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Interaction of human umbilical vein endothelial cells (HUVEC) with platelets *in vitro*: influence of platelet concentration and reactivity

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Abstract

Endothelialisation of polymer-based cardiovascular implants is one strategy to render biomaterials hemocompatible. The evaluation of the functionality and the confluence of an endothelial cell (EC) monolayer *in vitro* is therefore of crucial importance, because a non-functional or non-confluent EC monolayer can contribute to the failure of vascular grafts. Moreover, the comparison of different potential biomaterials regarding their ability to induce the formation of a functional confluent EC monolayer is of great value. Most of the currently reported *in vitro* studies focus on direct or indirect markers of EC behaviour. However, these studies still lack the final proof that the EC monolayer, which can be developed on polymers is confluent and functional. In this study, we investigated the suitability of an *in vitro* co-culture of human umbilical vein endothelial cells (HUVEC) with platelets to predict the functionality of an EC monolayer.

The interaction of platelets with HUVEC was evaluated depending on the concentration of the platelets in the added plasma and of the reactivity of the platelets to pharmacological stimuli. For this purpose, HUVEC were seeded in a 24 well plate. After three days of cultivation, platelets were added to the HUVEC cell culture medium to final concentrations of 200, 2,000 or 20,000 platelets/µl (n=7 each). The platelets were processed immediately after blood collection and added to the HUVEC culture after a 30 minutes resting period. As a first control, an EC monolayer just cultured with EC medium was used. As a second control EC supplemented with plasma without platelets were applied. The HUVEC monolayer was investigated microscopically after 1 hour of platelet exposition. The addition of thrombocytes to EC affected the EC adherence dependent on the initial cell seeding number of HUVEC, the platelet concentration, and also on the reactivity of platelets added. In both controls no significant EC detachment was detected.

The results demonstrated a significant influence of platelet concentration and reactivity on the adherence of EC in a static model.

Keywords: Endothelialisation, platelets, hemocompatibility, *in vitro* test system

1. Introduction

The patency of artificial vascular grafts used for replacement of atherosclerotic vessels is determined by factors such as thrombogenicity of biomaterials and intimal hyperplasia [8,31,35]. Improving the hemocompatibility of cardiovascular implants can be achieved by physical or chemical functionalisation of the material surfaces to minimise the adherence of proteins and blood cells [19,26,31]. The endothelialisation of biomaterials is another promising strategy to enhance the hemocompatibility [4]. Endothelial cells (EC) cover the inner face of blood vessels and are involved in different physiological processes such as regulation of the vessel tone thereby regulating the blood flow by secretion of vasoactive mediators (e.g. prostacyclin, NO, endothelin-1, eicosanoids) and controlling the permeability between blood and surrounding tissue [6,14,15,32,41]. Furthermore, EC possess antithrombotic features due to interfering in the coagulation cascade, suppressing the adherence and activation of platelets, stimulation of fibrinolysis, and additionally by the secretion of active agents and the expression of the glycocalix [13,38,40]. The glycocalix covers the luminal side of EC and is composed of proteoglycans, glycoprotein and glycosaminoglycan chains [21,36,39]. On average negatively charged glycocalix ensures that under physiological conditions no interaction between platelets and EC occurs [31]. Prerequisite for the undisturbed function of the endothelium is that the EC monolayer has to remain confluent and functional since injuries or endothelial denudation can initiate thrombus formation [27,38]. Hence, a developed EC monolayer on biomaterials as strategy to enhance the hemocompatibility of polymer-based materials has to be tested regarding the functionality and confluence of the accomplished EC monolayer.

The integrity and functionality of the EC monolayer *in vitro* can be influenced by the substrate material, on which the cells are cultivated [33]. For example, EC and platelet behaviour as well might be influenced by topographical formations, surface roughness or the elasticity of the substrate material [1].

In this study an *in vitro* test system in a static model was developed to investigate the confluence and functionality of an EC monolayer in a co-culture with platelets. This was performed using different numbers of HUVEC and platelets and furthermore thrombocytes with different reactivities mediated by variable storage times after blood sampling. In perspective, this model system could be used for the investigation and comparison of biomaterials regarding their qualities to support the formation of a confluent functional EC monolayer, which should enhance the hemocompatibility of polymer-based materials to overcome the limited patency of small diameter (< 6 mm) synthetic vascular grafts [31].

2. Material and Methods

To assess the impact of platelets on the detachment of EC 50,000, 20,000 or 10,000 human umbilical vein endothelial cells (HUVEC) were seeded on glass cover slips, commonly used as substrate material for HUVEC experiments, and incubated for one hour with 200, 2,000 or 20,000 fresh isolated platelets/µl in a co-culture system [22]. To analyze the platelet effect on EC *in vitro* the confluence of the HUVEC was determined microscopically. The functional efficiency of the thrombocytes was measured by induced platelet aggregation before experimental procedure. As control HUVEC cultivated in endothelial basal medium supplemented with EGM-2 SingleQuots® (EGM-2, Lonza, Basel, Switzerland) and HUVEC cultivated with plasma without platelets were used. Analysis started after three days of cultivation. According to the criteria of the Nordkem-workshop and the ethical guidelines of the Society for Clinical Hemorheology and Microcirculation blood was obtained from apparently healthy volunteers [2, 7]. The study protocol received an approval by the institutional committee of the Charité Berlin.

2.1. Cultivation of human umbilical vein endothelial cells

HUVEC (Lonza, Cologne, Germany) were cultured in EGM-2. HUVEC were cultivated at 37 °C and 5% CO_2 in polystyrene-based cell culture flasks (TPP, Trasadingen, Switzerland). For biological experiments HUVEC were first washed with PBS -/-, second trypsinised, washed and at least seeded in 24 well plates on glass cover slips with approximately $5*10^4$, $2*10^4$ and $1*10^4$ cells/well. Experimental procedure was performed with reaching 90%, 66% and 46% confluence of EC seeded initially with different concentrations. For this, the cultivation medium was replaced with fresh medium without platelets, fresh medium added with plasma alone or plasma adjusted to 200, 2,000 or 20,000 platelets/µl (n = 7). EC detachment was assessed after one hour of incubation, microscopically.

2.2. Blood collecting

The sampling of blood occurred as previously described [9]. Briefly, blood was collected by puncture of the uncongested cubital vein with slow aspiration. The blood samples were taken with monovettes (10 ml, Sarstedt) substituted with 1 ml anticoagulant citrated solution (0.106 mol/l final concentration) [5, 20]. After blood sampling the monovettes were inverted several times to mix blood and anticoagulant citrate to prevent thrombus formation.

2.3. Preparation of plasma and platelets

Platelets rich plasma (PRP) was generated as previously described [9]. In brief, after blood collecting the sample was centrifugated for 15 min at 120 g (Megafuge Heraeus, Thermo). Afterwards the supernatant was collected in a falcon tube. Before experimental procedure PRP was rested for a minimum time of 30 minutes.

Plasma without platelets was generated by centrifugation of 20 minutes with 2,000 g (Megafuge Heraeus, Thermo). Subsequently, the supernatant was collected in a falcon tube.

2.4. Reactivity of platelets

To analyse the functionality and reactivity of platelets to diverse agonists 30 minutes after resting of PRP different stimuli (ADP 200 µg; collagen 200 µg/ml; ristocetin 10 mg/ml) were added, to initiate the aggregation of the platelets. The induced platelet aggregation was detected by the change of light transmittance over a certain period of time.

2.5. Microscopical analysis of EC detachment

The assessment of the EC detachment after platelet addition was performed using Axiovert 40 C (Zeiss) in transmission (primary magnification 10x) after one hour of exposure. Per well four pictures were taken.

3. Results

3.1. Influence of the platelet concentration on HUVEC detachment

HUVEC seeded with 50,000 initially exhibited a confluence of about 90% ± 7% when cultivated with EC medium or medium completed with plasma but without platelets.

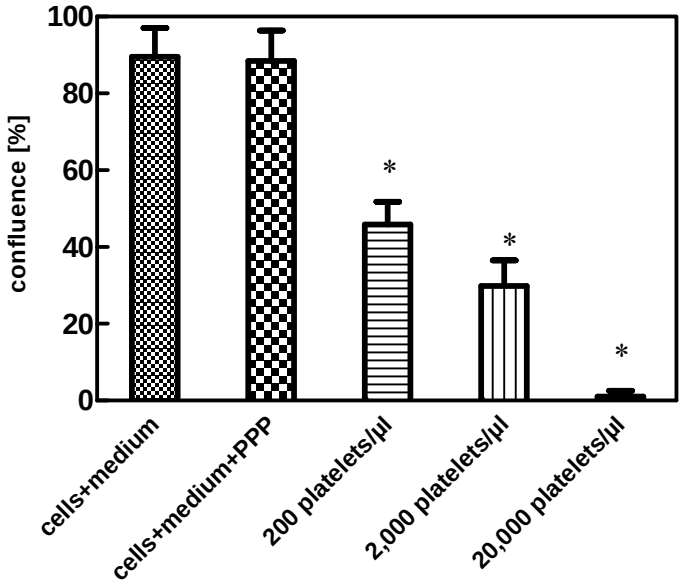

*Figure 1: Endothelial cell detachment depending on platelet concentration. HUVEC were seeded with 50,000 cells/well, initially. Platelet rich plasma was added once reaching a confluence of 90%. The platelet effect was assessed by analysis of HUVEC confluence with phase contrast microscopy. n=7, *p<0.05*

In contrast, the addition of 200 platelets/µl resulted in a detachment of HUVEC compared to control cells (45.9% ± 5% confluence, $p<0.05$). Moreover, adding 2,000 or 20,000 platelets/µl to cultivated HUVEC caused a significantly increased detachment with a confluence of 29.8% ± 6% and 1% ± 1%, respectively ($p<0.05$) In other words 49%, 67% or 99% of the seeded HUVEC detached in dependency of the platelet concentration. These results are presented in Fig.1.

An increase of HUVEC detachment was also preserved for HUVEC supplemented with 20,000 cells/well. Both controls presented a confluence of 66% ± 6% and 65% ± 6%. HUVEC substituted with 200 platelets/µl showed a confluence of 45% ± 8%, exposure of HUVEC with 2,000 platelets/µl resulted in 40% ± 7% and finally 20,000 platelets/µl in 33% ± 7% confluence. These platelet effects can be considered as significant ($p<0.05$) and are presented in Fig. 2.

HUVEC seeded with 10,000 cells/well and an initial confluence of approximately 45% ± 8% demonstrated a decline of cell confluence with 43% ± 7%, 41% ± 8% and 31% ± 6% for HUVEC supplemented with 200, 2,000 and 20,000 platelets/µl. In contrast to the other HUVEC densities mentioned before, just the addition of 20,000 platelets/µl to the cultivated HUVEC effected the adherence of the cells significant with a reduced EC monolayer of 31% ± 6% ($p<0.05$; Fig. 3).

Representative images of HUVEC and added platelets (200, 2,000, 20,000/µl) are visible in Fig. 4. The detachment of the HUVEC is apparent by the resulting cell confluence. Picture A

represents HUVEC without platelets and 90% confluence. Picture B, C and D show exemplarily 45%, 40% and 30 % cell confluence.

3.2 Analysis of platelet reactivity on HUVEC detachment

The reactivity of platelets declines over time after blood withdrawal. Therefore, it had to be assessed, whether the restricted reactivity to agonists influences the HUVEC detachment. EC confluence (initially seeded with 20,000 cells/well) was determined after co-culturing with 2,000 platelets/µl for one hour.

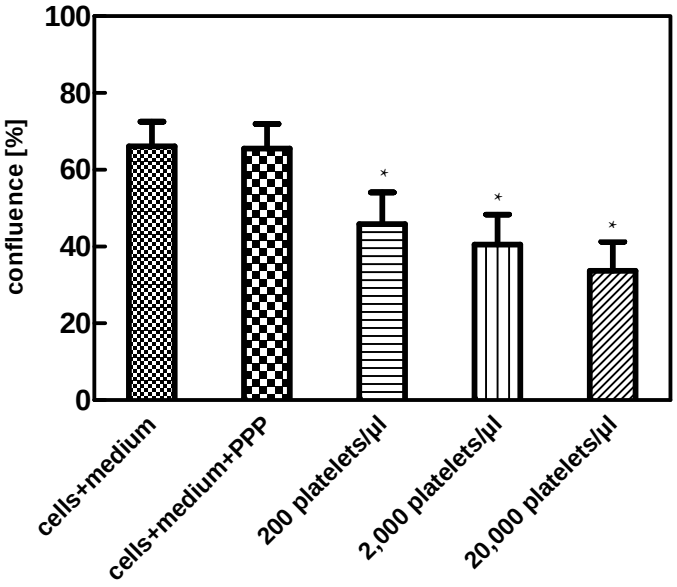

*Figure 2: Endothelial cell detachment depending on platelet concentration. HUVEC were seeded with 20,000 cells/well, initially. Platelet rich plasma was added once reaching a confluence of 66%. The platelet effect was assessed by analysis of HUVEC confluence with phase contrast microscopy. n=7, *p<0.05*

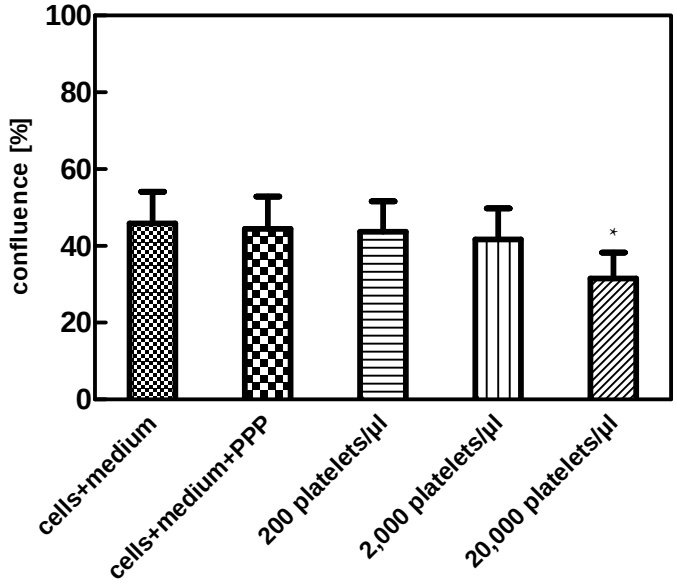

*Figure 3: Endothelial cell detachment depending on platelet concentration. HUVEC cells were seeded with 10,000 cells/well, initially. Platelet rich plasma was added once reaching a confluence of 46%. The platelet effect was assessed by analysis of HUVEC confluence with phase contrast microscopy. n=7, *p<0.05.*

Thrombocytes were added with decreasing reactivities. Prior to adding the platelets, the reactivity was determined by induced ristocetin-induced platelet aggregation (RIPA). RIPA demonstrated a platelet aggregation of 87% ± 3% for fresh isolated thrombocytes, 55% ± 12% for one hour stored platelets, 42% ± 12% for two hours stored and 34% ± 14% for three hours stored thrombocytes. The addition of platelets to the cultivated HUVEC was performed with reaching a confluence of round about 65% after three days of cultivation. The results are presented in Fig. 5.

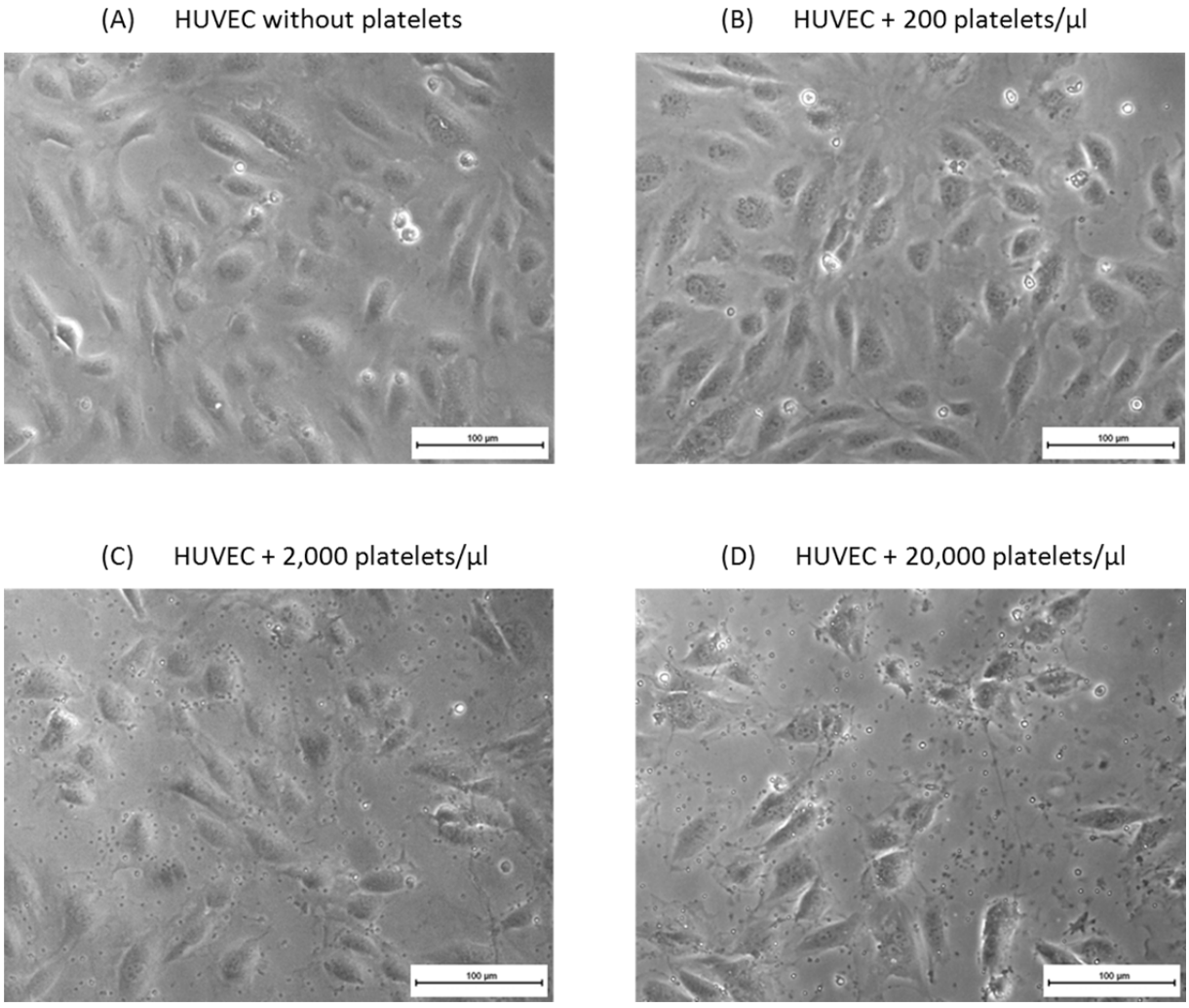

Figure 4: Representative images of HUVEC in co-culture with platelets. (A) HUVEC without platelets, (B) with 200 platelets/µl, (C) 2,000 platelets/µl, (D) 20,000 platelets/µl. Images were taken using Axiovert 40 C (Zeiss, primary magnification 20x, Scale bar: 100 µm.

After the incubation with fresh isolated platelets HUVEC demonstrated a dramatically loss of adherence leading to an analyzed confluence of 35% ± 10% and represents a deficit of nearly 50%. HUVEC added with thrombocytes stored for one, two or three hours presented a confluence of 42% ± 12%, 48% ± 15% and 54% ± 14%, which correlated with the ristocetin-induced platelet aggregation of the isolated thrombocytes.

4. Discussion

The intention of the study was the development of an in vitro system, which could be used for the assessment of endothelial cell-polymer interactions. In particular, it should allow to prove, whether EC seeded on a polymer still possess their antithrombotic capability [24]. Hence, it has to be stressed that just a functional confluent EC monolayer can maintain their diverse functions in the native blood vessel [16]. Vascular EC are located at the inner surface of blood vessels providing a nonthrombogenic surface by formation of antithrombotic and fibrinolytic structures and agents [10, 24, 32]. No interaction between platelets and EC take place in a health vascular system, although a close contact between both cell types is present in the circulation [30].

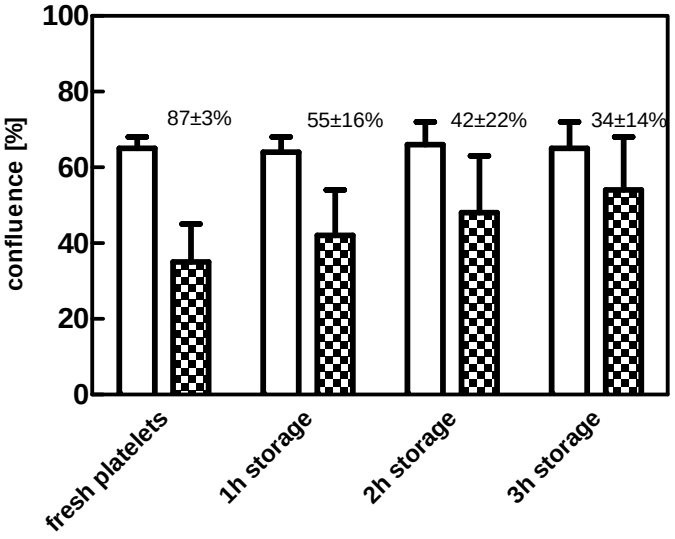

Figure 5: Effect of platelet reactivity on HUVEC adherence. HUVEC were seeded initially with 20,000 cells/well (white column). Supplementation of 2,000 platelets/µl fresh isolated, one hour, two hours or three hours stored (patterned column). RIPA is presented in %. The thrombocyte effect was assessed by analysis of the HUVEC confluence with phase contrast microscopy. n=7.

Therefore, the influence of the platelet concentration and reactivity on HUVEC was analyzed in a co-culture system. For this, HUVEC seeded with different initial numbers and in addition, platelet rich plasma with thrombocytes in various concentrations and reactivities (analyzed by induced aggregation assays) were co-cultured for one hour, thereafter, HUVEC detachment was quantified. The HUVEC adherence depended on the confluence of the HUVEC monolayer. Precisely, a confluence of 90% was associated with a higher detachment of EC compared to 66% and 45% initial EC density. Moreover, the concentration of the platelets in the PRP was also important for the HUVEC detachment. Lower EC confluences were detected with rising thrombocyte concentrations. Besides that, the impact of the platelet reactivity on HUVEC adherence was assessed. For this, the reactivity of the thrombocytes

added was characterised immediately before the experiments by ristocetin-induced platelet reactivity (RIPA). Using RIPA it could be demonstrated, that the reactivity of the platelets decreased over time as has been shown in former studies [23, 28, 34]. The lowest HUVEC detachment rate was observed for platelets with the lowest RIPA of 34% ± 14% (fresh isolated thrombocytes possessed a reactivity of 87% ± 3%). The results clearly indicate that the concentration of the thrombocytes and moreover the reactivity of the platelets were crucial influencing factors affecting the adherence of HUVEC. Platelets are reported taking part in hemostasis, thrombus formation and inflammation [27]. For this purpose the platelets have to be activated whereas different pathways are known [27]. During activation platelets release distinct soluble factors in the blood among others thrombin, ADP, serotonin, matrix metallo proteinases, fibrinogen, thrombospondine and collagenase thereby losing their property to react on agonists. Therefore, it can be assumed that the lower the reactivity of the platelets on agonists is, the higher will be the concentration of different secreted substances with the potential to influence the adherence of the HUVEC. In the *in vitro* system a close contact between HUVEC and platelets occurred due the co-culture of the two cells imitating the physical situation in native blood vessels. But, it has to be stressed that the developed experimental set up represents a static system with no blood flow. A direct consequence is the contact between both cell types normally *in vivo* separated by a thin cell free plasma layer. Due to this fact one possible implication for the detachment of HUVEC after exposure to platelets is the activation of the platelets by the direct contact to the HUVEC. This activation could induce the release of the above mentioned mediators, which then could initiate the HUVEC detachment. Collagenase secreted by platelets is involved in the proteolytic bulking of the subendothelial collagen. Therefore, this protease could be engaged in the detachment of the HUVEC observed in this study [17]. The decelerating HUVEC monolayer confluence in direct correlation to the platelet concentration is in line with this assumption. Another important detail was published by Suzuki et *al*. This group could demonstrate that a co-culture of human pancreatic cancer cell lines with activate platelets boosted the MMP9 secretion by tumor cells [37]. Matrix metalloproteinases (MMPs) are endopeptidases known to be involved in the degradation of the ECM formed by several components like collagens, elastin, fibronectin and proteoglycan and are also secreted by EC [12, 25, 37]. Potentially, the co-culture of platelets with HUVEC might induce the secretion of MMPs by EC which could then affect the lowered adherence of the HUVEC *in vitro*. In 1997 it was still mentioned that the generation of MMP2 mRNA is promoted by thrombin, which is secreted during the activation of platelets [12]. Thrombin as key enzyme in the human hemostasis is generated

also within the coagulation cascade by cleavage of the plasma protein prothrombin [29]. Formed thrombin can further promote the thrombin formation by platelet activation and is able to cleave components of the ECM such as fibronection, laminin and type V collagen [3, 18]. This modus operandi is used in the invasive growth of tumors and is in accordance to our obtained results. So the EC detachment could be a consequence of the ECM degradation mediated by thrombin, which could be formed due to activation of the platelets.

Also, fragment D, which develops during fibrinolysis as fibrinogen degradation product can affect the detachment process of EC from the substratum [11]. This degradation is as well thrombin mediated. Furthermore, it was reported that the generated fragment D induces the release of the plasminogen activator t-PA and u-PA (by EC). This would result in an increased plasmin activity. Plasmin, a known protease in the plasma, can degrade the ECM by fibrinogen cutting so that this enzyme also can be involved in the detachment of EC [11]. The release of t-PA and u-PA is further enhanced by thrombin formation [29]. All the further mentioned arguments clearly indicate in which way a co-culture of HUVEC with platelets could induce the observed EC detachment. Why platelets with a lower reactivity are associated with a decreased HUVEC detachment could presumably be explained with the fact, that previously activated platelets cannot be activated again. Besides that, mediators secreted by the platelets due to a former activation did not affect the EC due to the short half-life times. Several biochemical pathways are involved in the detachment of previously adherent EC after exposure to platelets, which will be controlled by the measurement of different parameters in future studies. To determine the EC adherence in dependency on different polymer-based biomaterials as cultivation substrates additional experiments have to be performed.

5. Conclusion

The addition of platelets to an EC monolayer in *vitro* affected the adherence of HUVEC on glass depending on the concentration and the reactivity of the thrombocytes added. Future studies shall indicate, whether the function and thereby the hemocompatibility of EC monolayers is influenced by the substrate material in static or dynamic conditions tested in co-cultures of thrombocytes and EC.

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