Journal of Biomechanics 47 (2014) 3400–3407

Contents lists available at ScienceDirect

Journal of Biomechanics

journal homepage: www.elsevier.com/locate/jbiomech
www.JBiomech.com

Alteration Young's moduli by protein 4.1 phosphorylation play a potential role in the deformability development of vertebrate erythrocytes

Fuzhou Tang, Xiaofeng Lei, Yanlian Xiong, Ruofeng Wang, Jinchun Mao, Xiang Wang *

Chongqing University Biomedical Engineering, Chongqing, PR China

ARTICLE INFO

Article history:
Accepted 16 July 2014

Keywords:
Young's modulus
Vertebrate erythrocyte
Protein 4.1 phosphorlation
Membrane skeleton protein affinity
Evolution of erythrocyte deformability
Amino acid sequences

ABSTRACT

The mechanical properties of vertebrate erythrocytes depend on their cytoskeletal protein networks. Membrane skeleton proteins spectrin and protein 4.1 (4.1R) cross-link with actin to maintain membrane stability under mechanical stress. Phosphorylation of 4.1R alters the affinity of 4.1R for spectrin–actin binding and this modulates the mechanical properties of human erythrocytes. In this study, phorbol 12-myristate-13-acetate (PMA)-induced phosphorylation of 4.1R was tested, erythrocyte deformability was determined and the erythrocyte elastic modulus was detected in human, chick, frog and fish. Furthermore, amino acid sequences of the functionally important domains of 4.1R were analyzed. Results showed that PMA-induced phosphorylation of 4.1R decreased erythrocyte deformability and this property was stable after 1 h. The values of Young's modulus alteration gradually decreased from human to fish (0.388 ± 0.035 kPa, 0.219 ± 0.022 kPa, 0.191 ± 0.036 kPa and 0.141 ± 0.007 kPa). Ser-312 and Ser-331 are located within the consensus sequence recognized by protein kinase C (PKC); however, Ser-331 in zebrafish was replaced by Ala-331. The sequence of the 8 aa motif from vertebrate 4.1R showed only one amino acid mutation in frog and numerous substitutions in fish. Analyses of Young's modulus suggested that the interaction between 4.1R with the spectrin–actin binding domain may have a special relationship with the development of erythrocyte deformability. In addition, amino acid mutations in 4.1R further supported this relationship. Thus, we hypothesize that alteration of membrane skeleton protein binding affinity may play a potential role in the development of erythrocyte deformability, and alteration of Young's modulus values may provide a method for determining the deformability development of vertebrate erythrocytes.

1. Introduction

The erythrocyte membrane cytoskeletal protein 4.1R, spectrin, and actin are crosslinked to form the ternary protein complex. This complex binds to the membrane protein, glycophorin, as well as to anion transporters and membrane phospholipids, and these complexes play crucial roles in maintaining the deformability and mechanical integrity of vertebrate erythrocytes (Mohandas and Chasis, 1993; Salomao et al., 2008). Phosphorylation of 4.1R modulates spectrin and actin affinity, and leads to decreased membrane mechanical stability (Manno et al., 2005). Phosphorylation of 4.1R in erythrocytes from people with essential hypertension, senescent erythrocytes, sickle erythrocytes and in erythrocytes from patients with chronic myelogenous leukemia has been shown to decrease the membrane mechanical stability (Apovo et al., 1989; Kundu et al., 1989; Postnov et al., 1988). Limited chymotryptic digestion of the human erythrocyte membrane 4.1R generates 30 , 16 , 10 , and 22/24 kDa fragments (Conboy, 1993). The 30 kDa N-terminal domain (FERM) binds to several proteins including the transmembrane proteins, glycophorin C (Gascard and Cohen, 1994) and band 3 (Lombardo et al., 1992). The phosphorylation of Ser-312 in the 16 kDa domain by PKC modulates interactions between the adjacent FERM and spectrin–actin binding (SAB) domain (Gauthier et al., 2011; Manno et al., 2005). The 10 kDa domain is associated with the SAB domain, working as the binding site for spectrin and actin (Hemming et al., 1995). The C-terminal 22/24 kDa domain interacts with the immunophilin FK506-binding protein, and other proteins (Walensky et al., 1998).

* Correspondence to: Key Laboratory of Biorheological Science and Technology, Ministry of Education, College of Bioengineering, Chongqing University, Chongqing 400030, PR China. Tel.: +86 23 65112877; fax: +86 23 65112877.
E-mail addresses: tangfuzhou@163.com (F. Tang), leixiaofeng0915@126.com (X. Lei), xyl0207@gmail.com (Y. Xiong), 394218768@qq.com (R. Wang), 243447033@qq.com (J. Mao), xwangchn@vip.sina.com (X. Wang).

http://dx.doi.org/10.1016/j.jbiomech.2014.07.022
0021-9290/

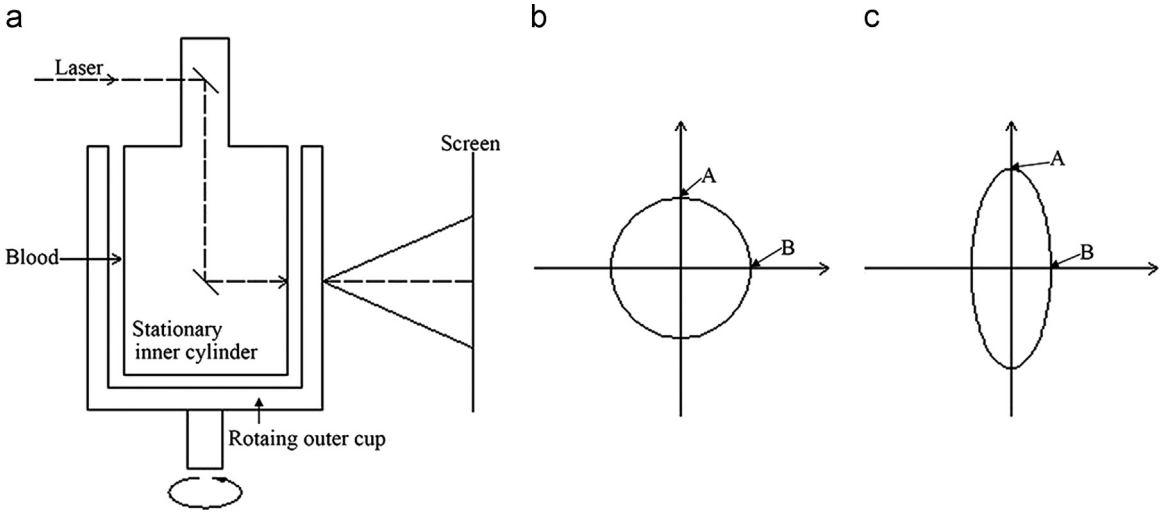

Fig. 1. Experimental setup of ektacytometer (a) and the diffraction pattern at no shear stress (b) or shear stress (c).

4.1R isoform diversity is regulated by extensive alternative splicing of 4.1R pre-mRNA (Conboy et al., 1991). In chick erythrocytes, the 77 kDa and 87 kDa 4.1R variants are dominant in immature erythroblasts, whereas the 100kDa and 115 kDa variants, which predominate in mature erythrocytes, result from terminal differentiation of such cells (Granger and Lazarides, 1984; Yew et al., 1987). The 56 kDa and 37 kDa 4.1R isoforms are present in *Xenopus laevis* oocytes, where they function as linker proteins between cytokeratin and the actin-based cytoskeleton (Carotenuto et al., 2009). In zebrafish erythrocytes, the 51, 25, and 14 kDa 4.1R isoforms are expressed, while the 30 kDa isoform binds uniquely to transmembrane proteins and calmodulin (Nunomura et al., 2007). Previous studies have documented the interactions between 4.1R and other cytoskeletal proteins in *X. laevis*, chick, and fish (Carotenuto et al., 2009; Granger and Lazarides, 1984; Nunomura et al., 2007). Selectively expressed 4.1R forms a ternary complex with spectrin and actin in erythroid cells. However, an important feature of skeletal protein expression is its high conservation between nucleated amphibian and enucleated mammalian erythroid cells (Winardi et al., 1995). Nucleated vertebrate erythrocytes have a protein kinase messenger expression system wherein PKC, cAMP-dependent protein kinase-induced phosphorylation, mediates membrane–cytoskeleton interactions and calcium–calmodulin interactions. Membrane protein phosphorylation modulates interactions between these membrane skeleton proteins, which alter skeletal protein function and decreases erythrocyte deformability (Proszkowiec-Weglarz et al., 2006; Saneyoshi et al., 2003).

PKC can directly alter the biomechanical properties of erythrocytes. From a biomechanics perspective, changes in the biomechanical properties of erythrocytes can reflect alterations in the erythrocyte skeleton and cellular membrane structure (Lim et al., 2006). The Young's modulus of enucleated mammalian erythroid cells and nucleated erythrocytes can be measured using atomic force microscopy (AFM) based on the Hertz model. AFM is used to measure the stiffness of cells firmly adhered to a substrate and to analyze the mechanical properties of the cells (Radmacher et al., 1996). Previous studies have documented a relationship between Young's modulus and the actin network of the cell (Sato et al., 2007; Kuznetsova et al., 2007). Rotsch et al. (1997) reported that chemical disassembly of the actin network through use of cytochalasin B and latrunculin A decreased the cell's elastic modulus. In contrast, nocodazole or colcemid treatment of fibroblasts resulted in a significant increase in their elastic modulus (Wu et al., 1998). Changes in cytoskeleton organization and the mechanical properties

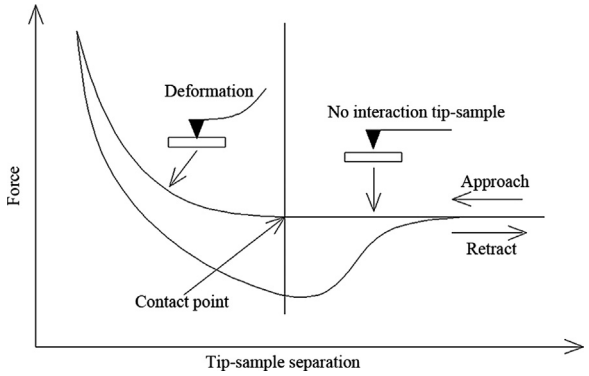

Fig. 2. Principle of measuring cellular mechanical properties with AFM probe.

of cells appear to be correlated with Young's moduli. Increases in Young's modulus can be caused by changes to the actin network; therefore, changes in Young's modulus can reflect alterations to the actin network structure of a cell. Manno et al. (2005) reported that phosphorylation decreased the affinity of P-4.1R to spectrin and actin. The decreased affinity of the protein-protein interactions induced a reduction in membrane cohesion, as shown by decreased mechanical stability of the membrane. Therefore, in the present study, changes in Young's modulus values were used to monitor alterations in the membrane skeleton protein binding affinity.

In this biomechanics study, AFM and amino acid sequence analysis were employed to investigate the relationship between alterations in the Young's modulus of vertebrate erythrocytes (modulated by PKC) and the development of erythrocyte deformability.

2. Methods

2.1. Erythrocytes collection

Human blood samples were collected from healthy volunteers, who provided their written informed consent to participate in the study. Animals used in this study were maintained in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). Blood samples from human and three other vertebrates were collected separately in heparinized tubes. Heart puncture was used on frogs, whereas the large vein in the wing of the chicks was used to extract samples. Fish blood was collected from the arteria caudalis by cutting the tail. The α-cellulose–Sigma Cell column was used to remove white cells and platelets.