Detection of erythrocytes in patient with elliptocytosis complicating ITP using atomic force microscopy

Xiaobo Xing\textsuperscript{a}, Hua Jin\textsuperscript{a}, Yuhong Lu\textsuperscript{b}, Qiulan Wang\textsuperscript{a}, Yunlong Pan\textsuperscript{b}, Jiye Cai\textsuperscript{a,\ast}, Haiyan Wang\textsuperscript{c}

\textsuperscript{a} Department of Chemistry, Jinan University, Guangzhou 510632, China
\textsuperscript{b} The First Affiliated Hospital, Jinan University, Guangzhou 510632, China
\textsuperscript{c} Guangzhou Institute of Measurement & Testing, Guangzhou 510030, China

\section*{A R T I C L E  I N F O}

Article history:
Received 9 August 2010
Accepted 10 August 2010

Keywords:
Erythrocyte
Elliptocytosis
Idiopathic thrombocytopenic purpura (ITP)
Atomic force microscopy
Morphologic information

\section*{A B S T R A C T}

The pathological changes of erythrocytes were detected at the nanometer scale, which was important for revealing the onset of diseases, early diagnosis, and effective therapies. Diseases may disturb the morphology and function of erythrocytes at molecular scale. There were dramatic surface deformations in topography of erythrocytes from a patient with elliptocytosis complicating idiopathic thrombocytopenic purpura (ITP). The overall shape and surface membrane of the healthy, pre- and post-therapeutic erythrocytes have been studied by high-resolution atomic force microscopy imaging. The results showed that we can detect healthy and pathological erythrocytes by the morphologic parameters of the length, width, ratio of length to width, peak, valley, valley-to-peak, surface fluctuation, and standard deviations of the erythrocytes. Therefore, the morphologic information of erythrocytes is very important indicator for diagnosing the healthy and disease, as well as evaluating therapeutic effect.

Crown Copyright © 2010 Published by Elsevier Ltd. All rights reserved.

\section*{1. Introduction}

Alterations of biophysical properties of blood cells contribute to the pathophysiology of hematologic diseases (Eaton and Hofrichter, 1995; Wilbur et al., 2007). Erythrocytes are a major component of blood for human, which transport oxygen to all parts of the body through hemoglobin. The shape and mechanical properties of the erythrocytes are determined by their membrane skeleton, a dynamic network of proteins associated with the plasma membrane. The deficiencies or defects in membrane skeletal proteins will disorder the morphology and function of the erythrocytes. Hence the typical biconcave shape of the erythrocytes can be altered by the effects of the pathophysiology (Jin et al., 2010). Therefore, any method to diagnose the onset of hematologic diseases must detect early subtle changes in the morphologic information of the erythrocytes at molecular scale before significant pathology occurs.

Elliptocytosis is a hemolytic disease related to defects within the erythrocyte membrane skeleton proteins such as spectrin and protein 4.1 (Baines, 2008; Dhermy et al., 1998; Nicolas et al., 1998). The resulting instability of the membrane skeleton can be detected by altered morphology and biophysical deformability of the erythrocytes. Adult idiopathic thrombocytopenic purpura (ITP) is an acquired organ-specific autoimmune hemorrhagic disease. The thrombocytopenia of ITP is mainly attributed to early destruction of platelets by the activated reticuloendothelial system, following their sensitization by antiplatelet glycoprotein autoantibodies. The presence of antibodies against platelet glycoproteins has traditionally been considered to play a central role, and several abnormalities involving the cellular mechanisms of immune modulation have been confirmed (Cines and Blanchette, 2002; McMillan, 2000). As a result, a patient with elliptocytosis complicating ITP would disorder the architecture and functions of the erythrocytes. Hence, the ability to detect erythrocytes of a patient with elliptocytosis complicating ITP at ultra-high resolution is very important for monitoring the progression of disease and developing effective therapies at molecular scale.

Owing to its ability to observe, manipulate and explore the functional components of the biological cell at nanometer resolution, atomic force microscopy (AFM) has emerged as a powerful tool for exploring biological structures at the molecular level, thereby contributing to improve our molecular understanding of their structure–function relationships (Stolz et al., 2009; Müller and Dufréne, 2008). For human, the morphological and biomechanical properties of their erythrocytes may be important in explaining the etiology of certain pathological situations (Jin et al., 2010). In this study, the morphological properties of erythrocytes from a patient with elliptocytosis complicating ITP are detailedly characterized at nanometer scale using AFM. The aim of the research is to develop a method for surface membrane parameter assessment, to work out the criteria for morphologic data comparison, and to use the method to evaluate the morphologic
parameters of erythrocytes for the healthy, disease, and therapeutic effect.

2. Materials and methods

2.1. Sample preparation

Blood samples were prepared from a 21-year-old female patient at the First Affiliated Hospital of Jinan University, with elliptocytosis complicating ITP. The clinical data is shown in Table 1. We selected three groups of blood samples: Group 1, healthy, control group, age 20–30; Group 2, pre-therapy; Group 3, the third month post-therapy. Each blood sample was separated into 3 parts: one part was used to take blood routine test, another was used to the Wright's stain, the third was diluted in phosphate buffered saline solution (pH = 7.4) and was introduced to freshly cleaved mica surfaces and then fixed with 1% glutaraldehyde for 10 min that also prevented the aggregation of spectrin–actin network elements of erythrocytes (Liu et al., 2005) drying in air for AFM scanning.

2.2. AFM measurement

AFM is a unique technology which offers cell topography analyses at nanometer scale (Duvshani-Eshet et al., 2006). An Autoprobe CP AFM (Veeco, USA) in contact mode widely used to measure the topography of cells (Dufrêne, 2008; Lamprecht et al., 2009; Jaroslawski et al., 2009), was applied to detect the immobilized erythrocytes at room temperature. Gold-coated silicon nitride tips (UL20B, Park Scientific Instruments) with a spring constant of 2.5 N/m and tip diameter of 20 nm were employed in all AFM experiments. The AFM cantilever and nearby cells could be visualized simultaneously by a microscope to locate the regions of interest. Single-cell imaging was repeated for ten cells, and each cell was scanned for three times. The instrument-equipped software IP 2.1 was performed to analyze the topography of the surface cell membrane. The analyzed area with AFM is 15 μm × 15 μm. The length (L) and width (W) means the maximum and minimum values of cell diameters, respectively. The peak (H) and valley (h) value means the maximum and minimum height values of the cell membrane surface, respectively. The peak-to-valley value ($R_{p-v}$) defines the difference between the maximum and minimum values of the cell surface in the analytical area. $R_s$ means the average surface fluctuation of the erythrocyte. The morphologic parameters between the different groups were compared using t test (SPSS 11). Means with $P$ values less than 0.05 were considered to be significantly different.

All data were mean values ± standard deviation (SD) taken from 10 different cells.

3. Results

The patient had elliptocytosis complicating ITP. According to the blood routine test result (Table 1), the patient had also iron deficiency symptom. The patient received iron supplement and immunodepression therapy, respectively, by oral administering polyferose capsules and corticosteroids. At the third month post-therapy, the patient’s situation was improved. The parameters of the blood routine (Table 1) in pre-therapeutic group are lower than that of normal group; and through three months therapy, red blood cell counts (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and ferritin content approach that of normal group except platelet counts (PLT).

Fig. 1 shows optical microscope images of Wright-Giemsa stain. As seen in Fig. 1A, the overall profiles of healthy erythrocytes appear uniform, exhibiting typical circular biconcave shapes with only a very small proportion having an irregular outline. While the overall shapes of the erythrocytes from the pre-therapeutic patient with elliptocytosis complicating ITP are remarkable deformities in all shapes of the erythrocytes from the pre-therapeutic patient, the elliptocyte, circle, and other shaped erythrocytes account for 9.5%, 89.6% and 0.9%, respectively, which approach that of control-healthy group with less than 15% of elliptocytes. Although, the overall shapes of the third month post-therapy erythrocytes (Fig. 1C) approach that of control-healthy group with less than 15% of elliptocytes. The patient received iron supplement and immunodepression therapy, respectively, by oral administering polyferose capsules and corticosteroids. At the third month post-therapy, the patient’s situation was improved. The parameters of the blood routine (Table 1) in pre-therapeutic group are lower than that of normal group; and through three months therapy, red blood cell counts (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and ferritin content approach that of normal group except platelet counts (PLT).

Fig. 1 shows optical microscope images of Wright-Giemsa stain. As seen in Fig. 1A, the overall profiles of healthy erythrocytes appear uniform, exhibiting typical circular biconcave shapes with only a very small proportion having an irregular outline. While the overall shapes of the erythrocytes from the pre-therapeutic patient with elliptocytosis complicating ITP are remarkable deformities in all shapes of the erythrocytes from the pre-therapeutic patient, the elliptocyte, circle, and other shaped erythrocytes account for 9.5%, 89.6% and 0.9%, respectively, which approach that of control-healthy group with less than 15% of elliptocytes. Although, the overall shapes of the third month post-therapy erythrocytes (Fig. 1C) approach that of control-healthy group with less than 15% of elliptocytes. The patient received iron supplement and immunodepression therapy, respectively, by oral administering polyferose capsules and corticosteroids. At the third month post-therapy, the patient’s situation was improved. The parameters of the blood routine (Table 1) in pre-therapeutic group are lower than that of normal group; and through three months therapy, red blood cell counts (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and ferritin content approach that of normal group except platelet counts (PLT).

All data were mean values ± standard deviation (SD) taken from 10 different cells.

Table 1

<table>
<thead>
<tr>
<th>Characteristics of blood routine test.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td><strong>RBC (×10^{12} L^{-1})</strong></td>
</tr>
<tr>
<td>Pre-therapy</td>
</tr>
<tr>
<td>Post-therapy</td>
</tr>
<tr>
<td>Healthy-control</td>
</tr>
</tbody>
</table>

X. Xing et al. / Micron 42 (2011) 42–46

Fig. 1. Optical microscopy images of blood smear with Wright-Giemsa stain from (A) control-healthy group; (B) pre-therapeutic group; and (C) the third month post-therapeutic group from a patient with elliptocytosis complicating ITP.
AFM surface topographic image of the healthy's erythrocyte. (A) Single cell; (B) height profile taken from Lines 1, 2, and 3 in Fig. 1A; (C) corresponding ultrastructure of membrane in (A).

$H = 1728 \pm 82$ nm, $h = 41 \pm 33$ nm, $R_{p-v} = 1687 \pm 79$ nm, and $R_a = 566 \pm 43$ nm.

AFM morphological images of the erythrocytes from pre-therapeutic group are shown in Fig. 3. Fig. 3A shows a typical elliptocyte to the length of 11.32 µm, the width of 6.78 µm, and the ratio of length to width of 1.67. Fig. 3B shows the height profiles taken from Line 1 and 2 in Fig. 3A, respectively. It is clear that the cell surface architecture in Fig. 3A has been seriously deformed, and the cell surface center also swelled. Even the structure of an erythrocyte appearing a normal circle disk was observed under optical microscopy. While a high-resolution AFM image in Fig. 3D shows a larger deformability in the surface membrane, and no longer a regular biconcave shape. As seen in Fig. 3A of pre-therapeutic group, the length, width, ratio of length to width, peak, valley, peak-to-valley value, average surface fluctuation of the erythrocytes are $L = 10.86 \pm 2.02$ µm, $W = 7.56 \pm 1.23$ µm, $r = 1.49 \pm 0.44$, $H = 1351 \pm 406$ nm, $h = 395 \pm 89$ nm, $R_{p-v} = 956 \pm 413$ nm, respectively. A larger change in ultrastructure of membrane proteins was observed for pre-therapy in Fig. 3C and F. The membrane protein was reorganized to a stripe patterns in one direction, corresponding to the asymmetric and deformed structure in erythrocytes. The statistic result shows the average surface fluctuation of $R_a = 1166 \pm 224$ nm, which is about two times of the healthy's erythrocytes $566 \pm 43$ nm. As a result, the defects of the membrane protein and skeleton at molecular scale result in the remarkable architecture deformation of erythrocytes.

The optical microscope image shows that post-therapeutic erythrocytes morphology was apparently normal (Fig. 1C). Fig. 4 shows AFM image of an erythrocyte at the third month post-therapy. As shown in Fig. 4A, the surface morphology of single cell has emerged a characteristic biconcave shape. For the post-therapeutic erythrocytes, the length ($L$), the width ($W$), the ratio of length to width ($r$), the peak ($H$), the valley ($h$), and the peak-to-valley ($R_{p-v}$) value are $8.89 \pm 0.94$ µm, $7.76 \pm 0.90$ µm, $1.13 \pm 0.22$, $1578 \pm 114$ nm, $79 \pm 38$ nm, and $1468 \pm 98$ nm (Fig. 5), respectively. The ultrastructure of the membrane protein (Fig. 4C) also appears a network structure, which is similar to that of the healthy's erythrocyte in Fig. 2C. At the third month post-therapy, the average surface fluctuation of $R_a = 674 \pm 53$ nm approximates that of healthy erythrocytes ($566 \pm 43$ nm). Similar topography and surface fluctuation features in post-therapeutic and healthy's erythrocytes indicate that the patient have recovered through three months' therapy.

4. Discussion

Our data implies that morphological changes occurring are distinct in the pathophysiology and therapy progression. AFM imaging results show the healthy's erythrocytes with typical biconcave
shape, exhibiting nanoscale network membrane proteins. For a patient with elliptocytosis complicating ITP, the structures of pre-therapeutic erythrocytes were of serious deformity; and through three-month therapy, the patients recovered and the topography feature of erythrocytes closed to that of control-healthy group.

Compared with the statistic morphologic data in Fig. 5, and the length and width of erythrocytes has a small difference for three groups. And the ratio of length to width (r) of pre-therapeutic cell (1.49 ± 0.44) is larger than that of post-therapeutic (1.13 ± 0.22) and healthy’s ones (1.10 ± 0.11). The height peak (H) of erythrocytes has a small difference (healthy: 1728 ± 82 nm; pre-therapy: 1351 ± 406 nm; the third month post-therapy: 1578 ± 114 nm). While the valley of height has a large difference (healthy: 41 ± 33 nm; pre-therapy: 395 ± 89 nm; post-therapy: 79 ± 38 nm), which show that the defects of the membrane protein and skeleton have induced the remarkable architecture deformation of erythrocytes. And the cell center of erythrocytes swells with a subsequent deformation of the topographic data (Fig. 4).

The ability to detect early changes in the morphological properties of erythrocytes in the healthy and patients, using AFM at the nanometer scale, has opened up the exciting prospect of using a simple nanodevice as a potential clinical tool. Therefore, for human, the morphological information of their erythrocytes is important indicator of well being. The ratio of length to width of r, the valley of h, the peak-to-valley of Rp–v, mean surface average surface fluctuation of Rv, and standard deviation of SD in the morphological information are important indicators for diagnosing the healthy, disease, and therapeutic effect. The next obvious step is to move this simple, yet effective microtool from the bench into the clinic, that is, to develop a user-friendly in situ AFM setup that allows for direct inspection of hematologic diseases through human erythrocytes. Moreover, the morphologic information of the erythrocytes may also serve as a sensitive diseases marker to detect the early subtle changes before significant pathological occur.

**Acknowledgements**

This work was supported by China’s 45th Postdoctoral Science Foundation (20090450099) and China’s National Natural Science Foundation (60578025, 30828028, 30772131). The authors thank Dr. Zhihong Liang for assistance in AFM measurements and Guan-qun Yang for his fruitful discussion.

**References**


