (0.001, 0.01, 0.1, 1, 10 and 100 pM, n = 2-6) had a significant impact on ASMC proliferation. The lack of effect by LTB₄ was confirmed by our finding that platelet-induced ASMC proliferation was not influenced by Captopril® (1, 5, 10 and 100 µM, n = 4), which inhibits the conversion of LTA₄ to LTB₄.

Figure 4. Effect of PLA₂ and 5-LOX-inhibitors on platelet-induced ASMC proliferation. The platelet-mediated (ASMC/platelet ratio 1/1,000) increase in ASMC proliferation was significantly inhibited by the 5-LOX inhibitor AA-861 (A) and the PLA₂-inhibitor ATK (B).

The two insets show that the highest concentration of both AA-861 and ATK significantly reduced basal ASMC proliferation, consequently, 100 µM was not used in the experiments where platelet-induced proliferation was studied.

Data are expressed as means ± SEM and Student t-test was used for statistical analysis.

(Observations from 9-14 separate cell passages and blood donors, except for experiments with 100µM, where n=3-4)

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

Reactive oxygen species (ROS) have been identified as important mediators of cell proliferation [18], and we found that production of these molecules, measured with the DCFDA, was increased in a ratio-dependent manner upon coincubation of ASMC with platelets (Figure 5). However, stimulation with the platelet-derived mitogenic factor PDGF-BB (50 and 150 ng/ml) which resulted in significant ASMC proliferation, did not induce any increased ROS production measured with the DCFDA probe (data not shown). To elucidate the involvement of NADPH-oxidase in ROS generation and platelet-induced ASMC proliferation, the oxidase inhibitors DPI and apocynin were used. Platelet-induced proliferation was significantly reduced by DPI, as shown by both the thymidine incorporation assay (Figure 6A) and the MTS assay (Figure 6A, inset), and according to both of those assays, the proliferation was also affected by apocynin (Figure 6B). The same concentrations of the two inhibitors had no significant impact on basal ASMC proliferation (data not shown). To reveal a potential relationship between arachidonic acid metabolism and ROS generation, we measured the ROS-production generated upon coincubation of platelets and ASMC in the presence of the PLA₂ and 5-LOX inhibitors. We found that both AA-861 (0.1-10 μ M; Figure 7A) and ATK (0.1-10 μ M; Figure 7B) significantly inhibited the increased ROS-production. The NADPH-oxidase-, PLA₂- and 5-LOX-inhibitors did not influence on ROS-production measured in a cell free system (xantinioxidase-hypoxantine or CuSO₄-H₂O₂ model) (data not shown).

Figure 5. Coincubation of ASMC and platelets resulted in increased generation of ROS, measured by the fluorescent probe DCFDA. The ASMC were coincubated with increasing numbers of platelets for 3 hours (from 6-7 separate cell passages and blood donors, except for ASMC/platelet ratio 1/1,000, where $n = 21$).

Data are expressed as means $\pm$ SEM and Student t -test was used for statistical analysis.

Figure 6. The effect of NADPH oxidase inhibitors on platelet-induced ASMC proliferation. ASMC were incubated with platelets in the absence or presence of the NADPH-oxidase inhibitor DPI (A) or apocynin (B). Thereafter, ASMC growth was analysed using the [³methyl-³H]-thymidine incorporation, which indicated that the platelet induced thymidine incorporation was inhibited by DPI and apocynin (n=2-4). The results were supported by data showing that platelet-mediated ASMC growth, analysed by the MTS-assay, was inhibited by apocynin and partially reduced by DPI (n=3-6; inset). One-way ANOVA followed by Dunnet's multiple comparison tests was used for statistical analysis.

Figure 7. Effects of PLA_2 and 5-LOX inhibitors on ROS-production, measured by the fluorescent probe DCFDA. The increased ROS-production generated after coincubation of ASMC and platelets (ASMC/platelet 1/1,000) was potently and significantly reduced by the 5-LOX inhibitor AA-861 (A) and the PLA_2 -inhibitor ATK (B). One-way ANOVA followed by Dunnet's multiple comparison tests was used for statistical analysis. Results from 8 separate cell passages and blood donors.

Discussion

The interest in platelets as important players in inflammatory airway diseases has increased during recent years [2, 3]. Platelets may play a vital role in asthma through their possible impact on airway remodeling [12] where increased growth of ASMC is a main feature. In the present study, we demonstrate by using different cell proliferation assays (i.e. MTS-assay, DNA measurements and thymidine incorporation) that platelets dose-dependently increase ASMC proliferation. The pro-proliferative effect of platelets was powerful, taking into account that the measurements were performed after only 24 hours.

It has previously been suggested that ROS participate during cell adhesion and proliferation [19, 32]. We found that ROS generation was enhanced in a co-culture of platelets and ASMC. Furthermore, inhibition of NADPH oxidase reduced the platelet-induced ASMC proliferation. Hence, we suggest that platelets contribute to increased ROS generation which is accompanied by an elevated proliferation of ASMC.

In accordance with earlier studies [33, 34], we showed that PDGF-BB caused ASMC proliferation. However, this effect was not associated with detectable ROS production. In addition, platelets, compared to high doses of external applied PDGF-BB, were more efficient in inducing ASMC proliferation. Furthermore, experiments performed in the presence of a mixture of platelet-inhibitors revealed that the drug treatment did not affect platelet-induced proliferation, indicating that the proliferative capacity of platelets involves additional mechanisms besides release of α -granule-stored PDGF-BB.

Many studies *in vitro* have shown that arachidonic acid metabolites influence on cell proliferation although the effects are cell-type specific [14-17, 35]. Cysteinyl leukotrienes, products of the 5-LOX pathway, have been implicated in various stages of airway inflammation, in the present study, we found that inhibition of PLA₂ and 5-LOX significantly reduced the ASMC proliferation caused by platelets. Western blot analysis also revealed that

the ASMC, but not platelets, express 5-LOX. Consequently, we suggest that the 5-LOX pathway is important in ASMC proliferation induced by platelets. However, addition of the leukotrienes LTC₄ and LTB₄ to ASMC cultures did not elicit ASMC proliferation per se. In accordance, it was recently shown that cysteinyl leukotrienes alone did not induce proliferation, but acted in synergy with other factors [36-38]. Some studies indicate for a link between eicosanoids and ROS production [14, 22-24]. We found that inhibition of PLA₂ and 5-LOX antagonized both the ROS generation and the ASMC proliferation induced by platelets. This demonstrates that the 5-LOX pathway is coupled to increased ROS production also in a co-culture of platelets and ASMC, and that this is important for stimulating proliferation. Enzymes such as lipoxygenase and cyclooxygenase may directly generate superoxide anion via a side reaction that depends on the presence of reducing co-substrates [32]. However, our results strongly suggest that the NADPH oxidase is the enzymatic source for ROS production. Consequently, it is likely that activation of the 5-LOX pathway causes a NADPH-oxidase dependent ROS production in co-cultures of platelets and ASMC. The exact signalling cross-talk and specific 5-LOX product(s) that contributes to NADPH-oxidase mediated ROS generation remains to be determined.

Microscopic examination showed that platelets bind to the ASMC suggesting that platelet/ASMC adhesion is an important event in initiating proliferation. In accordance, some previous studies have shown that the proliferative properties of platelets or platelet-derived microparticles involve membrane structures and is not solely dependent on alfa-granule-derived growth factors [39, 40]. The precise mechanisms underlying the binding between ASMC and platelets are however unknown.

From a clinical perspective, it has been shown that platelets in a symptomatic asthma group were found aggregated together with electron-dense fibrous material at the airway luminal edge [41]. Consequently, it is tempting to speculate that continuous recruitment and

inappropriate activity of platelets in the airways may contribute to pathological situations and increased proliferation of ASMC.

In conclusion, our findings demonstrate a pronounced capacity of platelets in inducing ASMC proliferation. Based on the results, we propose that the 5-LOX pathway and the NADPH-oxidase dependent ROS production are pivotal events in ASMC proliferation caused by platelets. This action of platelets represents a potential important and novel mechanism that may contribute to 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.

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