Platelet Adhesion and Mechanosensing on Collagen Coated Substrates

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Summary

Platelets play a critical role in maintaining homeostasis of the body. In order to protect the body from significant injury, platelets adhere to the vascular wall preventing critical vascular system failure. When the collagen is exposed on damaged endothelial cells, platelets adhere to the exposed protein and spread, increasing the size of the clot and preventing further damage to the body. For hemostatic diseases, biomechanical properties of platelets are an area of research critical to helping understand arterial diseases while also furthering the treatment of them. Platelet clot formation relies on the biomechanical aspects of the platelet surroundings as they adhere to the cell wall. It is hypothesized that an increase in the substrate stiffness on which platelets adhere to will increase the spreading area of the platelet in both static and dynamic environments. This paper shows a novel approach to better comprehend biomechanical properties of platelets relating to adhesion and spreading on varying substrate stiffness of collagen-coated polyacrylamide gels.

Chapter 1

Introduction

Platelets are cells produced from megakaryocytes in the bone marrow to aid in thrombosis and overall homeostasis of the human body. Platelets contribute to the hemostasis of the vascular system by varying mechanical aspects that are studied extensively today. When presented with vascular injury, platelets adhere to exposed adhesive domains of collagen on the extracellular matrix to induce thrombosis in the area. Additionally, platelets undergo conformational changes releasing granules and exposing phosphatidylserine (PS) to create a hemostatic plug. This adhesion of multiple platelets allows for a clot to be formed, working to prevent future hemorrhaging at the vascular injury site. When collagen on the extracellular matrix (ECM) is exposed, platelets bind to multiple molecules and proteins including laminin, fibrin, collagen, and Von Willebrand factor (vWF) to induce formation at the sight of the inury (1-3). In diseased patients, vessel wall stiffness increases, which can lead to an increased chance for cardiovascular diseases. However, how platelets adhere and spread on different substrate stiffness is not known.

Biomechanical properties of platelets are an aspect of research widely studied in the field of hematology as these properties are the basis for multiple aspects of hemostasis. Receptors glycoprotein VI (GPVI) and integrin α2β1 are the basis for platelet adhesion to exposed subendothelial proteins on the surface of the extracellular matrix. GPV1 allows for initial adhesion of the platelet to collagen substrate where integrin α2β1 is believed to have factors that attribute to the overall spreading of the platelet after adhesion (1). Once the platelets adhere to the ECM they continue to form a clot by releasing granules out of the activated platelets. Studies show that other cells can sense the mechanical environment they are attached to and therefore mechanotransduce signals to modify their activation, spreading, and adhesion. The biomechanical properties of platelets and their surroundings is not entirely understood, but

platelets, like other cells, have most of the basic cell components, therefore platelets may exhibit similar environmental mechanotransducing factors.

Previous studies have used varied substrate stiffness to show that platelets have a greater contractile force with an increase in stiffness of the substrate (2). Existing research however, does not analyze varying substrate stiffness and how platelet properties change to accommodate for the change. When platelets are presented into a dynamic environment, shear-stress on the platelets induces an increased activity of platelet adhesion. The platelet adhesion and spreading on varying stiffness of substrates in a dynamic environment allows for a comparison of the mechanosensing properties of single platelets on collagen and clot formation on the primarily adhered platelets. Research shows that there is a correlation between shear stress and the amount of platelet adhesion in a microfluidic chamber (4). These studies however have not determined all aspects of biomechanical properties of platelets flown through a microfluidic chamber leaving room to determine the mechanotransduction of platelets.

In hematology, biomechanical properties of cells and platelets allow for further understanding of varying characteristics of hemostasis and thrombosis in diseased patients. Here, we aim to learn the mechanosensing aspects of single platelet adhesion, spreading, and activity to collagen surfaces in a static aspect. Dynamically, we aim to study single platelet biomechanical properties along with clot formation in a microfluidic chamber to better understand the dynamic biomechanical properties. Along with the mechanotrasnduction of platelets, we aim to analyze the receptors and microvesicles associated with the spreading of the platelets on collagen substrates. With the results of the experiments, mechanical properties of platelets can be further understood to help in expanding the knowledge of hematological diseases and vessel stiffness associated with them.

Platelets are one of the most important factors in the formation of thrombosis in the blood. When exposed to the extracellular matrix of the cell, platelets aggregate according to certain factors allowing them to adhere to one another and the extracellular matrix creating a

hemostatic plug for vascular injury (5). Derived from megakaryocytes, platelets were not recognized as a key contributor in homeostasis of the human body until 1882. After the initial discovery, platelet formation was studied in relation to thrombus formation exclusively until the late 1900s where studies found a significant correlation between attributes of platelets and common blood disorders (6). Platelet biology and pathophysiology play a key role in understanding numerous biological functions and hemostatic diseases today.

Current research focuses on the biological properties of the platelet to determine aspects that could be used to treat hematological diseases. These properties include interaction with the extracellular matrix and the factors that contribute to the aggregation of platelets to form a hemostatic plug. It has long been accepted that the receptor GPV1 is used for initial adhesion while integrin factors of platelets are a key area of study for platelet adhesion. Integrin α2β1 was believed to be another factor in the initial adhesion of platelets on the fibronectin surface until recent studies showed that integrin α2β1 plays a pivotal role in the adhesion and spreading along with additional aggregation of the platelets (7). More studies are currently being done in order to entirely understand integrin receptors that could lead to possible therapeutic treatments for people with hemostatic diseases (8, 9). Another pharmacological aspect currently in research is the idea of an anti-platelet therapy for people currently at risk of having thrombosis. The only current treatment for this aspect of platelet formation is aspirin, but the lack of any other therapeutic ideas amounts for a large gap of knowledge that is driving research in the field (9, 10). The problem of anti-platelet therapies stems from the lack of knowledge in platelet pathophysiology such as the fundamental aspects of binding and aggregation at the site of thrombus.

An understanding of the basic molecular aspects of platelets is the key to determining the underlying causes of hemostatic diseases in the future. Current literature suggests that there are many factors that correlate to the adhesion and spreading of platelets along with the hypothesis that contact dependent aspects are the main driving cause for platelet thrombus formation (11).

Miocrovesicles and granules emitted from the platelets in contact with one or the other are said to determine features such as the size of the thrombus, contraction properties, and the time that the clot maintains adhesion in order to entirely heal the wound. Eph kinases and ephrins are some of the main factors in this close contact-dependent signaling from platelet to platelet and show aspects that institute clustering in the platelets along with interaction with the integrin sites of binding (8). These aspects are considered "late" activators of platelet formation and are still not entirely understood in the world of platelet adhesion and aggregation. Current research should be the connecting bridge from today's understandings of contact dependent aggregation of platelets into a new area of the field that allows for therapeutic medicine to be established and implemented into treatment.

Research regarding the mechanosensing traits of platelets relating to varying substrate stiffness is currently lacking. Most research correlating to this field of study was initially done during the first generation of research in the field of platelets and thrombus formation. Currently, factors that are being studied are associated with the study of aggregation and spreading of platelets in a study under flow similar to aspects presented to platelets in vivo (12). Previous studies have shown that platelets are affected by variables aspects in the vascular system. Shear stress and shear rate are influences of increased platelet aggregation and spreading when those platelets are presented with a vascular injury and exposed to extracellular matrix of the cells. These platelets have shown to congregate in the outer parts of the vessel that follows the hypothesis that more intense shear stresses are near the platelets allowing for quick activation of the platelets. In these studies, different substrates for platelets are coated in the surface to see particular effects of this change. In particular, collagen is shown to have specific receptors that help regulate the aggregation of platelets in flow. Studies on GPV1 have shown that this protein is one of the most significant factors of collagen binding (1, 3, 5). In order to determine properties of hemostatic diseases, the basis of platelets needs to be defined for situations both in

vitro and in vivo. Though research has been conducted on this subject, it is missing large parts of information that are critical for the understanding of hematological diseases.

Mechanosensing properties and mechanotransduction of platelets are currently lacking in information. Research for the basics of platelet adhesion and factors correlating to adhesion show potential for therapeutics in hemostatic injuries and diseases, but branches such as anti-platelet therapy have not developed farther in the past years. One aspect of basic biological properties that is significantly absent is the analysis of the spreading of platelets after initial adhesion. It is shown that an increased stiffness of vascular walls correlates to hemostatic diseases, but in order to treat such diseases more research should be conducted and implemented into treatments in order to allow for a pharmacological production to reduce the change of thrombosis in humans. More research can also lead to a larger understanding of the lack of thrombosis in patients along with increased risks for thrombosis of patients with specific hemostatic diseases. With research done on the biomechanical properties of platelets, new understanding of hemostatic diseases can allow for better treatment and care for patients.

Chapter 2

Materials and Methods

In order to determine the precise biomechanical traits correlating to platelet adhesion three experiments should be done. The first experiment is a static look at platelet adhesion. Polyachrylamide gels will be adhered to plasma treated glass slides in order to grow collagen on top of the surface. The glass slides are plasma treated and incubated with both saline solution followed by a glutaldehyde solution in order to maintain a connection of polyachrylamide gels. After the gels adhere to the slide, collagen is then spread along the surface of the gels with an overnight incubation at 100 μg/mL concentration. Afterwards, platelets were then isolated and incubated with collagen coated substrates for 1 hour at a concentration of 5.5×10^6 platelets/mL. Afterwards, platelets were stained with plasma membrane dye, Cellmaks Orange and imaged through confocal microscopy. In order to determine the average spreading area, Image-J software was used, determining the average spreading area of adhered platelets on varying substrate stiffness.

To further the understanding of the mechanism behind platelet adhesion and spreading, inhibitors of multiple aspects of platelets in the coagulation cascade were tester. MLCK, ROCK and latruculin A were used as a pre-treatment method to inhibit the multiple pathways of actomyosin activity in activated platelets. These platelets were treated for 1 hour prior to incubation with the collagen and then allowed to incubate. After this, the platelets were stained and compared to a control (DMSO) for the average spreading area using methods described before.

With the activation of platelets, other factors correlating to clot formation become activated in different amounts. For platelets, integrin $\alpha_{IIb}\beta_3$ is considered one of the primary adhesion factors of platelets on collagen. To test if platelets show a trend of increased integrin

$\alpha_{IIb}\beta_3$ with the similar trend of platelet spreading, PAC-1 was used to stain for the activated integrin and then the area of the intensity was measured via confocal microscopy and compared to the intensity of the lower level.

Finally, phosphatidylserine exposure is a main indicator of the level of activation in platelets. In order to test this activation, platelets were stained with Annexin-V and quantified under confocal microscopy to obtain the amount of PS exposure per platelets.

Chapter 3

Results

For all stiffnesses, platelets adhered and spread showing no difference in the number of platelets on the gel for all of the varying stiffness. Platelets adhered at a rate of 5000 to 9000 platelets/mm^2 per 1 hour of incubation. Though the number of platelets didn't change, the properties of these platelets did. There is a significant increase of platelet spreading for gels greater than 5.0 kPa compared to stiffness of less than 5.0 kPa. These platelets spread to an area of 30-40 μm^2 on the softer gels while spreading on stiffer gels at a range of 50-60 μm^2 showing significance in the spreading area.

In order to determine the activation of the platelets spread on collagen coated substrated, actomyosin activity was inhibited. Pathways involving MLCK and ROCK were inhibited using ML-7 and Y-27632 respectively for one hour before incubation with collagen coated gels. Treatment with ML-7 completely repressed the stiffness mediated spreading of platelets on PA gels. On the other hand though, Y-27632 increased the stiffness mediated spreading of platelets where platelets adhered to the gels and spread significantly more compared to the control DMSO. This shows that platelet spreading is activated through the MLCK signaling pathway found in platelets. Additonally, platelets were inhibited with latrunculin A to inhibit actin polymerization. This showed a decrease similar to that of ML-7 showing an additional regulation of platelet spreading by actin polymerization and therefore proves actomyosin pathways are involved in substrate-stiffness mediated platelet spreading and adhering.

Platelet activation and adhesion to collagen induces a calcium depedent activation of the platelets to change the shape and size of each platelet. Due to importance of calcium intake to platelet adherence and spreading, calcium levels were tested to confirm a difference in the platelet adherence and spreading. Results of varying calcium concentrations show that a lower

concentration of calcium significantly increased the spreading area of platelets on all substrates compared to the control. Additionally, a higher concentration of calcium inhibited the spreading of platelets on all substrates also causing platelets increased activation and bleb formation.

Finally, outside-in signaling of platelet adherence and spreading was tested through the activation of two proteins. Both aIIb3 integrins and exposed PS were used to determine the activation state of platelets. As they bind to the exposed collagen, platelets activate individual aIIbb3 integrins. After adhesion, the activation of a platelet ends in the exposure of PS on the outside of the membrane as a biomarker to determine the activation of each platelet. To test integrin activation, PAC-1-FITC binds to activated integrins and fluorescence was quantified for each stiffness. The intensity of the fluorescence did not change from stiffness to stiffness showing that the initial adhesion to the substrate does not affect the spreading. Conversely though, PS exposure of adherent platelets increased with increasing substrate stiffness. On 0.5 kPa gels, 10% of platelets exposed PS while 5 and 50 kPa gels showed a percentage higher than 40-50 %.

Chapter 4

Discussion

In order to obtain the results present in this paper, platelets were adhered to Type-I rat collagen on varied stiffness PA gels. These gels allowed for a controlled environment of regulated microenvironments mechanics for the platelets to adhere to and spread. In doing this, the adhesion of collagen was not affected and was administered to the gel surface at a constant density of 100 μg/mL. This was made possible by the manipulation of the PA gels due to the crosslinking density of the solution, which in turn does not change the overall gel mechanical properties, and changes only the stiffness of the gel. Therefore, the gels allowed for a separation of biochemical cues normally found in the subendothelium layer and instead focus on the mechanotransduction factors associated with the platelets and the substrate stiffness. In order to obtain the varying stiffness of the gels, crosslinking manipulation of the polyacrylamide network allows for the gels to obtain varying stifnesses from ranges of 0.25 kPa to 100 kPa used in the study.This environment setting allows for the focus to be solely on the interactions of collagen, platelets, the microenvironment stiffness, and the mechanotransduced signals between the three.

When platelets are exposed to subendothelial collagen, the platelets begin to adhere and spread on the substrate surface. In our experiment, the platelets spread on collagen coated PA gels with varying results. We found that for stiffness of PA gels below 5.0 kPa, platelets did not spread as much compared to the stiffer gels. This shows us that the individual factor of environmental stiffness can cause the platelets to react and respond differently. Therefore, platelets must mechanotransduce the signals of its environment to be able to respond to different situations. These signals are completely separated from the biochemical factors that are already known and therefore using this information, platelet activity can be better predicted in situations where there is known to be stiffer arterial walls such as in disease patients, such as ones with atherosclerosis, and with increased age. The implications of our findings, while helping to better

understand a diseased state, also help in the understanding of a development of a clot and mechanical pathways involved in platelet activation, spreading, and adhesion.

These mechanical pathways involved in platelet adhesion and spreading were tested against each other. Our findings showed that specific myosin actin and actin polymerizations pathways involved with MLCK inhibition help to mediate the spreading of adhered platelets on our collagen coated PA gels. ROCK inhibition, on the other hand, did not have this same effect but rather increased the platelet spreading area for softer gels compared to the control DMSO. This implies that though MLCK inhibition actin pathways invoke decreased spreading, a different pathway helps to create more spreading on varied PA gels. Another aspect of the mechanisms of platelet spreading is the Ca2+ component. In order for platelets to use the myosin actin pathway to spread, platelets rely on Ca2+ to help in the process of activation and spreading. We showed that there was a trend of decreased amount of Ca2+ to increase amount of spreading for adhered platelets. This result indicates that Ca2+ plays a crucial role in the contractile forces found in platelet activation. Without the presence of Ca2+, the lack of calcium inhibits the ability for the platelet to contract and bleb, instead letting the platelet spread greater than regular. With an increase in Ca2+, the myosin actin pathway contracts the platelets with a greater contractile force than in a normal concentration of Ca2+ therefore indicating that Ca2+ mediates platelet contractile forces and therefore helps to regulates platelet spreading. With both PAC-1-FITC staining along with Annexin V, our results showed that platelet activation is not closely related to αIIbβ3 integrins but is mediated by a different pathway different to that of PAC-1.To that end, substrate stiffness does not mediate the αIIbβ3 integrins to an activated state but rather is only deterministic on initial platelet adhesion. For Annexin V, the PS exposure, procoagulant activity is shown to greatly increase as the platelet becomes more activated. The flippase of the PS from the inner of the cell to the outside indicates that an increase in thrombin production will occur along with the production of more granules and vesicles. These actions promote the creation of a

clot and the attraction of other platelets to the already adhered platelets. As activation increases, platelets tend to spread and attach to one another forming a hemostatic plug to prevent injury.

Our results prove that with increased substrate stiffness, platelets will continue to spread at a greater area than on softer gels (<5.0 kPa). In doing this, the signals are mechanotransduced into the platelet determining different characteristics of the platelet. Myosin-actin and actin polymerization determine the spreading of the platelet along with the inclusion of Ca2+ creating a mechanistic pathway to determine size and spreading. Platelet activation is determined by substrate stiffness which is shown by the increased PS exposure as the substrate stiffness increase. By understanding the underlying pathway and characteristics of platelets when exposed to purely mechanical factors, a better understanding of the mechanical signals involved in platelet activation can help to better understand platelet activation, spreading, and adhesion in vivo. Also, the implications of our study will help to better understand the effects of diseases with increased vessel wall stiffness along with the effect of increased stiffness with age. In the identification of how platelets react to varying substrate stiffnesses, we determined platelet characteristics for better understanding of clot formation and hemostasis along with determining the mechanotransduction factors related to platelet activation, spreading, and adherence.

Altogether, this study shows the ability of platelets to sense their surrounding environmental factors. The mechanical properties associated are mechanotransduced through the platelets to change the overall physiology of the clot. Platelets are driven by varying stiffness to change activation of the platelet along with adhesion and spreading on collagen. Additionally, platelets experience a stiffness-mediated signaling, which is mediated by actin polymerization pathways of the cell. These affects are increased by the reduction of Ca^{2+} in the extracellular space. With these findings, there is a better understanding of platelet activation and spreading and how substrate stiffness mediates mechanotransduction of this environment. The results also show the implications of how stiffened arterial walls could lead to increased risk for thrombosis with an increase in age and with atherosclerosis. Additionally, the mechanisms for platelet

spreading and activation were explored to show the similarity of platelets to other cell types in the ability to mechanotransduce signals for the surrounding environment. Further studies will show a better understanding of platelet spreading and activation in a more dynamic flow environment while also understanding more in depth on the overall characteristics of platelet spreading and activation.

Appendix A

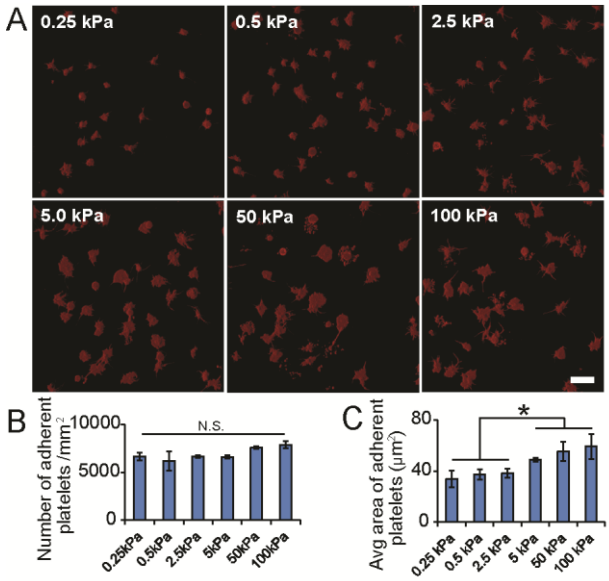

Figure 1: Substrate stiffness mediates platelet adhesion but not platelet spreading on collagen

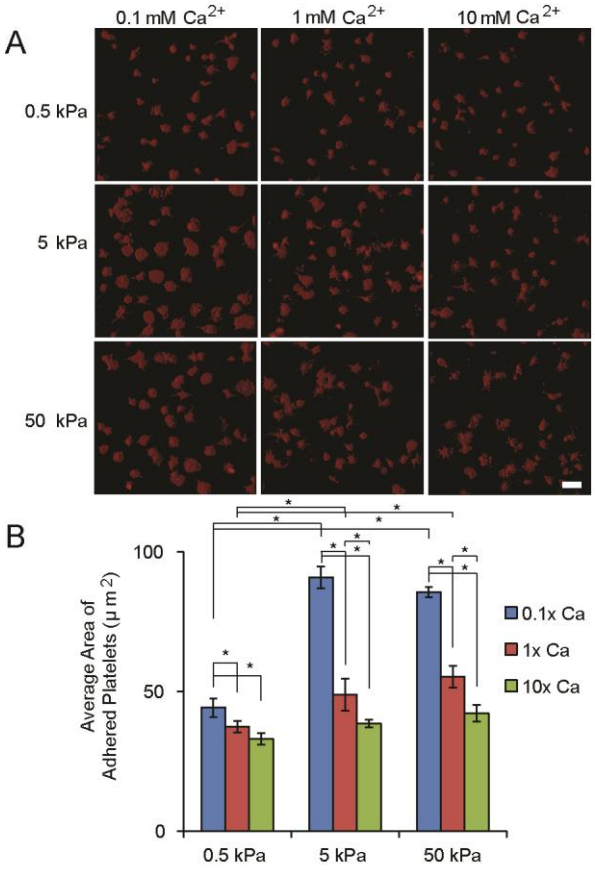

Figure 3: Extracellular Ca^{2+} concentration affects substrate stiffness mediated spreading on collagen.

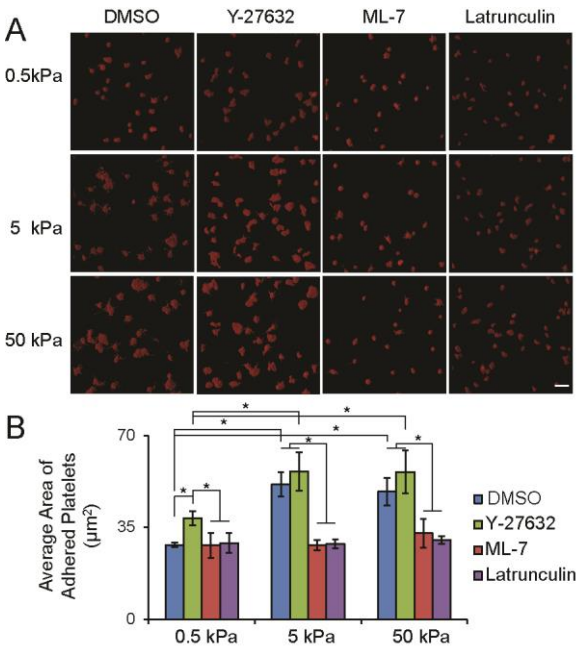

Figure 2: Substrate stiffness mediated platelet spreading on collagen is regulated by actomyosin activity.

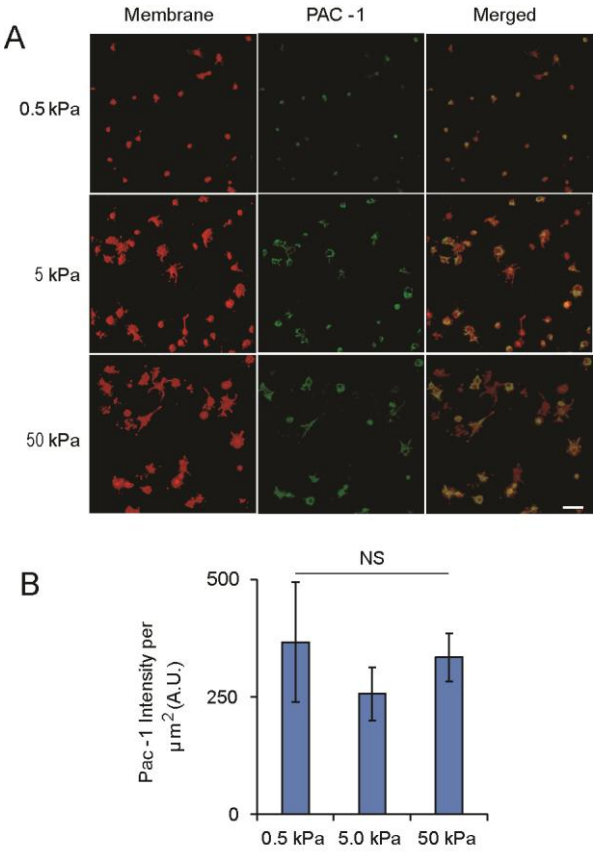

Figure 4: Integrin $\alpha_{IIb}\beta_3$ activation of adherent platelets on collagen is not mediated by substrate stiffness.

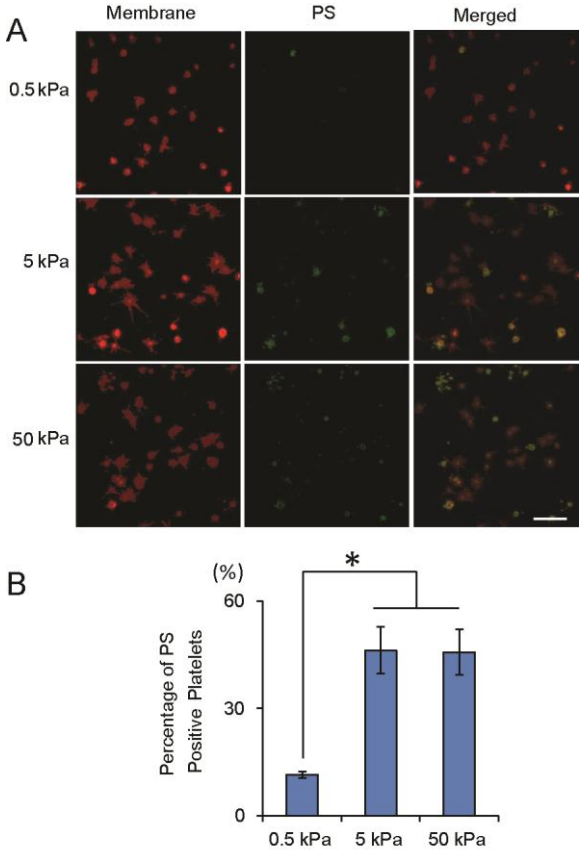

Figure 5: Substrate stiffness mediates PS exposure of adherent platelets on collagen.

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