Effect of platelets on fibroblasts: clinical relevance and test of an experimental model

Author: Nasim Hedayati
Tutor: Mikael Ivarsson
Title: Associate Professor, Örebro University
Abstract

Chronic wounds have become a more focused research topic worldwide. A defect has been noticed during one of the four phases of wound healing. Fibroblasts, which play an important role during the proliferation phase by producing collagen, has been shown to be stimulated by TGF-β1 that is stored in the platelets. Platelet-rich plasma is a well-studied treatment and is applied topically to the effected area. This has proven to have great results in the healing process. Four donors were selected and ELISA was used in order to measure the amount of TGF-β1 in co-cultures of fibroblasts and platelets. After 24 hours of coculture, a significant proliferation of fibroblasts was noticed. Even though large variations between the donors were noticed in the results, an increase of TGF-β1 could still be noticed between the control group and the coculture, which supported the hypothesis that platelets have a relevant effect on fibroblast proliferation.

Keywords: Chronic wounds, platelet-rich plasma, TGF-β1, fibroblast cells, coculture, ELISA
Background

Chronic wounds represent an increasing problem worldwide. Because of the worldwide problem with overweight, there is an annual increase of diabetic patients; this in turn has cause for an increase of people who suffer from diabetic foot ulcers. Also other disease like cardiovascular disorders has increased over the last years, which also increases the risks of suffering from a chronic wound. If the patient also has a compromised immune system, their odds are even worse since all these factors contribute to an escalation of chronic wounds. The elderly are similarly very vulnerable since they usually are less mobile and some are even bedridden, which leads to an attenuated circulation [1].

There are three types of chronic wounds, venous leg ulcers, diabetic foot ulcers and pressure ulcers. The venous leg ulcers appear because the venous valves in the legs are non working and this leads to hypertension in the veins, which in turn increases the pressure in the capillaries and leads to edema. A venous pressure above 45 mmHg usually leads to leg ulcers. Diabetic foot ulcers are caused by repeated pressure on neuropathic and often ischemic foot. Pressure ulcers, which also are a commonly appearing chronic wound, are caused by constant pressure on sensitive parts of the body with important blood vessels. This usually affect patient that are immobile, such as coma patients or those on bed rest [1].

The chronic wounds do not have a “normal” definition since every case is individual and unique. Chronic wounds are very unpredictable. They can be caused by an acute wound that have been exposed to compression or tissue damage with devastating effects on the body’s own wound healing mechanisms. A chronic wound does not usually heal since the wound never closes completely and it keeps reappearing. An example of a chronic wound caused by a disease is the global epidemic, diabetes, and its diabetic foot ulcers. For wounds in general, they can be divided in either acute or chronic. These terms can indicate both the reason for its appearance and the time it takes to heal. Under the category acute wounds are those that are cause by a trauma, surgery, burns or insect bites etc. These have a timeframe for how long the healing process will take [2].

For a wound to proceed to normal healing, there are some important criteria, which needs to be fulfilled. These include, a good circulation, nutrition, immune status and to avoid mechanical force. Depending of the severity and the size of the injury, it usually takes about
three to 14 days for a wound to heal. This process is divided in the four phases of wound healing: coagulation, inflammation, proliferation and remodeling [3].

In the first phase, the blood coagulates to prevent bleeding from the open wound. During the inflammation phase, neutrophils and macrophages are recruited to the site to phagocyte the bacteria. It is during this phase that it is important to have a proper immune system and enough growth factors. In the third phase, the so-called proliferation phase, the fibroblasts are important since they are the ones who produce collagen matrix. Also new blood vessels invade forming granulation tissue and epidermal cells migrate to close up the open wound. For this process to work it is important for the collagen deposition to work, in turn dependent on e.g. presence of specific nutrients such as vitamins. The last and fourth phase, which is the remodeling, collagen is built along the surface of the wound. In this phase also, the cells that are no longer required or fulfill a purpose are removed by programed cell death or apoptosis. In the case of a chronic wound, at least one of the mechanisms during these four phases are not functioning properly and is the cause for the long healing process [3]. A chronic wound is normally stuck in the inflammation phase, which means that the tissue is repeatedly recruiting neutrophils to the affected area and is releasing cytotoxic enzymes, free radicals and inflammatory mediators, which keeps damaging the tissue [1].

Transforming growth factor beta 1 (TGF-β1) is a biologically active peptide, which has an important role in tissue repair and is part of the cytokine super family. Many studies have shown that TGF-β1 is one of the strongest stimulators when it comes to wound healing since it stimulates collagen synthesis in fibroblasts, which is also important in the healing process [4,5]. According to previous studies, it has been noticed that platelets are a major storage site for TGF-β1. Since platelets probably also play a role in tissue repair, TGF-β1 derived from platelets may have a physiological role in the regeneration of wounds [6].

In particular, it appears that fibroblasts are dysfunctional in a chronic wound. They show signs of early cellular senescence, which means that the cell undergoes a biochemical change and a diploid cell stops replicating. Otherwise usually cells undergo irreversible replicative senescence with age [7,8]. A relatively new method used for improving tissue regeneration is the use of platelet-rich plasma (PRP), which is a blood derivate rich of platelets. Isolation of PRP is carried out by centrifugation of the patients’ own blood which means that there are no risks with immunological reactions since the blood comes from the patient himself. PRP has been used for improving wound healing in patients with burn damage or those with chronic
wounds that will not close. This is proven to work since the even small amounts of PRP carries a large amount of TGF-β1 and other growth factors such as platelet-derived factor (PDGF) that stimulates the synthesis of collagen and extra cellular matrix (ECM) by fibroblast cells [9,10,11]. Other studies has shown that the combination of PRP, fibrin glue and collagen matrix improves wound healing significantly and have achieved great results since the combination of the three produces a complex of biological and molecular occurrences needed for cell migration, proliferation and remodeling [12].

In order to measure the fibroblast proliferation, metabolic labeling assay can be applied where [³⁵S] methionine is used to enable counting of the fibroblasts. Other methods such as couler counters, which measure the cells in a cell suspension fluid and colorimetric assays where cells are dyed and then detected with a spectrophotometer, are often used in the field. The latter method is more time and cost efficient but with some less accuracy [13,14,15].

Enzyme-linked immunosorbent assay (ELISA) is a quantitative analytic method, which shows the interaction between antigen and antibodies through color changes that are achieved by the addition of an enzyme-linked conjugate. An enzyme substrate is used to detect the presence and concentration of molecules in a biological fluid. This method can be used to measure low concentrations of hormones and proteins etc. since the affinity of an antibody to its antigen is very strong, while binding to other structures are weak. There are different kinds of ELISA using different ways to detect the antigen of interest. The most common methods are direct ELISA, indirect ELISA, competitive ELISA and sandwich ELISA. The methods are described schematically below (Fig 1) [16].

Figure 1: Illustrates the procedures taken during the different methods of ELISA where a) demonstrates the direct ELISA method, b) demonstrates the indirect method of ELISA, c) shows the sandwich ELISA and d) illustrates the competitive ELISA method.

http://www.abnova.com/images/content/support//ELISA.gif
Aim

The aim of this study was to set up an experimental model for platelet – fibroblast interactions, and measure the amount of TGF-β1 in co-cultures of fibroblasts and platelets with ELISA. Another aim was to investigate previous studies regarding clinical use of platelets/platelet rich plasma for improving wound healing.

Material and methods

Cell culture

Previously sampled during a skin biopsy, normal skin fibroblasts were collected from the freezer and thawed. To ensure a sterile environment for the cells, the following procedures were carried out in a laminar air hood. Thawed fibroblast cells were added to Dulbecco’s modified eagle’s medium (DMEM) cell culture medium supplemented with 10% fetal bovine serum (FBS) and 0.5 ml gentamicin in a centrifuge tube. The cells were centrifuged for five minutes at 250 x g. The supernatant was removed and the cell pellets were transferred to flasks containing DMEM medium. Cell culturing was continued in a cell culture flask.

Trypsinization of fibroblasts, reseeding in 6-well plates and platelet isolation

After 72 hours of cell culturing, detachment of fibroblasts from the flasks was performed by trypsinization. After removal of the cell culture medium and washing with phosphate buffered saline (PBS), trypsin (0.25%) was added to cells under sterile conditions. The bottle was then incubated at 37°C for five minutes. After addition of more cell culture medium, the cells were transferred to a tube, which was centrifuged at 250 x g for another five minutes. The supernatant was removed and the cells resuspended in fresh cell culture medium. 200 000 fibroblasts in 2 ml medium were transferred to each of 16 wells of six well plates. After 24 hours, the medium was changed to serum free DMEM medium and the cells were serum-starved for another 24 hours before start of cocultures.

For platelet isolation, blood was drawn from four blood donors after informed consent. Samples were then anonymous during the proceeding steps. The blood from each donor was collected in two plastic tubes with sodium heparin (Venosafe VF-109SH, 9 ml, Terumo). The blood was then diluted with acid-citrate-dextrose (ACD) and centrifuged at 220 x g at 22°C for 20 minutes. The centrifuge was set at a slow start and stop motion to prevent activation of
the thrombocytes. The upper part of the PRP was transferred to a new tube and centrifuged again at 480 x g and 22°C for another 20 minutes, again with slow start and stop motion to achieve a pellet with platelets. The supernatant was removed and the pellet was resuspended in serum free DMEM medium. Diluted 1:100 with serum free DMEM and 10 ul transferred to a Bürker chamber for counting of cells.

**Co-culture of activated platelets and fibroblasts**

The volume of serum free DMEM was adjusted to 2.7 ml in each well containing the fibroblasts. The inserts (3.0 µm pore size, Cell Culture Insert, Falcon) were placed inside the wells of the two plates that were going to contain platelets. 20 µg/ml Fibrillar collagen (Chrono-Par Collagen, Chrono-Log Corp, Havertown) was added to the inserts containing 25x10⁶ platelets in 1.7 ml serum free DMEM, which were thereby activated. Platelets from the four donors were added to inserts in duplicate, thus giving rise to eight cocultures. These were matched with eight controls without inserts/platelets (in total 16 cultures). The plates were left to rest on a vibrating platform shaker for 15 minutes prior to incubation at 37°C for 24 hours. After 24 hours supernatants from the medium covering the cells were collected and frozen at -80°C.

**Enzyme-linked immunosorbent assay**

TGF-β1 sandwich ELISA was performed with a kit (Opt-EIA, ELISA, Becton Dickson AB). The 96 micro wells were coated with Capture Antibody and left in the refrigerator over-night. On day two the wells were washed and blocked with Assay Diluent, incubated for an hour and then washed again. The standard solution, samples and the control were prepared and added in triplets to the wells and incubated for another two hours. Then they were washed and the “working detector” containing detection antibody and streptavidin-horse radish peroxide was added to each well and incubated for one hour. After the last wash, substrate solution (Tetramethylbenzidine TMB and Hydrogen Peroxide, The BD Pharmingen™ TMB Substrate Reagent Set) was added and the plate was incubated in the dark to induce the color for the spectrophotometric reading. The stop solution was added and the plate was read at 450 nm and 540 nm in a spectrophotometer (Thermo Labsystems, Multiskan Ascent, Thermo Fisher Scientific Inc.).
A schematic drawing of the ELISA plate read in the spectrophotometer can be seen below (Fig 2).

![Schematic drawing of the ELISA plate](image)

**Figure 2:** Schematic drawing of the ELISA plate with 96 micro wells. The red numbers 0-8000 are the standard concentration of TGF-β1 (0-8000 pg/ml). The green numbers one to eight shows the control group, which were not cultured with platelets. The purple numbers nine to 16 shows the samples from cocultures with platelets. Each donor was read at two triplicates as shown above. Nine and ten, 11 and 12, 13 and 14, 15 and 16 are from the same donor respectively.

The absorbance value from the 540 nm reading was subtracted from the achieved value at 450nm for each well the mean value for each standard, coculture and the control sample was calculated and added to GraphPad Prism (GraphPad Software, Inc. USA) for constructing a diagram.

**Statistical Analysis**

ELISA results are shown as mean +/- SEM. In order to compare the control group with the donor group a statistical analysis was made by unpaired t test were p< 0.05 was set for significant difference.

**Results**

Inspection of the cultures after 24 hours revealed normal morphology of the fibroblasts in all wells, indicating that they were still healthy. Interesting, fibroblasts in co-cultures appeared more densely packed than the ones without platelets (Fig 3). Concentrations of TGF-β1 in the medium covering the fibroblasts are shown in Table I. The mean TGF-β1 concentrations in cocultures were higher than in the controls, but variation was large (Fig 4).
The mean TGF-β1 concentration was 114±/− 12 pg/ml in the controls, and 393+/− 174 pg/ml in the platelet co-cultures. The difference between the groups was not statistically significant (p = 0.13).

Figure 3: Demonstrates the fibroblasts after 24 hours of incubation in the control group, which doesn’t contain any platelets (a) and the denser fibroblast in the co-cultures containing platelets, fibrillar collagen and DMEM (b).

Table 1: Shows mean TGF-β1 concentrations for the control group without platelets (1-8) and the mean TGF-β1 concentrations for the coculture group containing platelets (9-16).

<table>
<thead>
<tr>
<th>Fibroblasts in control group (2 x 10⁵)</th>
<th>TGF-β1 concentration (pg/ml)</th>
<th>Coculture of fibroblasts with platelets</th>
<th>TGF-β1 concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>119,277</td>
<td>9¹</td>
<td>1208,217</td>
</tr>
<tr>
<td>2</td>
<td>127,597</td>
<td>10¹</td>
<td>1369,327</td>
</tr>
<tr>
<td>3</td>
<td>119,277</td>
<td>11²</td>
<td>149,167</td>
</tr>
<tr>
<td>4</td>
<td>119,277</td>
<td>12²</td>
<td>49,167</td>
</tr>
<tr>
<td>5</td>
<td>49,087</td>
<td>13³</td>
<td>153,607</td>
</tr>
<tr>
<td>6</td>
<td>149,167</td>
<td>14³</td>
<td>190,097</td>
</tr>
<tr>
<td>7</td>
<td>66,007</td>
<td>15⁴</td>
<td>33,295</td>
</tr>
<tr>
<td>8</td>
<td>162,637</td>
<td>16⁴</td>
<td>-14,184</td>
</tr>
</tbody>
</table>

¹ Containing platelets from donor one
² Containing platelets from donor two
³ Containing platelets from donor three
⁴ Containing platelets from donor four
Discussion

There was evidence of increased fibroblast proliferation in the co-cultures after 24 hours of incubation, compared to in the absence of platelets (Fig 3). This is in agreement with earlier studies [15], which supports the theory that fibroblasts proliferate in the presence of platelet-derived factors such as e.g. TGF-β1 [5]. Applied clinically on a chronic wound, platelets thus are likely to improve fibroblasts that are stuck in a nonfunctioning state during the proliferation phase. This would also facilitate the production of collagen and the wound presumably restarts the healing process [4,6].

In the results from ELISA it appears that the fibroblasts had produced a certain amount of TGF-β1 (around 100 pg/ml). From this level there was a tendency towards an increase of TGF-β1 concentration in the co-culture group. However, there was a large variation between the different donors although there was a tendency towards increase of the concentration compared to the control group.

The reason for the large differences could be many, but we encountered some problems during the platelet isolation and activation process. Also when the pellet from donor four was going to be resuspended in serum free DMEM during the last step of platelet isolation, the
pellet was hard and difficult to resuspend. A probable reason for this is that the platelets had been activated too soon.

These are possible reasons for the variability in TGF-β1 concentration as showed by ELISA for the co-cultures; control levels were more stable. The results are not very dependable because of the large variations in the coculture group.

During the three co-culture trials that we did, we encountered some problems that made us stop the experiments before proceeding to ELISA measurements of TGF-β1. One issue was the origin of collagen to activate the platelets – first we used denatured collagen instead of fibrillar collagen, and this caused a gelation of the collagen in the wells. Another issue was the concentration of Ca²⁺, which was added to the platelets upon start of the cocultures - it was found that we had added 10-fold the concentration due to a dilution error. This caused the fibroblasts to die during the 24-hour cultures, more in the monocultures than in the cocultures. The whole issue was, however, unnecessary since in fact the medium in itself contain enough Ca²⁺ to activate platelets (>1 mM). Thus, in the third attempt, no extra Ca²⁺ was added along with fibrillar collagen to activate the platelets.

Considering the large variations between the different samples and mean standard error with a p value at 0.13, it could be beneficial to redo the ELISA in order to ensure accurate results before continuing with other experiment. Other secreted factors may also be relevant to measure. One such is PDGF, and it may be that this cytokine is triggering the cells to proliferate in the cocultures. Other factors may also be relevant, but the experimental set-up must first be reliable to go further with other studies on the fibroblasts response. These studies will include analysis of gene and protein expression in the fibroblasts, especially with relevance to proliferation and ECM production [10]. In particular, global approaches such whole genome microarrays and proteomics are interesting to do. Analysis of fibroblast proliferation can be performed by e.g. metabolic labeling and calculation of growth-rates [13].

**Conclusion**

Using platelet-fibroblast cocultures, we were able to detect an effect of platelets on the growth of fibroblasts. There was also a trend of increased TGF-β1 levels in platelet cocultures. The results confirm earlier studies on the effect of platelets on fibroblast growth. Further studies on fibroblasts response of platelets are called for, but overall our results, as well as others,
suggests that treatment of chronic wounds with activated platelets (e.g. platelet-rich-plasma) may be an option for improving wound healing in afflicted patients.
References


