

Research

Open Access

## Platelets stimulate fibroblast-mediated contraction of collagen gels

Ulrika Zagai\*<sup>1</sup>, Karin Fredriksson<sup>1</sup>, Stephen I Rennard<sup>2</sup>, Joachim Lundahl<sup>1</sup>  
and C Magnus Sköld<sup>1</sup>

Address: <sup>1</sup>Department of Medicine, Karolinska Hospital, Stockholm, Sweden and <sup>2</sup>Department of Internal Medicine, University of Nebraska Medical Center, Omaha, NE, USA

Email: Ulrika Zagai\* - [ulrika.zagai@ks.se](mailto:ulrika.zagai@ks.se); Karin Fredriksson - [karin.fredriksson@ks.se](mailto:karin.fredriksson@ks.se); Stephen I Rennard - [ulrika.zagai@ks.se](mailto:ulrika.zagai@ks.se); Joachim Lundahl - [joachim.lundahl@ks.se](mailto:joachim.lundahl@ks.se); C Magnus Sköld - [magnus.skold@ks.se](mailto:magnus.skold@ks.se)

\* Corresponding author

Published: 17 October 2003

Received: 29 May 2003

*Respiratory Research* 2003, 4:13

Accepted: 17 October 2003

This article is available from: <http://respiratory-research.com/content/4/1/13>

© 2003 Zagai et al; licensee BioMed Central Ltd. This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.

### Abstract

**Background:** Platelets are thought to play a role in a variety of inflammatory conditions in the lung, some of which may lead to fibrosis. In the current study we tested the hypothesis that whole platelets and platelet lysate can mediate remodelling of extracellular matrix *in vitro* by affecting fibroblast-mediated contraction of a collagen gel. We also sought to determine to what extent platelet-derived growth factor (PDGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) contribute to this effect.

**Methods:** Washed platelets, isolated from healthy blood donors, and platelet lysate (freezing and thawing), were cast together with human lung fibroblasts in three-dimensional collagen gels. The gels were then released and cultured for four days. PDGF and TGF- $\beta_1$  concentrations were measured in culture supernatants by ELISA.

**Results:** Both platelets and platelet lysate augmented fibroblast-mediated gel contraction in a time and concentration dependent manner ( $19.9\% \pm 0.1$  (mean  $\pm$  SEM) of initial area vs.  $48.0\% \pm 0.4$  at 48 hours;  $P < 0.001$  and  $41.5\% \pm 0.6$  vs.  $60.6\% \pm 0.3$  at 48 hours;  $P < 0.001$ , respectively). Fixed platelets had no effect in the system. Both TGF- $\beta_1$  and PDGF-AA/AB were released in co-culture. PDGF-AA/AB had a maximum release at 24 hours whereas TGF- $\beta_1$  release increased with longer culture periods. Neutralising antibodies to these mediators partially inhibited platelet-induced gel contraction.

**Conclusion:** We conclude that platelets may promote remodelling of extracellular matrix *in vitro* and that PDGF and TGF- $\beta$  partially mediate this effect, also indicating a role for other mediators. The findings may be an important mechanism in regulating repair processes after injury.

### Introduction

Platelets have a major function in maintaining homeostasis by initiating the coagulation process. In addition, activated platelets have a capacity to participate in complex

cellular interactions. For instance, platelets constitute and release a variety of mediators that can modulate endothelial permeability and recruit inflammatory cells [1]. This local inflammatory process also enables circulating

platelets to enter the extra vascular milieu and to adhere to exposed matrix via integrins that bind to collagen and laminin [2].

Fibroblasts are the major type of mesenchymal cells present in the connective tissue matrix [3]. Besides being a structural cell, the fibroblast can secrete a number of inflammatory mediators, which have the potential to drive fibrotic tissue remodelling. This is a complex process consisting of recruitment and proliferation of fibroblasts and production of extracellular matrix components. Part of this process includes contraction of matrix and can be present not only in scar formation but also in most fibrotic conditions [4,5]. Culturing of fibroblasts in three-dimensional native type I collagen gels has been used to model this contractile process and is considered to be one aspect of fibrotic tissue remodelling [6,7].

In certain conditions, platelets may accumulate as a part of the inflammatory response. For instance, in acute respiratory distress syndrome (ARDS) platelets are initially sequestered in the pulmonary microvasculature where they release substances that promote vaso- and bronchoconstriction [8]. Histologically ARDS is characterised by an intense inflammation in the lung, which may progress to pulmonary fibrosis.

Platelet-derived growth factor (PDGF) and transforming growth factor- $\beta$  (TGF- $\beta$ ) constitute two potential platelet-associated mediators promoting fibrosis. Previous studies have shown that both these mediators can stimulate fibroblast-mediated contraction of collagen gels [4,9,10]. In the current study we tested the hypothesis that whole platelets and platelet lysate could augment contraction of collagen gels *in vitro*. We also wanted to evaluate the relative contribution of PDGF and TGF- $\beta$  in mediating this effect.

## Materials and methods

### Materials

Type I collagen (rat-tail tendon collagen, RTTC) was extracted according to a previously published method [11]. Briefly, tendons were excised from rat-tails, and the tendon sheath and other connective tissues were carefully removed. After repeated washing with Tris-buffered saline (0.9% NaCl, 10 mM Tris, pH 7.5) the tendons were washed in increasing concentrations of ethanol. Type I collagen was then extracted in 6 mM acetic acid. Protein concentration was determined by weighing a lyophilised aliquot from each lot of collagen. The RTTC was stored at 4°C until use.

### Cell culture

Human foetal lung fibroblasts (HFL1) were obtained from the American Type Culture Collection (Rockville,

MD, USA). The cells were cultured on 100-mm tissue culture dishes (FALCON; Franklin Lakes, NJ, USA) with Dulbecco's Modified Eagle Medium (D-MEM; GIBCOBRL/Lifetechnologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; GIBCOBRL/Lifetechnologies), 50 U/mL penicillin G sodium, 50  $\mu$ g/mL streptomycin sulphate (penicillin-streptomycin, GIBCOBRL/Lifetechnologies) and 1  $\mu$ g/mL amphotericin B (fungizone, GIBCOBRL/Lifetechnologies). The fibroblasts were passaged twice a week and were used between the 15<sup>th</sup> and 20<sup>th</sup> passage. Confluent fibroblasts were trypsinised (Trypsin-EDTA; GIBCOBRL/Lifetechnologies, 0.05% trypsin 0.53 mM EDTA), resuspended in D-MEM without serum and used for collagen gel culture.

### Platelets

Platelets were obtained from healthy blood donors by an apheresis procedure, using a blood cell separator (Cobe BCT Inc, Lakewood, CO, USA) and a closed-system apheresis kit (extended life platelet dual needle set, Cobe). This system generates a leukocyte-depleted concentrate ( $< 1 \times 10^6$  leukocytes/L). The platelet concentrates were stored in a computerised environmental chamber (Melco Engineering, CA, USA) at 21.6–22.5°C with constant linear agitation, 60 rpm. Samples were drawn under sterile conditions from each bag. The platelets were centrifuged at  $900 \times g$  for 15 minutes and then washed four times ( $850 \times g$ , 10 min) in 5 mL sterile phosphate-buffered saline (PBS) to get rid of plasma. Finally, the platelets were resuspended in PBS and counted in a Micro Diff II Hematology analyzer (Beckman Coulter; Fullerton, CA, USA).

### Fixation and lysing of platelets

Washed platelets were resuspended in 4% paraformaldehyde (PFA), incubated for 10 minutes in room temperature and washed three times ( $900 \times g$ , 5 min) in PBS. The fixed cells were resuspended in PBS to various concentrations. To obtain platelet lysate the platelets were lysed by freezing ( $-20^\circ\text{C}$ ) and thawing three times. The cell debris was removed by centrifugation and the platelet lysate (supernatant) was stored in  $-20^\circ\text{C}$  until use. The lysate contained significant concentrations of PDGF and TGF- $\beta$  (approximately 500 ng/mL lysate for both, measured by ELISA, see below).

### Collagen gel contraction assay

Collagen gels were prepared as previously described [12]. Briefly, distilled water, RTTC,  $4 \times$  D-MEM, fibroblast- and platelet-suspensions were mixed together to a final concentration of 0.75 mg/mL of collagen,  $3 \times 10^5$  fibroblasts/mL and physiologic ionic strength of  $1 \times$  D-MEM. Platelets were added in various concentrations from  $1 \times 10^6$ – $1 \times 10^8$  platelets/mL gel. The different mixtures were kept on ice during the preparation. Five hundred and fifty  $\mu$ L of

the gel solution were then cast into each well of a 24-well tissue culture plate with a 2-cm<sup>2</sup> growth area (FALCON). Gelation occurred within 30 minutes at 37°C.

The ability of co-cultured platelets to affect fibroblast-mediated collagen gel contraction was determined by the slow contraction assay of Bell *et al.* [6]. Fibroblasts and platelets in co-culture were prepared as described above. After gelation the gels were released from the surface of the culture well using a sterile spatula and transferred into 60-mm tissue culture dishes (FALCON) containing 5 mL of serum-free D-MEM. The floating gels were incubated at 37°C and 5% CO<sub>2</sub> for four days and the gel area were measured daily. The same preparation procedure was performed when fixed platelets or platelet lysate were tested in the system.

#### **Measurement of gel area**

In all the experiments, the area of the collagen gels was measured by an image analyser system (LEICA Microsystems AG; Wetzlar, Germany). The images were recorded by a video camera comprising a zoom lens mounted approximately 15 cm above a lighted stage. Sixty-mm culture dishes rested on this stage, and the gels were observed directly. No special mounting was required for stability. The area of the gels was then captured and processed by computer software from LEICA Microsystems AG and collagen gel contraction in the horizontal axes was determined. Thickness was not measured and contraction in the vertical area was not assessed. With the described method, the area of each gel could be measured while sterility was preserved.

#### **DNA content**

In order to estimate cell numbers in the gels, the DNA content was assayed fluorometrically with Hoechst dye 33258 (SIGMA-ALDRICH, St Louis, Missouri, USA) by a modification of a previously published method [13]. Briefly, the gels were dissolved in 0.5 mL collagenase (GIBCOBRL/Lifetechnologies, 0.25 mg/mL) by incubation at 37°C for 2–3 h [14]. Cells were then collected by centrifugation (1500 × g, 10 min). After freezing and thawing once, 1 mL of ddH<sub>2</sub>O was added to each sample and cells were sonicated. The DNA content was determined by adding 2 mL of TNE buffer (10 mM Tris, 3 M NaCl, and 1.5 mM EDTA, pH 7.4) containing 2 µg/mL of Hoechst dye 33258. Fluorescence intensities were measured with a fluorescence spectrometer (Fluorescan 2; Lab-systems, Finland) with an excitation wavelength at 355 nm and an emission wavelength at 460 nm.

#### **Determination of PDGF-AA/AB and TGF-β<sub>1</sub> in culture medium**

Floating gels containing fibroblasts and co-culture of fibroblasts and viable, fixed or lysed platelets were cul-

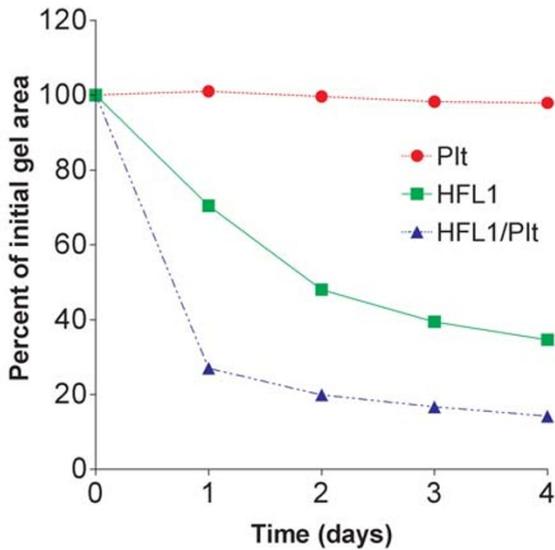
tured in 5 mL serum free D-MEM in 60-mm tissue dishes (FALCON). After two days the media surrounding the gels were collected and the samples were analysed for PDGF-AB and TGF-β<sub>1</sub> by commercially available quantitative sandwich enzyme immunoassay, ELISA (PDGF-AB; Quantikine<sup>®</sup>, Human PDGF-AB Immunoassay, TGF-β<sub>1</sub>; Quantikine<sup>®</sup>, Human TGF-β<sub>1</sub> Immunoassay, R&D Systems, Minneapolis, MN, USA).

In additional time-course experiments PDGF-AB, PDGF-AA (PDGF-AA; Quantikine<sup>®</sup>, Human PDGF-AB Immunoassay, R&D Systems) and TGF-β<sub>1</sub> were determined in the surrounding media from co-culture of fibroblast and whole platelets after 2, 12, 24 and 48 hours of culture by ELISA. The TGF-β<sub>1</sub> ELISA was performed as previously described by Kim *et al.* [15]. Briefly, microtiter plates (VWR International, Stockholm, Sweden) were coated with monoclonal anti-human TGF-β<sub>1</sub> antibodies (clone 9016, R&D Systems). Standard and samples were added and incubated followed by addition of biotinylated anti-TGF-β<sub>1</sub> antibodies (200 ng/mL, R&D Systems). Then HRP-streptavidin (1:200, R&D Systems) was applied. Finally the substrate TMB (3,3',5,5'-tetra methylbenzidine, SIGMA-ALDRICH) was added and allowed to develop. TGF-β<sub>1</sub> was measured with or without activation by acidification in both assays. The optical density (OD) was determined by a spectrophotometer (Anthos 2001, Lab-design, Täby, Sweden) by measuring absorbance at 450 nm.

#### **Neutralisation of human PDGF and TGF-β bioactivity**

Neutralising antibodies to PDGF and TGF-β were used to evaluate the relative contribution of these two agents to fibroblast-mediated collagen gel contraction induced by platelets. Anti-human PDGF antibody, pan-specific anti-TGF-β polyclonal antibody (R&D Systems, Europe, Ltd., Abingdon, Oxon, UK) were pre-incubated with platelets 30 minutes in room temperature before mixed in the gels. Antibodies were also added to the media surrounding the gels (final concentration 25 µg anti-PDGF/mL and 50 µg anti-TGF-β/mL). An irrelevant rabbit IgG antibody (R&D Systems) was also tested (final concentration 50 µg/mL). Gels were cultured for two days and gel area was measured as described in *Measurement of gel area*.

To check the efficiency of the blocking antibodies in the gels PDGF and TGF-β were added to collagen gels and surrounding medium. A concentration of PDGF and TGF-β equal to that assayed in fibroblast/platelet co-culture (see Figure 7, HFL1/Plt) augmented fibroblast-mediated collagen gel contraction by approximately five percent. This augmentation could be neutralised by the addition of antibodies to PDGF and TGF-β.



**Figure 1**  
Time dependent augmentation of fibroblast-mediated collagen gel contraction induced by platelets (Plt). Fibroblasts (HFL1:  $3 \times 10^5$ /mL gel) and Plt ( $10^8$ /mL gel) or the two cell types together (HFL1/Plt) were cast into a 24-well tissue culture plate. The assay was performed by releasing the gels into medium immediately after gelation. The area of each gel was measured by an image analyser on four consecutive days. Vertical axis: gel area expressed as percentage of original gel size. Horizontal axis: time (days). Data are presented as mean of triplicate. Standard errors of mean ( $\pm$  SEM) were less than 1%.  $P < 0.001$ : HFL1 vs. HFL1/Plt on day 2.

**Table 1: Platelets augment fibroblast-mediated contraction of collagen gels.**

Cells in collagen gels	Area of gels (% of initial gel area)	SEM	n
Fibroblasts	46.1	$\pm 1.8$	10
Platelets	98.5***	$\pm 1.1$	8
Fibroblasts/Platelets	28.4***	$\pm 2.6$	10

Area was determined after 2 days of culture. Concentration of fibroblasts and platelets was  $3 \times 10^5$  respective  $10^8$  cells/mL gel. Values are means  $\pm$  SEM, each including triplicate of gels, n= number of individual experiments. \*\*\*  $P < 0.001$  vs. fibroblasts.

**Statistical methods**

Data are presented as mean of three replica gels for each condition and standard error of mean ( $\pm$  SEM). As the batch of RTTC, the number of cell passages and culture conditions can affect the gel contraction, the data shown in each figure are taken from a representative experiment

of which each was repeated on multiple occasions. Additional data are presented as mean and standard deviation ( $\pm$  SD). Groups of data were evaluated by analysis of variance (ANOVA). Differences between two groups of data were analysed by student's t-test.

**Results**

**Effects of platelets on fibroblast-mediated contraction of collagen gels**

Repeated experiments, containing a co-culture of fibroblasts ( $3 \times 10^5$  cells/mL) and platelets ( $1 \times 10^8$  cells/mL) demonstrated a significant ( $P < 0.001$ ) augmentation of fibroblast-mediated collagen gel contraction (Table 1). The gel area, when measured on four consecutive days, revealed that the augmentation of contraction was time dependent (Figure 1). Thus, gels containing fibroblasts and platelets contracted more at each single point than control gels, containing only fibroblasts. The floating gels contracted most during the first 48 hours. After two days, only a small additional contraction was observed suggesting that the gels have reached a final size. Platelets alone did not induce any contraction.

The augmentation of gel contraction was dependent on the number of platelets added (Figure 2). The lowest concentration of platelets that augmented gel contraction was  $1 \times 10^7$  platelets/mL gel. Adding more than  $1 \times 10^8$  platelets/mL gel did not result in any additional augmentation of contraction (data not shown).

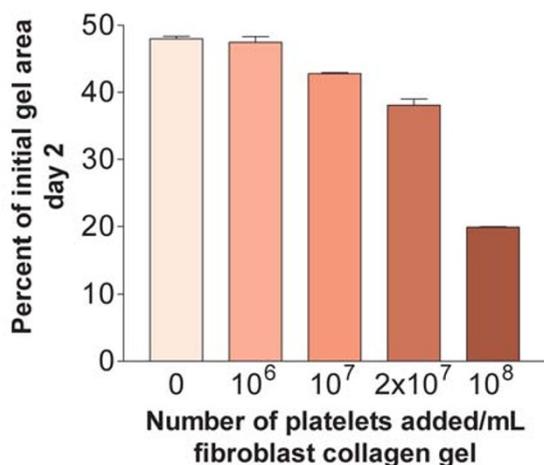
**Effects of platelet fixation and platelet lysate on fibroblast-mediated contraction of collagen gels**

To evaluate if the platelet-induced augmentation of fibroblast-mediated collagen gel contraction was due to the presence of the platelet structure *per se*, platelets were fixed with paraformaldehyde and tested in the gel contraction assay.

Fixed platelets co-cultured with fibroblasts did not augment gel contraction compared to gels cultured with fibroblasts and non-fixed platelets (Figure 3). This indicates that viable functional platelets are required to stimulate fibroblast-mediated collagen gel contraction, either by cell-cell contact or by releasing soluble factors in the co-culture system. To test the hypothesis, whether the effect was mediated by platelet associated soluble factors, supernatant from lysed platelets was tested for gel contraction. The lysate stimulated the gel contraction in a time and concentration dependent manner, suggesting that cell-cell contact is not mandatory (Figure 4).

**DNA content**

The amount of DNA in collagen gels was determined to assess whether the increased fibroblast-mediated contraction induced by platelets was due to cell prolifera-



**Figure 2**

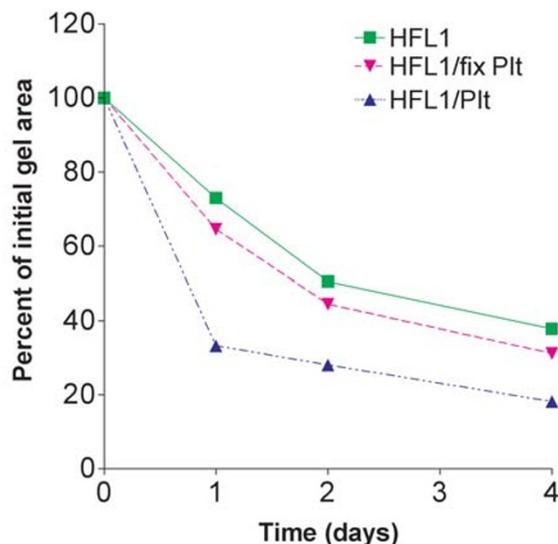
Concentration dependent augmentation of fibroblast-mediated collagen gel contraction. Increasing numbers of platelets were mixed together with fibroblasts in a three-dimensional collagen gel. After gelation, the gels were released and the area of the floating gels was measured after 48 hours of culture. Vertical axis: gel area expressed as percentage of original gel size. Horizontal axis: number of platelets added to gels. Data shown as mean of triplicate  $\pm$  SEM.  $P < 0.001$ : 0 (HFLI) vs.  $10^7$  at all data points.

tion in the culture system. Both co-cultures of whole platelets and platelet lysate resulted in a significant increase in DNA content compared to fibroblasts alone after two days of culture (OD-value:  $240.1 \pm 41.0$  (mean  $\pm$  SD) and  $249.6 \pm 19.3$  vs.  $176.6 \pm 15.1$ ;  $P < 0.01$  and  $P < 0.001$  respectively, Figure 5).

According to a standard curve (data not shown), this increase in DNA content equals an increase in cell number from 270 000 cells/mL gel to approximately 450 000 cells/mL gel. To evaluate to what extent this increase in cell number augments gel contraction, various numbers of fibroblasts embedded in collagen gels were assayed for contraction. As shown in Figure 6, and as previously reported by Bell *et al.* [6], there was a dose dependent increase in fibroblast-mediated collagen gel contraction. According to the platelet-induced proliferation in our system (see Figure 5), not more than 10–15 percent augmentation of fibroblast-mediated collagen gel contraction could be explained by an increased cell number.

#### Concentration of PDGF and TGF- $\beta$

Both PDGF and TGF- $\beta$  can augment fibroblast-mediated collagen gel contraction. Therefore, we assayed culture supernatant for these mediators by ELISA. First, the con-

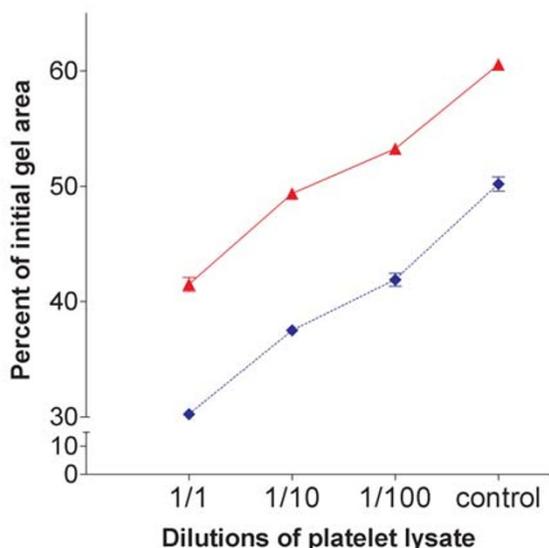


**Figure 3**

Fixed platelets do not augment fibroblast-mediated collagen gel contraction. Fibroblasts, platelets, fixed platelets (PFA 10 min) or combinations of cell types were cast into collagen gels. The gels were released into medium and the area was measured on four consecutive days. Vertical axis: gel area expressed as percentage of original gel size. Horizontal axis: time (days). Data shown as mean of triplicate.  $\pm$  SEM were less than 1%.  $P < 0.001$ : HFLI/Plt vs. HFLI/fix Plt at all data points.

centrations of cytokines in all culture conditions were determined after two days of culture. The concentration of PDGF-AB in surrounding media in samples containing only fibroblasts, was not detectable (Figure 7a), while it was increased in fibroblast/platelet and fibroblast/platelet lysate co-cultures ( $454 \pm 13$  pg/mL (mean  $\pm$  SD) and  $1061 \pm 6$  pg/mL respectively;  $P < 0.001$ ). The concentration of PDGF-AB in co-culture of fixed platelets and fibroblasts was significantly lower ( $44 \pm 1$  pg/mL;  $P < 0.001$ ) than in fibroblast/platelet co-culture.

In samples with fibroblasts or fibroblasts and fixed platelets the concentration of TGF- $\beta_1$  was  $258 \pm 7$  pg/mL (mean  $\pm$  SD) after two days of culture (Figure 7b). When fibroblasts and unfixed platelets were co-cultured, the concentration of TGF- $\beta_1$  was  $1962 \pm 21$  pg/mL ( $P < 0.001$  vs. fibroblast and fibroblast/fixed platelets). The concentration of TGF- $\beta$  was further increased ( $3561 \pm 60$  pg/mL) when fibroblasts were cultured with platelet lysate.



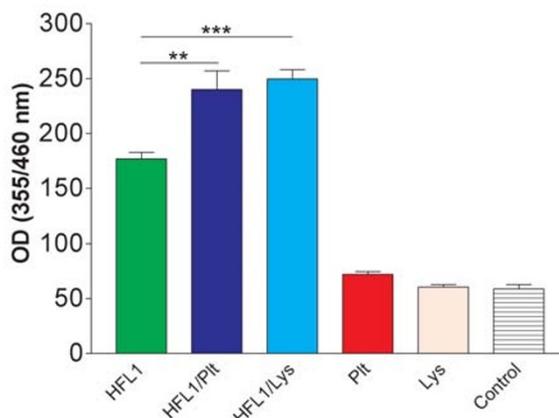
**Figure 4**

Time and concentration dependent effect of platelet lysate on fibroblast mediated collagen gel contraction. Various dilutions of platelet lysate were mixed together with fibroblasts in collagen gels. After gelation, the gels were released and the area of the floating gels was measured after 2 (solid line) and 4 (dotted line) days. Vertical axis: gel area expressed as percentage of original gel size. Horizontal axis: dilutions of platelet lysate, control contain no lysate. Data presented as mean of triplicate.  $\pm$  SEM were less than 1%.  $P < 0.001$ : HFLI vs. platelet lysate at all data points.

In order to investigate the time course of PDGF and TGF- $\beta_1$  release in fibroblast/platelet co-culture, media from additional experiments were collected after 2, 12, 24 and 48 hours. PDGF-AB was released in higher concentrations at 24 hours compared to PDGF-AA ( $444 \pm 4$  pg/mL vs.  $257 \pm 7$  pg/mL, Figure 8). After 48 hours the PDGF release was lower for both isoforms ( $167 \pm 7$  pg/mL and  $115 \pm 21$  pg/mL, respectively). There were no detectable levels of PDGF when fibroblasts were cultured alone (data not shown). In contrast, TGF- $\beta_1$ -release increased with increasing length of culture. At 48 hours the concentration was  $1888 \pm 18$  pg/mL in co-culture, while fibroblasts alone released almost the same amount ( $665 \pm 9$  pg/mL) at all measured time points (Figure 9).

#### Neutralisation of PDGF and TGF- $\beta$ bioactivity

To assess the impact of PDGF and TGF- $\beta$  on platelet-induced augmentation of gel contraction, neutralising antibodies were added to the culture system. Addition of neutralising antibodies to PDGF and TGF- $\beta$  respectively did partially inhibit the platelet-induced augmentation of



**Figure 5**

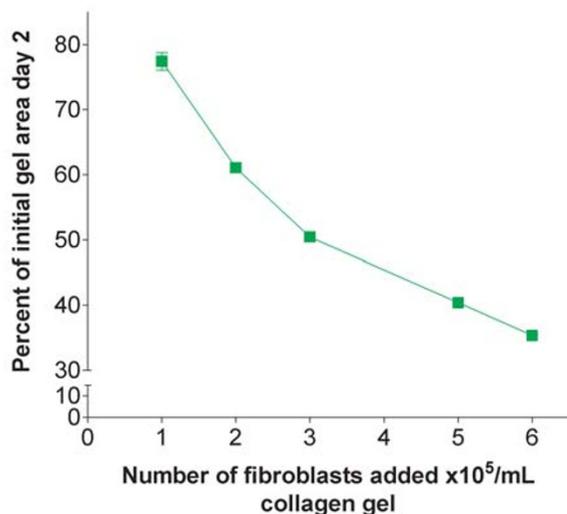
Cell numbers in collagen gels estimated by DNA content. Floating collagen gels containing 300 000 fibroblasts/mL gel (HFLI), HFLI and  $10^8$  platelets/mL gel (HFLI/Pit) or fibroblasts and undiluted platelet lysate, (HFLI/Lys) were cultured for two days. The gels were then dissolved in collagenase and DNA contents in pellets were determined by a fluorometric assay. Control is collagen gels without cells or lysate. Vertical axis: Optical Density (OD) measured at wavelength 355/460 nm. Data shown as means  $\pm$  SD ( $n = 6$ ). \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

gel contraction (Figure 8). When both antibodies were added together an inhibition of 11 percent was observed. Irrelevant antibody (rabbit IgG) did not significantly inhibit the platelet-induced augmentation.

#### Discussion

In the present study we demonstrate that both whole platelets and platelet lysate can stimulate fibroblast-mediated collagen gel contraction. We were also able to show that the stimulating effect by platelets partially could be attributed to the pro-fibrotic mediators PDGF and TGF- $\beta$ .

Fibroblasts are believed to be the key cell in tissue remodelling and in the formation of fibrosis. We used an *in vitro* model for tissue remodelling, fibroblast-mediated contraction of collagen gels, originally described by Bell *et al.* [6]. Even though the culture system is a simplification of *in vivo* conditions, regarded as only one aspect of tissue remodelling, the system has been used to elucidate effects of potential fibrotic mediators [16–19]. In the three-dimensional collagen gel system the ability of fibroblasts to contract the gels is dependent on a variety of factors including fibroblast strain, cell density, collagen concentration and the presence of soluble factors [4,9]. For instance, serum is a potent stimulus of fibroblast-



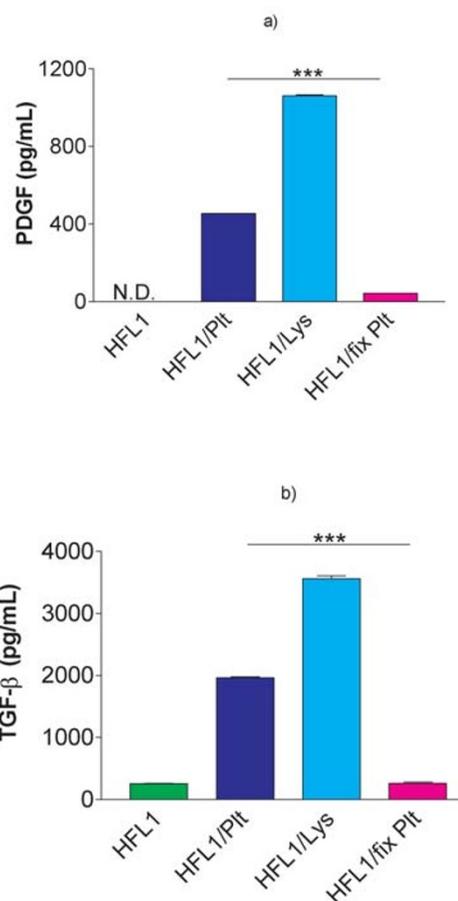
**Figure 6**

Fibroblast-mediated collagen gel contraction is dependent of cell concentration. Increasing number of fibroblasts (HFL1) were added to three-dimensional collagen gels. After gelation, the gels were released and the area of the floating gels was measured after 48 h of culture. Vertical axis: gel area expressed as percentage of original gel size. Horizontal axis: number of fibroblasts added to gels. Data shown as mean of gel triplicate and  $\pm$  SEM.

mediated collagen gel contraction and has previously been used in a number of studies [20–22].

In the current study we demonstrate that platelets in co-culture with fibroblasts can augment contraction of three-dimensional collagen gels. We found that the stimulation was concentration dependent and that a platelet concentration of  $1 \times 10^8$  cells/mL gel induced the most pronounced effect. Under normal physiological conditions the platelet count in the bloodstream is about  $3 \times 10^8$  cells/mL and their lifespan is 7–10 days. In addition, the number of circulating platelets increases significantly during inflammation. Therefore we assumed that the number of platelets present in tissue after injury is probably not less than  $10^8$  cells/mL. Two lines of evidence suggest that the stimulatory effect can be mediated by soluble factors. Firstly, fixed platelets had no augmentative effect e.g. the presence of the platelet structure *per se* did not stimulate contraction. Secondly, a soluble platelet lysate had a pronounced augmentative effect, suggesting that stimulating factors are released from platelets.

Two pro-fibrotic platelet-associated mediators are PDGF and TGF- $\beta$  and increased concentrations of these media-

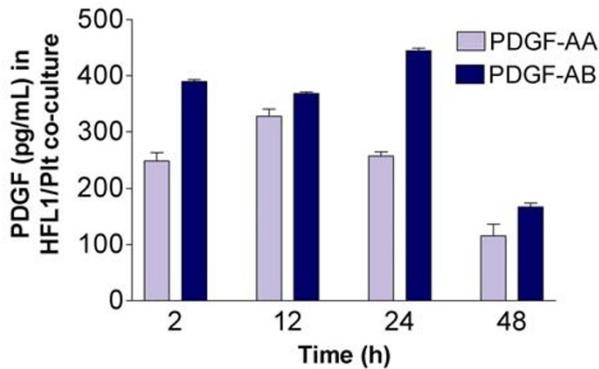


**Figure 7**

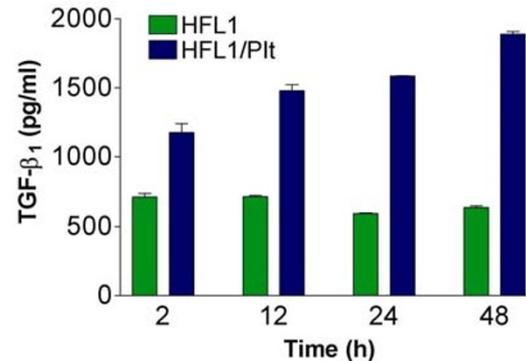
Release of PDGF-AB and TGF- $\beta_1$  in co-culture. Fibroblasts (HFL1) or fibroblasts together with platelets (Plt), fixed platelets (fix Plt) or platelet lysate (Lys) were cast into collagen gels. After two days of culture, the media surrounding the gels were collected and analysed for a) PDGF-AB and b) TGF- $\beta_1$  (ELISA). Vertical axis: total concentration of PDGF-AB respectively TGF- $\beta_1$  (pg/mL). Data shown as mean of duplicates  $\pm$  SD. \*\*\* P < 0.001.

tors have been detected in inflammatory and fibrotic conditions *in vivo* [23–26]. Since these mediators are known to augment fibroblast-mediated collagen gel contraction [4,9] we sought to determine the relative contribution of them in the contraction assay. PDGF and TGF- $\beta$  were present in significant levels in culture supernatants and adding neutralising antibodies resulted in a partial inhibition of the platelet-induced augmentation.

We also determined two isoforms of PDGF. PDGF-AA is reported to be the most abundant form released from

**Figure 8**

Time-dependent release of PDGF-AA and -AB in fibroblast/platelet co-culture. Fibroblasts (HFL1) together with platelets (Plt) were cast into collagen gels. After 2, 12, 24 or 48 hours of culture, the medium surrounding the gels were collected and analysed for PDGF-AA and PDGF-AB by ELISA. Vertical axis: concentration of PDGF (pg/mL) in medium. Data shown as mean of duplicates  $\pm$  SD.

**Figure 9**

Time-dependent release of TGF- $\beta_1$  in fibroblast/platelet co-culture. Fibroblasts (HFL1) or fibroblasts together with platelets (Plt) were cast into collagen gels. After 2, 12, 24 or 48 hours of culture, the medium surrounding the gels were collected and analysed for TGF- $\beta_1$  by ELISA. Vertical axis: concentration of TGF- $\beta_1$  (pg/mL) in medium. Data shown as mean of duplicates  $\pm$  SD.

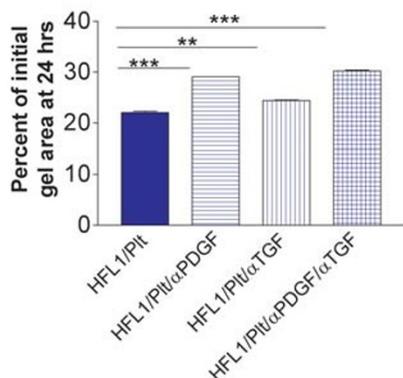
platelets [27]. However, we found more released PDGF-AB than PDGF-AA in fibroblast/platelet co-culture. TGF- $\beta$ , frequently present in co-culture, has been shown to down regulate the PDGF- $\alpha$ -receptor on human fibroblasts [28,29]. Since PDGF-AA only bind the  $\alpha$ -receptor, while PDGF-AB bind both  $\alpha$ - and  $\beta$ -receptors we believe that the observed effect on fibroblast-mediated contraction is predominantly a PDGF-AB effect.

The release of PDGF was highest during the first 24 hours of culture and decreased significantly with increasing length of culture. In contrast, TGF- $\beta_1$  increased with longer culture periods at measured points during the first 48 hours. One plausible explanation for this difference in kinetics is that PDGF is only released from platelets, while TGF- $\beta_1$  also can be produced by fibroblasts in response to stimuli during the culture period.

The involvement of these two mediators in the fibroblast-mediated gel contraction assay was confirmed by adding PDGF and TGF- $\beta$  in the same concentrations as assayed in fibroblast/platelet co-culture, resulting in a small increase of contraction, which could be abolished by neutralising antibodies. In addition, by using excess of antibodies to PDGF and TGF- $\beta$  we were able to inhibit a part of the platelet-induced contraction. Collectively, these data suggest that PDGF and TGF- $\beta$  constitute two mediators released from platelets, which partially contribute to the contraction process and that additional platelet-derived mediators or mechanisms can not be excluded.

One possible mechanism involved is cell proliferation. PDGF has a number of effects on mesenchymal cells including stimulation of proliferation [12,30]. Since an altered cell concentration can influence collagen gel contraction [6] we assayed DNA as measure for cell number. The small amounts of DNA in the control, platelet and platelet lysate gels may be DNA fragments originating from the collagen preparation. The increased amount of DNA found when platelets and platelet lysate were cultured together with fibroblasts may in our assay explain approximately 10–15 percent of the increased contraction. However, not even the additive effect of PDGF, TGF- $\beta$  and proliferation can fully explain the total stimulation induced by platelets. Other mechanisms such as up-regulation of integrin receptors, altered fibroblast phenotype and production/secretion of other stimulating mediators must therefore be considered.

Platelets are a rich source of potential pro-fibrotic mediators [1] that may have a potential to interact with fibroblasts in our *in vitro* model. For instance, Mio *et al.* [31] recently showed that lysophosphatidic acid, LPA, which is released from activated platelets, can augment fibroblast-mediated gel contraction. Another inflammatory mediator released by platelets is epidermal growth factor (EGF), a mesenchymal mitogen that stimulate excess deposition of collagen [8]. Thus, a number of platelet-associated



**Figure 10**

Antibodies to PDGF ( $\alpha$ PDGF) and TGF- $\beta$  ( $\alpha$ TGF) partially block platelet-induced stimulation of fibroblast-mediated collagen gel contraction. Collagen gels containing fibroblasts (HFL1) or fibroblasts and platelets (Plt) with or without antibodies were prepared. After gelation, the gels were released into media containing either antibodies to PDGF, TGF- $\beta$  or the antibodies together. After 24 hours of culture the gel area was measured by an image analyser. Vertical axis: gel area expressed as percentage of initial gel area. Data presented as mean of triplicates  $\pm$  SEM. \*\* P < 0.01, \*\*\* P < 0.001.

mediators are likely to contribute to the pro-fibrotic effect observed in the current study. Whether these factors are directly, or through interaction, involving multiple mediators and/or cells are only speculations. Nevertheless our data point out that platelets accumulated in the lung may be pivotal for the imbalance between inflammatory and fibrotic cytokines thus promoting the subsequent fibrotic process.

## Conclusion

We conclude that platelets have the potential to stimulate remodelling of extracellular matrix *in vitro* and that PDGF and TGF- $\beta$  may mediate part of this effect. This may be an important mechanism in regulating repair processes after injury. We therefore suggest that platelet/mesenchymal cell interactions may be one important mechanism leading to pulmonary fibrosis.

## Abbreviations

ARDS acute respiratory distress syndrome

DNA deoxyribonucleic acid

D-MEM dulbecco's modified eagle medium

EDTA ethylenediamine tetra-acetic acid

ELISA enzyme-linked immunosorbent assay

FBS fetal bovine serum

HFL1 human foetal lung fibroblasts

IgG immunoglobulin G

LPA lysophosphatidic acid

OD optical density

PBS phosphate-buffered saline

PDGF platelet-derived growth factor

PFA paraformaldehyde

Plt platelets

TGF- $\beta$  transforming growth factor- $\beta$

TNE tris-NaCl-EDTA

RTTC rat-tail tendon collagen

SD standard deviation

SEM standard error of mean

## Acknowledgements

The authors would like to thank Professor Rolf Lewensohn at the Cancer Centrum Karolinska, for letting us use equipment for DNA analysis. The study was supported by grants from the Swedish Heart-Lung Foundation, The Cancer and Allergy Foundation, The Swedish Foundation for Health Care Science and Allergy Research and Karolinska Institutet.

## References

1. Heffner JE and Repine JE: **Platelets**. *The Lung: Scientific Foundations* 2nd edition. Edited by: Crystal RG and West JB. Philadelphia, Lippincott-Raven Publishers; 1997:947-959.
2. Ganong WF: **Review of medical physiology**. 18th edition. Stamford, Connecticut, Appleton & Lange; 1997:586-601.
3. Sime PJ, Tremblay GM, Xing Z, Sarnstrand BO and Gaudie J: **Asthma**. *Interstitial and bronchial fibroblasts* Edited by: Barnes PJ, Grunstein MM, Leff AR and Woolcock AJ. Philadelphia, Lippincott-Raven Publishers; 1997:475-489.
4. Montesano R and Orci L: **Transforming growth factor beta stimulates collagen-matrix contraction by fibroblasts: implications for wound healing**. *Proc Natl Acad Sci U S A* 1988, **85**:4894-4897.
5. Clark RA: **Regulation of fibroplasia in cutaneous wound repair**. *Am J Med Sci* 1993, **306**:42-48.
6. Bell E, Ivarsson B and Merrill C: **Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro**. *Proc Natl Acad Sci U S A* 1979, **76**:1274-1278.

7. Grinnell F: **Fibroblasts, myofibroblasts, and wound contraction.** *J Cell Biol* 1994, **124**:401-404.
8. Hasleton PS and Roberts TE: **Adult respiratory distress syndrome - an update.** *Histopathology* 1999, **34**:285-294.
9. Gullberg D, Tingstrom A, Thuresson AC, Olsson L, Terracio L, Borg TK and Rubin K: **Beta 1 integrin-mediated collagen gel contraction is stimulated by PDGF.** *Exp Cell Res* 1990, **186**:264-272.
10. Ikuno Y and Kazlauskas A: **TGFbeta1-dependent contraction of fibroblasts is mediated by the PDGFalpha receptor.** *Invest Ophthalmol Vis Sci* 2002, **43**:41-46.
11. Elsdale T and Bard J: **Collagen substrata for studies on cell behavior.** *J Cell Biol* 1972, **54**:626-637.
12. Mio T, Adachi Y, Romberger DJ, Ertl RF and Rennard SI: **Regulation of fibroblast proliferation in three-dimensional collagen gel matrix.** *In Vitro Cell Dev Biol Anim* 1996, **32**:427-433.
13. Labarca C and Paigen K: **A simple, rapid, and sensitive DNA assay procedure.** *Anal Biochem* 1980, **102**:344-352.
14. Liu X, Umino T, Cano M, Ertl R, Veys T, Spurzem J, Romberger D and Rennard SI: **Human bronchial epithelial cells can contract type I collagen gels.** *Am J Physiol* 1998, **274**:L58-65.
15. Kim H, Liu X, Kobayashi T, Kohyama T, Wen FQ, Romberger DJ, Conner H, Gilmour PS, Donaldson K, MacNee W and Rennard SI: **Ultrafine carbon black particles inhibit human lung fibroblast-mediated collagen gel contraction.** *Am J Respir Cell Mol Biol* 2003, **28**:111-121.
16. Fukamizu H and Grinnell F: **Spatial organization of extracellular matrix and fibroblast activity: effects of serum, transforming growth factor beta, and fibronectin.** *Exp Cell Res* 1990, **190**:276-282.
17. Adachi Y, Mio T, Takigawa K, Striz I, Romberger DJ, Spurzem JR and Rennard SI: **Fibronectin production by cultured human lung fibroblasts in three-dimensional collagen gel culture.** *In Vitro Cell Dev Biol Anim* 1998, **34**:203-210.
18. Ohga E, Matsuse T, Teramoto S and Ouchi Y: **Activin receptors are expressed on human lung fibroblast and activin A facilitates fibroblast-mediated collagen gel contraction.** *Life Sci* 2000, **66**:1603-1613.
19. Mio T, Liu X, Toews ML, Adachi Y, Romberger DJ, Spurzem JR and Rennard SI: **Bradykinin augments fibroblast-mediated contraction of released collagen gels.** *Am J Physiol Lung Cell Mol Physiol* 2001, **281**:L164-71.
20. Guidry C: **Fibroblast contraction of collagen gels requires activation of protein kinase C.** *J Cell Physiol* 1993, **155**:358-367.
21. Zhang HY, Gharaee-Kermani M, Zhang K, Karmiol S and Phan SH: **Lung fibroblast alpha-smooth muscle actin expression and contractile phenotype in bleomycin-induced pulmonary fibrosis.** *Am J Pathol* 1996, **148**:527-537.
22. Lijnen P, Petrov V and Fagard R: **In vitro assay of collagen gel contraction by cardiac fibroblasts in serum-free conditions.** *Methods Find Exp Clin Pharmacol* 2001, **23**:377-382.
23. Denis M: **Neutralization of transforming growth factor-beta 1 in a mouse model of immune-induced lung fibrosis.** *Immunology* 1994, **82**:584-590.
24. Bergmann M, Tiroke A, Schafer H, Barth J and Haverich A: **Gene expression of profibrotic mediators in bronchiolitis obliterans syndrome after lung transplantation.** *Scand Cardiovasc J* 1998, **32**:97-103.
25. Haque MF, Harris M, Meghji S and Barrett AW: **Immunolocalization of cytokines and growth factors in oral submucous fibrosis.** *Cytokine* 1998, **10**:713-719.
26. Hoyle GW, Li J, Finkelstein JB, Eisenberg T, Liu JY, Lasky JA, Athas G, Morris GF and Brody AR: **Emphysematous lesions, inflammation, and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor.** *Am J Pathol* 1999, **154**:1763-1775.
27. Soma Y, Dvonch V and Grotendorst GR: **Platelet-derived growth factor AA homodimer is the predominant isoform in human platelets and acute human wound fluid.** *Faseb J* 1992, **6**:2996-3001.
28. Bonner JC, Badgett A, Lindroos PM and Osornio-Vargas AR: **Transforming growth factor beta 1 downregulates the platelet-derived growth factor alpha-receptor subtype on human lung fibroblasts in vitro.** *Am J Respir Cell Mol Biol* 1995, **13**:496-505.
29. Soma Y, Mizoguchi M, Yamane K, Yazawa N, Kubo M, Ihn H, Kikuchi K and Tamaki K: **Specific inhibition of human skin fibroblast chemotaxis to platelet-derived growth factor A-chain homodimer by transforming growth factor-beta1.** *Arch Dermatol Res* 2002, **293**:609-613.
30. Price WA: **PDGF-BB regulates IGF-mediated IGFBP-4 proteolysis in fetal lung fibroblasts.** *Exp Lung Res* 2001, **27**:655-674.
31. Mio T, Liu X, Toews ML and Rennard SI: **Lysophosphatidic acid augments fibroblast-mediated contraction of released collagen gels.** *J Lab Clin Med* 2002, **139**:20-27.

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:  
[http://www.biomedcentral.com/info/publishing\\_adv.asp](http://www.biomedcentral.com/info/publishing_adv.asp)

