基于AFM的药物刺激前后淋巴瘤活细胞的形貌及弹性的变化

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摘要: 原子力显微镜(AFM)的发明为研究单个活细胞的形貌结构及物理特性提供了新的技术手段。然而, 由于缺少合适的固定方法, 利用AFM对动物悬浮活细胞的形貌进行高分辨率成像还面临着巨大的挑战。本文提出一种基于微柱阵列和静电吸附相结合的动物悬浮细胞固定方法。通过微柱阵列的机械钳制和多聚赖氨酸的静电吸附实现了对单个淋巴瘤B细胞的固定, 并在此基础上利用AFM动态观测了不同浓度Rituximab刺激下淋巴瘤B细胞的表面形貌及弹性的变化。经过0.2 mg·mL−1的Rituximab刺激2 h后, 细胞表面的褶皱增加, 细胞的杨氏模量从196 kPa减小到183 kPa。经过0.5 mg·mL−1的Rituximab刺激2 h后, 细胞形貌发生显著变化并出现突起结构, 细胞的杨氏模量从234 kPa减小到175 kPa。实验结果表明淋巴瘤细胞形貌和弹性变化的幅度随着Rituximab刺激浓度的增加而增加, 加深了对Rituximab作用效果的认识。

关键词: 原子力显微镜; 淋巴瘤; 弹性; 力曲线; 杨氏模量

Drug-Induced Changes of Topography and Elasticity in Living B Lymphoma Cells Based on Atomic Force Microscopy

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Abstract: Atomic force microscopy (AFM) provides a means for characterizing the surface topography and biophysical properties of individual living cells under near-physiological conditions. However, owing to the lack of adequate cellular immobilization methods, AFM imaging of living, suspended mammalian cells is still a big challenge. In this paper, a method is presented for immobilizing individual living B lymphoma cells that combines mechanical trapping with pillar arrays and electrostatic adsorption with poly-L-lysine. In this way, the topography and elasticity changes of individual B lymphoma cells that were stimulated with different concentrations of Rituximab were observed and measured dynamically. When the cell is...
stimulated by 0.2 mg·mL−1 Rituximab for 2 h, the cell topography becomes more corrugated and Young’s modulus decreases from 196 to 183 kPa. When the cell is stimulated by 0.5 mg·mL−1 Rituximab for 2 h, the cell topography changes more significantly and some tubercles appear, and Young’s modulus decreases from 234 to 175 kPa. These results thus provide a unique insight into the effects of Rituximab on individual cells.

Key Words: Atomic force microscopy; Lymphoma; Elasticity; Force curve; Young’s modulus

1 Introduction
The introduction of atomic force microscopy (AFM) in 1986 eventually led researchers in the early 1990s to investigate the topography of individual biomolecules and living cells under near-physiological conditions. Traditional microscopy images the topography of a sample via reflected or transmitted light or electron beams. In contrast, AFM renders topographic images by directly scanning a sharp tip attached to the end of a cantilever over the sample surface, while monitoring the changes in cantilever deflection. A feedback loop maintains a constant deflection between the cantilever and the sample by vertically moving an actuator to keep the interaction forces between the tip and the sample constant. The main advantage of AFM over other imaging techniques is that it can provide the topography of biological samples (such as cells, proteins, DNA) at sub-nanometer resolution under physiological conditions, without the need for staining or fixation. Thus AFM has been widely applied in the life sciences.

In early AFM studies, researchers scanned air-dried cells that lost all their activity. Then researchers used chemically fixed cells; however, chemical fixation may alter cellular structures. Hence it became desirable to image living cells under near-physiological conditions (in buffer solutions or in culture medium). The prerequisite for imaging living cells in liquids is to immobilize them on a solid support in order that they withstand the lateral forces exerted by the scanning tip. The topography of adherent cells that are spread over a substrate can easily be obtained with a small scan force (<100 pN), otherwise the cantilever deflection is smaller than for hard surfaces. The result is a linear force curve (Fig.1(c)). For soft samples such as cells, the tip indents the sample and the cantilever deflection is smaller than for hard surfaces. The result is a curved approach force curve (Fig.1(c)) and reflects the cell elasticity. The approach curve is analyzed to compute Young’s modulus of the cell, rather than the retraction curve because the latter is affected by adhesion forces between the tip and cellular surface.

In this paper, we present a cellular immobilization method based on a pillar array and electrostatic adsorption in order to obtain the topography and elasticity changes of individual B lymphoma cells stimulated with different concentrations of Rituximab.

2 Experimental
2.1 Pillar array
Pillar arrays are fabricated by photolithography on a silicon substrate (525 μm thickness, 100 mm diameter). The space between pillars is 10 μm. The height of the pillars is 5 μm and the diameter is 10 μm. Fig.2(a) is a schematic of how the cells are immobilized with a pillar array—one cell is trapped by four pillars.
pills (one pillar unit). Fig.2(b) shows the scanning electron microscopy (SEM) images of the pillar array while Fig.2(c) is the AFM three-dimensional topography.

2.2 Sample preparation

B lymphoma cells 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 as follows. A pillar array 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 a drop of the solution was placed onto the poly-L-lysine-coated pillar array and incubated for 1 min. For AFM imaging, the pillar array was attached to a glass slide using a small piece of double-sided adhesive tape and placed into a petri dish containing PBS.

2.3 AFM imaging and elasticity measurements

AFM imaging and elasticity measurements were performed using a Dimension 3100 AFM (Veeco, USA). SiN cantilevers with a nominal spring constant of 0.12 N·m⁻¹ were used; the spring constant was calibrated with the Thermal Tune Adapter (Veeco Company, USA). Force curves were performed on pillars to calibrate the deflection sensitivity of the probe. The experiments were performed in PBS at room temperature. The AFM probe was guided onto the pillar-trapped cells with the aid of a charge-coupled device (CCD) camera. Imaging was performed in tapping mode with a drive frequency of 9 kHz, and the scan rate was 0.3 Hz for 256 × 256 pixels. Elasticity measurements were performed in contact mode.

The topography of the entire trapped cell was scanned before zooming into local areas for higher-resolution images. Force curves were performed at these selected local sites on the cell surface, all at the same loading rate of 1.99 μm·s⁻¹. To examine the effects of Rituximab, 400 μL (1 mL) of Rituximab solution (10 mg·mL⁻¹, obtained from Affiliated Hospital of Military Medical Academy of Sciences) was added into the petri dish and allowed to incubate for 2 h. 20 mL PBS was also added into the petri dish. For a control experiment, 1 mL human immunoglobulin G (IgG) solution (10 mg·mL⁻¹) was added instead and incubated for 2 h. Following incubation, the cell topography was re-acquired as were the force curves.

3 Results and discussion

With the immobilization method based on pillar arrays and electrostatic adsorption, single lymphoma cells could be easily imaged by AFM. Fig.3 shows AFM images of the topography changes of a trapped B lymphoma cell before and after stimulation with 0.2 mg·mL⁻¹ Rituximab. Fig.3(a, b, c) shows the topography image, amplitude image, and three-dimensional topography image of the trapped cell before Rituximab stimulation. The scan size was 40 μm. Because the substrate was coated with positively charged poly-L-lysine, the negatively charged cells readily adsorbed. In addition, pillars surrounding each cell helped it to resist the lateral forces exerted by the probe during scanning, thus avoiding displacement of the cell. In Fig.3(d, e) the scan size was reduced to 3 μm for the topography image and amplitude image of a local area of the cell surface. We can see that the topography is a little corrugated. Individual protein components of the cell-surface machinery cannot be observed in the AFM images, because of the soft, flexible and dynamic nature of the cell surface. Following incubation in Rituximab, topography images and amplitude images of the same local area were reacquired (Fig.3(f, g)). We can see that the topography becomes more corrugated (denoted by the arrows in Fig.3(g)).

Fig.4 shows the AFM images of the topography of a trapped B lymphoma cell before and after stimulation with 0.5 mg·mL⁻¹ Rituximab. Fig.4(a, b, c) shows the 40 μm topography image, amplitude image, and three-dimensional topography image of the trapped cell before stimulation. Fig.4(d, e) shows the topography image and amplitude image of the local area of the cell surface before stimulation, and Fig.4(f, g) shows the topography image and amplitude image of the same local area after stimulation. We can see that the topography becomes notably more corrugated after stimulation and some tubercles appear (denoted by the arrows in Fig.4(g)). Comparing these topography changes with those of the cell stimulated with 0.2 mg·mL⁻¹ Rituximab, we can see that the 0.5 mg·mL⁻¹ Ritux-
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Imab-induced changes are more significant. Fig. 5 shows the process of computing Young’s modulus from force curves. Fig. 5(a) shows a typical force curve obtained on the silicon pillars. The dotted line is the approach curve, and the solid line is the retraction curve. When the tip contacts the hard silicon pillar, the deformation of the pillar is very small. Hence the force curve obtained on pillar is a two-segment straight line. In contrast, the force curve obtained on a cell surface is curved (Fig. 5(b)) because the tip indents the cell surface. Young’s modulus of the cell was computed after converting the approach curves into indentation curves, by applying the Hertz model:

\[ F_{\text{sphere}} = \frac{4ER^2\delta^{1/2}}{3(1-\nu^2)} \]  
\[ F_{\text{cone}} = \frac{2E\delta}{\pi(1-\nu^2)} \tan \theta \]

where \( \nu \) is the Poisson ratio of the sample (0.5), \( \delta \) is the indentation depth, \( R \) is the radius of curvature of the AFM tip, \( \theta \) is

Fig. 3  AFM images of the topography changes of a trapped B lymphoma cell stimulated with 0.2 mg·mL⁻¹ Rituximab
(a) topography image of a cell; (b) amplitude image of a cell; (c) three-dimensional topography image of a cell; (d) topography image of a local area before Rituximab stimulation; (e) amplitude image of a local area before Rituximab stimulation; (f) topography image of a local area after Rituximab stimulation;
(g) amplitude image of a local area after Rituximab stimulation

Fig. 4  AFM images of the topography changes of a trapped B lymphoma cell stimulated with 0.5 mg·mL⁻¹ Rituximab
(a) topography image of a cell; (b) amplitude image of a cell; (c) three-dimensional topography image of a cell; (d) topography image of a local area before Rituximab stimulation; (e) amplitude image of a local area before Rituximab stimulation; (f) topography image of a local area after Rituximab stimulation;
(g) amplitude image of a local area after Rituximab stimulation
the half-opening angle of the AFM tip, $E$ is Young’s modulus, and $F$ is the loading force. There are several hundred effective data points in an approach curve and each data point is used to compute a value of Young’s modulus. After computing these values, a histogram was constructed (Fig. 5(c)). Gaussian fitting indicates that Young’s modulus of the cell is 0.203 MPa. After putting this value and the indentation depth into the Hertz model (Eqs. (1) and (2)), the theoretical indentation curve was constructed (Fig. 5(d)). We can see that it is consistent with the experimental indentation curve, indicating that the Hertz model is appropriate for describing the indentation of lymphoma cells by the AFM tip.

Fig. 6 shows the elasticity changes of lymphoma cells after stimulation by Rituximab at different concentrations. Fig. 6(a, b) shows the changes of cell elasticity stimulated with 0.2 mg · mL⁻¹ Rituximab. Before the stimulation, seven force curves were obtained and Young’s modulus was computed for each as discussed above. A Gaussian fitting of a histogram as shown in

![Fig. 5 Computing Young’s modulus for a cell from force curves](image)

(a) force curves obtained on a pillar; (b) force curves obtained on a cell surface; (c) histogram and Gaussian fitting of the values of Young’s modulus computed from an approach force curve; (d) contrast of experimental indentation curve and theoretical indentation curve

![Fig. 6 Elasticity changes of a cell stimulated with different concentrations of Rituximab](image)

(a) Young’s modulus before 0.2 mg · mL⁻¹ Rituximab stimulation; (b) Young’s modulus after 0.2 mg · mL⁻¹ Rituximab stimulation; (c) Young’s modulus before 0.5 mg · mL⁻¹ Rituximab stimulation; (d) Young’s modulus after 0.5 mg · mL⁻¹ Rituximab stimulation; (e) Young’s modulus before 0.5 mg · mL⁻¹ IgG stimulation; (f) Young’s modulus after 0.5 mg · mL⁻¹ IgG stimulation
Comparing the topography changes of the trapped cells we can see that as the concentration of Rituximab increases, the topography of the cell surface changes significantly. Also, Young’s modulus decreases as the concentration of Rituximab increases. Rituximab binds to CD20 on the B lymphoma cell surface and can induce apoptosis. The binding of Rituximab to CD20 leads to the downregulation of p38 MAPK, NF-kB, and ERK1/2, which results in the downregulation of Bel-2. Then Cyt C is released from the mitochondria and the apoptosis pathway is activated. This intracellular signal transduction that is induced by Rituximab binding may result in the changes of the topography and elasticity of the cell. Investigations that is induced by Rituximab binding may result in the changes of cell elasticity stimulated with 0.5 mg·mL−1 Rituximab. Before the stimulation, six force curves were obtained and a Gaussian fitting of a histogram of the Young’s modulus shown in Fig.6(c) indicates that Young’s modulus is (0.234 ± 0.027) MPa. After the stimulation, six force curves were obtained and the Gaussian fitting indicated that Young’s modulus was (0.175 ± 0.013) MPa (Fig.6(d)). We can see that 0.5 mg·mL−1 Rituximab stimulation decreases Young’s modulus markedly. For a control experiment, we observed the elasticity changes of the cell after stimulation by 0.5 mg·mL−1 IgG. Before stimulation, Young’s modulus of the cell was (0.191 ± 0.019) MPa (Fig.6(e)). After stimulation, Young’s modulus became (0.188 ± 0.014) MPa (Fig.6(f)). Thus we can see that the elasticity is nearly unchanged after IgG stimulation. Unlike Rituximab, IgG does not bind to the lymphoma Raji cell surface. Thus the influence of Rituximab on Raji cells verifies a specific binding to CD20 on the cell surface.

Fig.6(a) indicates that the Young’s modulus of the cell was (0.1960 ± 0.0049) MPa. After 0.2 mg·mL−1 Rituximab stimulation, another seven force curves were obtained and the Gaussian fitting indicated that Young’s modulus was (0.1830 ± 0.0061) MPa (Fig.6(b)). We can thus see that the 0.2 mg·mL−1 Rituximab stimulation decreases Young’s modulus slightly. Fig.6(c, d) shows the changes of cell elasticity stimulated with 0.5 mg·mL−1 Rituximab. Before the stimulation, seven force curves were obtained and a Gaussian fitting of a histogram of the Young’s modulus shown in Fig.6(c) indicates that Young’s modulus is (0.234 ± 0.027) MPa. After the stimulation, six force curves were obtained and the Gaussian fitting indicated that Young’s modulus was (0.175 ± 0.013) MPa (Fig.6(d)). We can see that 0.5 mg·mL−1 Rituximab stimulation decreases Young’s modulus markedly. For a control experiment, we observed the elasticity changes of the cell after stimulation by 0.5 mg·mL−1 IgG. Before stimulation, Young’s modulus of the cell was (0.191 ± 0.019) MPa (Fig.6(e)). After stimulation, Young’s modulus became (0.188 ± 0.014) MPa (Fig.6(f)). Thus we can see that the elasticity is nearly unchanged after IgG stimulation. Unlike Rituximab, IgG does not bind to the lymphoma Raji cell surface. Thus the influence of Rituximab on Raji cells verifies a specific binding to CD20 on the cell surface.

4 Conclusions
In summary, rapid developments in biological AFM have allowed researchers to investigate the behavior of single living cells. Using AFM to understand the correlation between intracellular chemical processes and cellular physical properties will significantly improve our understanding of physiological events. In this paper, an immobilization method for suspended mammalian cells based on a pillar array and electrostatic adsorption is presented. The method enabled topography and elasticity changes of individual B lymphoma cells to be observed and measured dynamically with AFM. The results verify the efficacy of the pillar-based immobilization method and indicate that as the concentration of Rituximab increases, the topography of the cell becomes rougher and softer.

References


