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Detecting the Micro/nano Physical Properties of Single Lymphoma Cells with Atomic Force Microscopy

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***Abstract*—Non-Hodgkin's lymphoma (NHL) is the most common adult hematological cancer. With the advent of combination therapy of chemotherapy and the monoclonal anti-CD20 antibody Rituximab, the substantial advancement in the treatment of B-cell malignancies has been achieved. In the clinical treatment of NHL, however, there are still many patients who are not sensitive to the therapy of rituximab. Hence investigating the interactions between rituximab and lymphoma cells is crucial for us to understand the actions of rituximab and design drugs with better efficacies. Traditional biochemical methods for cell detection require the various pretreatments of the cell, destroying the structures of cells. This paper uses atomic force microscopy (AFM) to label-free characterize the micro/nano physical properties of single lymphoma cells, including cell morphology, cell elasticity, and molecular interactions on the cell surface. The study improved our understanding of the rituximab actions.**

Keywords—Atomic force microscopy; Lymphoma; Image; Elasticity; Force curve

I. INTRODUCTION

Non-Hodgkin's lymphoma (NHL) is the most common hematological malignancy in adult, whereas B-cell lymphomas accounting for 85% of all NHLs [1]. The most substantial advancement in the treatment of B-cell malignancieshas been achieved due to the invention of the monoclonal CD20 antibody rituximab [2-4]. Rituximab targeted therapy combined with traditional chemotherapy/radiotherapy can significantly improve the survival rate of patients with lymphoma [5]. This combination technology has become the mainstream option for B cell lymphoma treatment. As a chimeric monoclonal antibody (mAb) produced by recombinant technology [6], Rituximab binds specifically to CD20, an antigen expressed by most human B lymphocytes. CD20 is a cell-surface marker expressed on mature B cells and most malignant B cells [7]. The binding of Rituximab to CD20 can lead to the dissolution of target cells. In recent years, the clinical efficacy of Rituximab targeted therapy in B cell lymphoma has been found to be remarkably different on different patient [8]. Therefore, it is of great significance to understand the mechanism of Rituximab treatment in order to enhance the efficacy of Rituximab. A deeper understanding of the molecular mechanism of therapeutic effect will provide useful guidance for the treatment of major diseases.

The advent of atomic force microscopy (AFM) makes it possible to study the morphology and molecular specific binding interactions on living cells [9]. AFM has many advantages, including nanometer spatial resolution, outstanding controllability, little damage to the sample being measured, and rapid and facile sample preparation [10]. Single molecule force spectroscopy(SMFS) based on AFM can be used to detect the interaction between biomolecules in the physiological environment by recording force curves on cells with tips carrying ligands which can specifically bind to the receptors on cell surface [11-13]. In the force curve mode, the functionalized tip was controlled to approach and touch the cell surface until the maximal loading force was achieved. Then the tip retracted from the cell surface. During the contact between cell and AFM tip, if the ligands on AFM tip bind to the receptors on cell surface, then the receptor-ligand complex is pulled during the retraction. When the loading force is large than the binding stregnth of the complex, the complex ruptures and a specific unbinding peak occurs in the force curve. Through the analysis of force curve, the interaction force between receptors and ligands can be calculated.

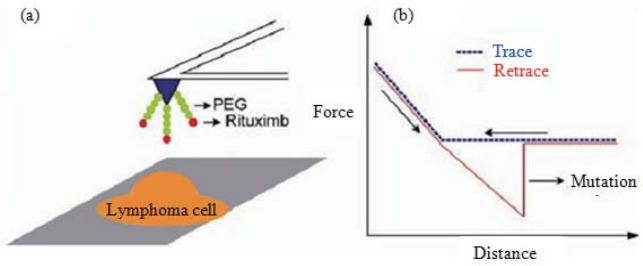

Fig.1. Measurement of interaction force between CD20 and Rituximab by AFM single molecule force spectroscopy. (a)Measured the interaction between CD20 and Rituximab on the surface of lymphoma cells;(b) Typical force curve.

In this paper, we used AFM to investigate the multiple physical properties of single lymphoma cells. The AFM probe

is covered with Rituximab molecules. The functional probe is approached onto lymphoma cells to form CD20-Rituximab combination. The binding force is measured by recording the force curve as the probe retracted from the cells. Based on the numerous needlepoint probes interacting with lymphoma cells, the contact area of the probe and the lymphoma cells is greatly increased, and subsequently the possibility of CD20 interacting with Rituximab is promoted. And the error of the experimental results is largely reduced. This research work will lay a foundation for further study of Rituximab resistance differences associated with interaction force between CD20-Rituximab. The Rituximab curative effect for people individual differences provide a new research idea, which laid a foundation for the accurate treatment of individual patients.

II. Materials and Methods

A. Sample Preparation

B lymphoma cells acquired from the Raji cell line were cultured at 37 ° C (5% CO2) in RPMI-1640 culture medium containing 10% fetal bovine serum. The sample preparation was performed as follows. The petri dish was covered with poly-*L*-lysine solution and air-dried at room temperature. Cells were harvested by centrifuging a suspension for 5 min at 1000 r·min-1. After removal of the supernatant, fresh phosphate buffered saline (PBS) was added and 100 μl of the solution was placed onto the poly-*L*-lysine-coated petri dish and incubated for 20 min, rinsed, incubated in 0.5% glutaraldehyde for 30min, rinsed, and saved in PBS.

B. Imaging of AFM

Experiments were conducted using the Dimension3100 AFM (Veeco, USA) and the probe used for Bruker's DNP probe. The nominal spring constant of the employed cantilever was 0.01 N/m. The sample was placed in the petri dish containing PBS. Using the optical microscope system AFM probe is located to the cell. Scanning frequency is 0.3 Hz, and the scanning line is set to 256, sampling points of the scanning line is set to 256, scanning force is set to 50 pN.

C. Detection of Elasticity

Experiments were conducted using the Dimension3100 AFM and the Bruker's MLCT-O probe, whose nominal spring constant is 0.01 N/m. The living Raji cells were immobilized on substrates coated with poly-*L*-lysine. The experiments were performed in PBS at room temperature. The AFM probe was guided onto the cells with the aid of a charge-coupled device (CCD) camera. Elasticity measurements were performed in contact mode, and the scan rate was 0.5 Hz for 256×256 pixels.

D. Preparation of Functional Probe

According to the method of reference [14], Pyramid-tiplessed silicon nitride cantilevers (Bruker, MLCT-O) for this experiments were functionalized with NHS-PEG-MAL as follows. Cantilevers were covered with a layer of amino in the argon environment, using APTES and N, N-diisopropylethylamine for 1h. Placing the amino-covered cantilevers in the NHS-PEG-MAL for 2~3 h, so that the PEG molecule and the surface of the cantilevers combine to form a stable compound. Using protein adhesion molecule SATP to bind Rituximab with PEG molecule. Then placed the cantilevers inside the hydroxylamine buffer for 1 h. The functionalized probe was stored at 4℃ in PBS for use.

E. Detection of Bonding Force between CD20 and Rituximab

Using the functionalized probe to detect B lymphoma cell surface CD20 antigen and Rituximab specific binding reaction. In the force curve model, the force curve was measured on the surface of Raji cell using the functional probe. When the probe approached the cell surface, the CD20 protein on cell surface was specifically combined with Rituximab. When the probe retracts from the cell surface, CD20 separate from Rituximab, there was a distinct mutation peak in the force curves, the peak corresponds to the specific binding of CD20 and Rituximab. 500 force curves were recorded on each cell. To prove the specificity blocking experiments measured CD20-Rituximab binding force, enough antibodies are added to cell surface binding sites for shielding CD20 antigen molecules, and then the function of measuring probe was employed after the binding of CD20-Rituximab again.

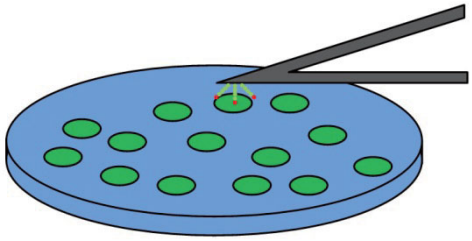

Fig.2. Diagram of molecule force between CD20 and Rotuximab

F. Statistical Methods

There is interaction between functional probe measuring cell surface receptor and ligand molecules in the modification. Therefore, the mutation peaks in the force curves may contain several intermolecular binding. Values for detachment forces are evaluated using box-and-whisker plots and frequency distributions. For SMFS, frequency(%) is defined as the total number of all non-zero force points divided by the total number of sampled sites for each experimental condition. We use Poisson analysis to calculate single pair intermolecular forces[15]. The principle is presented as follows:

$$F = \frac{\sigma^2}{\mu}$$

In a measurement process, measurement variance σ^2 and average value of μ, can be calculated according to single receptor-ligand *F*. At the same loading rate of multiple measurements, if measuring results of σ^2-μ can fit a straight line, the slope of the straight line is the binding force single receptor ligand pairs.

III. Results

With the electrostatic adsorption and chemically immobilization, single Raji cell could be easily imaged in PBS by AFM. Fig.3(a, b, c) shows the typical topography image, amplitude image, and three-dimensional topography image of the Raji cell. The scan size is 30 μm, scan rate is 0.3Hz.

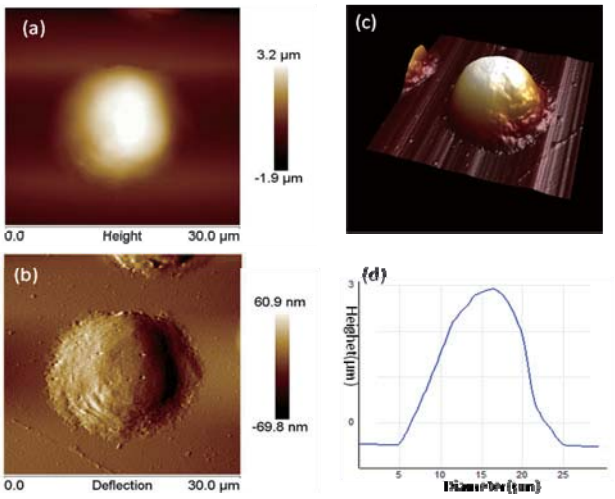

Fig.3. Typical AFM images of Raji cell. Topography image of a cell; (b) Amplitude image of a cell; (c) Three-dimensional topography image of a cell;(d) Profile curves.

We can obtained data of depth, roughness and horizontal distance from the image of AFM. Analyzed 10 lymphoma cells to obtain the statistical values (Mean±SEM) of depth was about 2.73±0.65μm, roughness was 469.8±113.18nm, horizontal distance was 19.58±3.22μm.

After fixing living cells using poly lysine, AFM was adopted to detect force curve. 100 force curves were acquired on each cell of the selected 5 cells. Fig.4 (a) presents typical force curves obtained using AFM on the cell. The blue line is the approach curve, and the red line is the retraction curve. When the tip contacts the substrate, the deformation of the petri dish is very small. Hence the force curve obtained on substrate is a two segment straight line. In contrast, the force curve obtained on a cell surface is bent (Fig.4 (b)), because the tip indents into the cell surface.

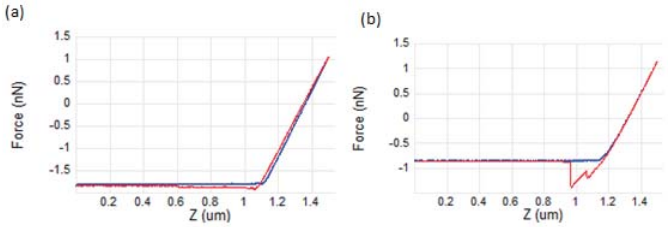

Fig.4. typical force curve. (a) force curves obtained on petri dish; (b) force curves obtained on a cell surface.

Young′s modulus (E) of the cell was computed after converting the approach curves into indentation curves, by applying the model. The formula [16] is as follows:

$$F = \frac{4ER^{1/2}\delta^{3/2}}{3(1-\nu^2)}$$

where, R is the radius of cell, δ is the indentation depth of probe on cell, ν is the Poisson's ratio of cell, which is set to be 0.33 here. After calculation, the young's modulus of lymphoma cells is evaluated to be about 586 Pa.

Then the modified Rituximab probe is used to detect B lymphoma cell surface. CD20 antigen on the cell surface specificity binds to Rituximab. The fixed living cells was used for detection of single molecule binding force. Fig.5(a) is typical force curve. There was a distinct mutation peak in retraction curve. Fig. 5(b) shows the same position of force curve obtained after adding Rituximab in PBS to block the CD20s on the cell, showing that the peak vanished.

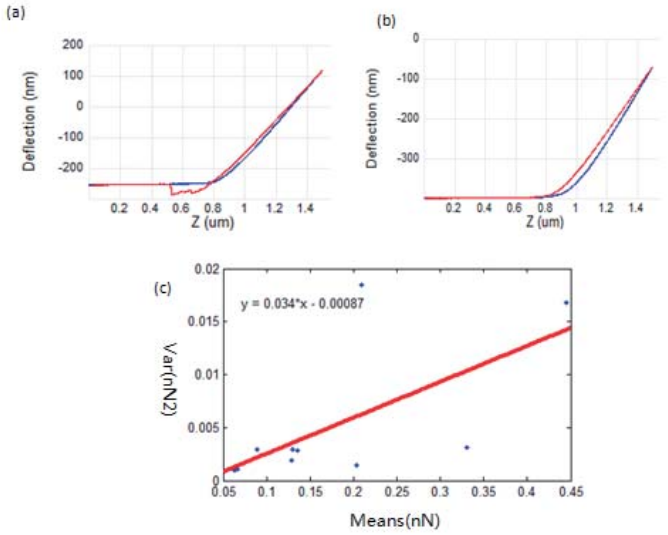

Fig. 5. Measurement of lymphoma Raji cells on the surface of the interactions between CD20 and Rituximab. (a) typical force curve; (b) After joining the Rituximab to obtain the force curve; (c) Calculation of single pair CD20-Rituximab interaction by Poisson analysis.

Force curve were recorded on ten lymphoma cells using the functional probe. For each cell, 100 force curves were recorded. The mean and variance of the binding forces for the six cells were calculated. The means value were 0.1286, 0.0660, 0.2092, 0.1359, 0.3303, 0.4447, 0.2030, 0.0635, 0.1298, 0.0893 respectively. The corresponding variance value were 0.0019, 0.0011, 0.0185, 0.0028, 0.0031, 0.0168, 0.0014, 0.0010, 0.0029, 0.0026. The mean-variance points were linearly fitted (Fitting equation is $f(x)=p1\times x+p2$). Fig. 5C shows that the fitting results is $p1=0.034$, $p2=0.00087$. The slope of fitting line shows that the interaction force of single pair CD20-Rituximab is 34 pN (Fig.5 (c)).

IV. Conclusion

In this paper, the surface morphology of lymphoma cells was imaged by AFM imaging, and the mechanical properties of the cells were detected by AFM indenting. The interactions between CD20 and Rituximab on the surface of lymphoma B cells were probed and the binding force of single CD20-rituximab was extracted by single molecule force spectroscopy. The study laid the foundation for further study on the molecular mechanism of Rituximab effect.

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