PLATELET CONTRACTILE FORCE ASSAY USING MICROPOLTS AND THE
ROLE OF NONMUSCLE MYOSIN IIA REGULATION

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ABSTRACT
In this paper, the critical role of myosin IIA in platelet force generation is demonstrated using a novel force measurement tool. Microfabricated arrays of flexible posts were used in combination with pharmaceutical inhibitors to assay platelet forces. The results demonstrate a promising approach to identify pharmaceutical drugs that aid in treatment of platelet disorders and cardiovascular diseases.

KEYWORDS: Microposts, myosin IIA, platelets, soft lithography

INTRODUCTION
The ability for platelets to generate force is critical in the formation of platelet aggregates and their ability to maintain hemostasis [1]. However, few technologies exist that can quantify the generation of platelet forces during clotting, which is an important diagnostic in trauma medicine or cardiovascular disease. We have recently developed a technique to measure platelet forces using arrays of microposts [2]. In this paper, we report an advancement of this technique by demonstrating its ability to assay pharmacological inhibitors of nonmuscle myosin IIA for investigating its role in platelet force generation. Microposts offer more precision than macroscale techniques to measure platelet forces like thromboelastography[3] or retractometry [4] because microcontact printing can be used to control the platelet-matrix interactions and the microscale dimensions of the posts reduce the effect of the surrounding fibrin meshwork in obscuring the measurement of platelet forces. In addition, our technique can be used to screen pharmacological inhibitors in a high throughput manner, which enables significantly more data points than previous work on measuring platelet forces using atomic force microscopy [5].

In this study, pharmacological inhibitors - ML-7, Y-27632 and blebbistatin - were used to interfere with myosin activity. Phosphorylation of myosin regulatory light chain (MLC) causes a conformational change in the structure of myosin that allows individual myosins to assemble together as bipolar filaments. These bipolar filaments can then bind with actin to generate cytoskeletal tension through myosin’s ATPase activity [6]. Biochemical regulators of myosin phosphorylation – Rho kinase (ROCK) and myosin light chain kinase (MLCK) – are essential for platelets to generate force (Fig. 1). Specifically, we used Y-27632 to inhibit ROCK, ML-7 to inhibit MLCK, and blebbistatin to inhibit myosin’s ATPase activity.

METHODS
An array of vertical microposts that were 6 µm tall and 2.3 µm in diameter and spaced 9 µm apart were used in this study. The array were made from polydimethylsiloxane (PDMS) through a replica-molding process from a SU-8 silicon master that has similar sized features of microposts. Soft lithography was used to replicate the features of SU-8 silicon master multiple times so that many experiments with platelets could be performed with identical arrays. Fibronectin was adsorbed to a flat PDMS stamps for 1 hour, dried with nitrogen, and then placed in conformal contact with the tips of the microposts in order to transfer the protein onto the tip of the posts (Fig. 2). Prior to stamping, the microposts were treated with ozone for 7 minutes to enable protein transfer. The next step was to label the array with a fluorescence dye in order to allow for fluorescence microscopy imaging; for this purpose micropost arrays were stained with conjugated BSA-AF594 for 1 hour. A solution of 0.2% Pluronic F-127 was used to block the sidewalls of the posts as well as the base of the array from additional protein adsorption. When platelets were later added to the arrays, they attached onto the tips of the posts and formed a clot structure.
Bio-functionalized substrates were submerged in Tyrode buffer. Platelets were then added to the buffer and allowed to settle to the bottom for 30 minutes in order to form aggregates. After a washing step to remove the unattached platelets, thrombin (1U/ml) was added for 30 minutes to activate the platelets. Each myosin inhibitor was added for 15 minutes and then the platelets on the array of microposts were immediately fixed and stained.

Each micropost acts as a simple cantilever and the force of the platelets can be detected by the deflection of the tip of the micropost (Fig. 3). Deflection of each post was measured using fluorescence images obtained from the tip and base of the substrate for each particular aggregate. A MATLAB code was used to analyze the deflection of the microposts and calculate the platelet forces based upon the known spring constant of the microposts in the array.

Results and Discussion

Two distinct pathways are involved in the regulation of actin-myosin interactions and cell contraction. One of which is through MLCK activity and the other is through ROCK activity [7]. In this work both kinases were inhibited to investigate their individual effect on the platelet aggregate force generation.

ROCK activation results in MLC phosphorylation: the kinase can directly phosphorylate MLC and can also inhibit MLC-phosphatase [7]. To investigate the effect of myosin phosphorylation on the platelet forces, Y-27632 at different concentrations was used to inhibit ROCK. The strength of the forces on the microposts by the platelets decreased as the concentration of Y-27632 was increased, demonstrating the importance of myosin activity and ROCK in the process of force generation in platelet aggregates (Fig. 4A).

Similarly, the role of MLCK was investigated by inhibition of the kinase using ML-7 [8]. MLCK can directly phosphorylate MLC in a manner that is independent of ROCK activity. Different concentrations of ML-7 were used in the assay and platelet forces were observed with decrease with the concentration of ML-7 (Fig. 4B). Together, these results with Y-27632 and ML-7 provides a strong evidence that inhibition of myosin activity affects the force-generating activity of the actin-myosin contractile system in platelet aggregates.

Finally, blebbistatin was used to inhibit actin-myosin force generation by affecting myosin’s ATPase activity. Blebbistatin can bind to the large cleft of myosin, which stabilizes the binding of ADP to myosin and prevents the turnover of ATP that is necessary for its myosin’s motor activity [9]. Similar to before, different concentrations of blebbistatin were used and platelet forces were measured using microposts. Platelet forces reduced as the concentration of blebbistatin was increased (Fig. 5). These results confirm that inhibiting myosin’s ATPase activity leads to reduced forces in platelet aggregates.
CONCLUSION
Together, this data aids in understanding the critical role of myosin IIA in platelet force generation. Furthermore, a new technology was used in combination with pharmaceutical inhibitors to assay platelet function, which demonstrates a promising approach to identify pharmaceutical drugs that aid in treatment of platelet disorders and cardiovascular diseases.

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REFERENCES

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