Effects of methotrexate on the viscoelastic properties of single cells probed by atomic force microscopy

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Abstract Methotrexate is a commonly used anti-cancer chemotherapy drug. Cellular mechanical properties are fundamental parameters that reflect the physiological state of a cell. However, so far the role of cellular mechanical properties in the actions of methotrexate is still unclear. In recent years, probing the behaviors of single cells with the use of atomic force microscopy (AFM) has contributed much to the field of cell biomechanics. In this work, with the use of AFM, the effects of methotrexate on the viscoelastic properties of four types of cells were quantitatively investigated. The inhibitory and cytotoxic effects of methotrexate on the proliferation of cells were observed by optical and fluorescence microscopy. AFM indenting was used to measure the changes of cellular viscoelastic properties (Young's modulus and relaxation time) by using both conical tip and spherical tip, quantitatively showing that the stimulation of methotrexate resulted in a significant decrease of both cellular Young's modulus and relaxation times. The morphological changes of cells induced by methotrexate were visualized by AFM imaging. The study improves our understanding of methotrexate action and offers a novel way to quantify drug actions at the single-cell level by measuring cellular viscoelastic properties, which may have potential impacts on developing label-free methods for drug evaluation.

Keywords Atomic force microscopy · Viscoelastic properties · Methotrexate · Cell · Young's modulus · Relaxation time

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1 Introduction

Studies in biomechanics and biophysics of cancer cells in the past decade have shown that cellular mechanical properties are fundamental parameters that reflect the physiological state of a cell [1]. During the process of cancer metastasis, circulating tumor cells (CTCs) must squeeze through the extracellular matrix and endothelial cell–cell junctions to enter the vascular system [2]. Besides, the CTCs should overcome the fluid shear effects of blood to adhere to the vascular endothelium of distant organs [3]. These behaviors (detachment, intravasation, adhesion to blood vessel wall, and extravasation [2]) are closely related to the mechanical properties of cancer cells, such as deformability, cytoadherence, locomotion, and viscoelastic and frictional properties [1, 4]. Studies have shown that cellular mechanical properties are effective label-free biomarkers for indicating the physiological states of cells, including cancer induction [5], cancer metastasis [6], cell mitosis [7], bacterial resistance [8], and so on. For example, cancer cells are softer than their normal counterparts [5] and aggressive cancer cells are even softer than indolent cancer cells [6, 9]. The soft feature enables the CTCs to penetrate obstacles (e.g., basement membrane, extracellular matrix, and blood vessel wall) in the human body during metastasis [10]. It is increasingly apparent that progress in biomechanics and biophysics of cancer cells will have potential impacts on diverse fields such as drug screening, trauma evaluation, cancer diagnosis, and personalized medicine [11]. There are several tools that can measure cellular mechanical properties, including micropipette aspiration, microfluidics, optical tweezers, magnetic twisting, and atomic force microscopy (AFM) [1, 11]. Of these tools, due to its unique advantages (nanometer spatial resolution and piconewton force sensitivity in aqueous conditions), AFM is the most commonly used one [12–14].

Methotrexate is an anti-cancer chemotherapy drug that is commonly used in clinical practice [15]. Methotrexate prevents cancer cells from sustaining purine and pyrimidine systems, which eventually inhibits the synthesis of DNA [16]. So far, to our knowledge, the role of cellular mechanical properties in methotrexate's killing mechanism is still unclear. In this work, with the use of AFM, the effects of methotrexate on the viscoelastic properties (Young's modulus, relaxation time) of four types of cells (C2C12 cells, L929 cells, A549 cells, and HEK 293 cells) were quantitatively investigated. Optical and fluorescence microscopy were first performed to qualitatively investigate the effects of methotrexate on cells. Then, AFM indenting was performed to measure the changes of cellular Young's modulus and relaxation time during the actions of methotrexate using both conical tips and spherical tips. Finally, AFM imaging was applied to visualize the morphological changes of cells induced by methotrexate.

2 Materials and methods

2.1 Cell lines and agents

C2C12 (mouse myoblast cell line), L929 (mouse fibroblast cell line), A549 (human lung cancer cell line), and HEK 293 (human embryonic kidney cell line) cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell culture medium

was purchased from Hyclone Laboratories (Logan, UT, USA). C2C12 cells were cultured in DMEM medium (high glucose) containing 10% fetal bovine serum, 1% penicillin-streptomycin solution, sodium pyruvate, and glutamine at 37 °C (5% CO₂). L929 and HEK 293 cells were cultured in DMEM medium (high glucose) containing 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37 °C (5% CO₂). A549 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution at 37 °C (5% CO₂). Methotrexate (25 mg/ml) was obtained from the Affiliated Hospital of the Military Medical Academy of Sciences (Beijing, China).

2.2 Optical and fluorescence microscopy

Optical and fluorescence microscopy were performed to qualitatively investigate the effects of methotrexate on the proliferation of cells. Cells were grown in Petri dishes for 24 h before optical and fluorescence microscopy experiments. Then, cells were digested by trypsin and harvested as cell suspensions. Then, 1 ml of cell suspension was added to a fresh sterile Petri dish and then 4 ml of cell culture medium containing methotrexate was added to the same Petri dish. The final concentration of methotrexate was 25 µg/ml or 50 µg/ml. For control experiments, 1 ml of cell suspension from the same batch was added to another Petri dish and then 4 ml of cell culture medium that did not contain methotrexate was added. Cells (from the methotrexate group and from the control group) were then incubated at 37 °C (5% CO₂) for 24 h or 48 h. After the incubation, the cell culture medium was removed from the Petri dishes and propidium iodide (PI) staining solution (Ruizekang Technology Co., Beijing, China) was added and incubated for 3 min. After washing the Petri dishes with phosphate buffered saline (PBS) three times, cells in PBS were observed using a fluorescence microscope (Ti, Nikon, Tokyo, Japan).

2.3 AFM imaging

AFM experiments were performed using a Bioscope Catalyst AFM (Bruker, Santa Barbara, CA, USA), which was set on an inverted microscope (Ti, Nikon, Tokyo, Japan). The type of probe used for AFM imaging was MLCT and a cantilever with nominal spring constant 0.03 N/m was used. AFM images were recorded on living C2C12 cells (from the methotrexate group and from the control group) in cell culture medium. The imaging mode was contact mode. The scan force was 1 nN. The scan rate was 0.5 Hz. The scan line was 256 and the sampling point for each scan line was 256.

2.4 Spherical probe preparation

The spherical probe was fabricated by gluing a sphere to the cantilever of an AFM probe according to the reference [10]. The detailed process of spherical probe preparation is as follows (Fig. 1). (1) An AFM probe was mounted onto the head of AFM and the laser signal reflected off the probe cantilever was adjusted. (2) A drop of the polystyrene sphere solution (the sphere diameter was ~20 µm) was placed on a fresh glass slide and a drop of two-part epoxy adhesive (Araldite, USA) was placed on another position of the same glass slide by using a toothpick. (3) Under the guidance of optical microscopy, the AFM cantilever was moved to contact the epoxy adhesive and then retracted immediately. (4) The AFM cantilever was moved to contact a single sphere for 10 s and then retracted. Figure 1a, b show the optical

Fig. 1 Spherical probe preparation. **a, b** Under the guidance of optical microscopy, a sphere was glued to the end of the AFM cantilever. Before (**a**) and after (**b**) gluing a sphere to the AFM cantilever. **c** SEM image of a fabricated spherical probe

images before (Fig. 1a) and after (Fig. 1b) gluing a sphere to the cantilever of the AFM probe. The prepared spherical probes were placed in a probe box (Bruker, Santa Barbara, CA, USA) for 24 h at room temperature for the hardening of epoxy adhesive. Figure 1c is the SEM (Zeiss, Germany) image of a fabricated spherical probe, clearly showing that a sphere was glued to the cantilever of the AFM probe.

2.5 AFM single-cell viscoelasticity measurement

Indenting and stress-relaxation experiments were performed to measure the cellular Young's modulus and relaxation time. Both a conical probe and a spherical probe were used. The nominal spring constant of the conical probe was 0.03 N/m and the nominal spring constant of the spherical probe was 0.12 N/m. The exact spring constants were calibrated by the thermal noise method [17]. Under the guidance of optical microscopy, AFM tips were engaged to the central area of individual cells. Then, approach-reside-retract cycle of AFM tip in the vertical direction was carried out in the force ramp mode, as shown in Fig. 2. During the approach-reside-retract cycle, the AFM tip far away from the cell (Fig. 2a) firstly approached and contacted the cell until the maximal loading force was achieved (Fig. 2b). Then the tip resided for a period of time during which the vertical distance between AFM probe and substrate was maintained unchanged (Fig. 2c). After the residence, the tip retracted from the cell and returned to its original position (Fig. 2a). During the approach-reside-retract cycle, force curves were recorded with the Nanoscope Manipulation Software (Bruker, Santa Barbara, CA, USA) in the relative trigger mode. The trigger threshold (the maximal force exerted by the AFM probe) was 5 nN for the conical tip and 20 nN for the spherical tip. The approaching velocity of the AFM tip was 12 μm/s. The residence time of the tip on the cell after the tip achieved the maximal loading force was set to 2 s. Cellular relaxation occurred during the residence stage. The deflections of the AFM cantilever during the relaxation were recorded via an oscilloscope (LeCroy, New York, USA) to obtain the stress-relaxation curves. Cellular Young's modulus was extracted from force curves, while cellular relaxation time was extracted from the stress-relaxation curves.

For each cell type (C2C12, L929, A549, HEK 293), drug group (with methotrexate) and control group (without methotrexate) from the same batch of cells were prepared. After

Fig. 2 Schematic diagram of AFM single-cell viscoelasticity measurement in approach-reside-retract cycle. The AFM probe is driven by a piezoelectric ceramic driver to move vertically. The vertical displacement (the distance between probe and substrate) of the AFM probe is recorded by the piezoelectric ceramic driver. The deflection of the cantilever is detected by a four-quadrant position sensitivity detector. **a** The AFM tip is initially far away from the cell. **b** The tip vertically approaches and contacts the cell until the maximal loading force is achieved. **c** The tip then resides for a period of time (during the residence the distance between probe and substrate is maintained unchanged) and then retracts to its original position in **a**. During the residence, the cellular relaxation process occurs

culturing cells at 37 °C (5% CO₂) for 24 h, the cell Petri dishes (drug group and control group) were placed on the sample stage of AFM for mechanical measurements. First, the conical tip was used to obtain force curves and stress-relaxation curves on ten cells in the Petri dish. Then the spherical tip was used to obtain force curves and stress-relaxation curves on ten cells in the same Petri dish. For each cell, ~10 force curves were obtained and ~10 stress-relaxation curves were recorded at the central area of the cell.

2.6 Data analysis

The Hertz–Sneddon model was applied to the approach force curves to extract the cellular Young’s modulus (Hertz model was used for spherical tip and Sneddon model was used for conical tip) [18]:

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

$$F_{conical} = \frac{2E\delta^2 \tan\theta}{\pi(1-\nu^2)} \quad (2)$$

where ν is the Poisson ratio of the cell (cells are considered as incompressible material and thus $\nu = 0.5$), F is the applied loading force of tip, δ is the indentation depth, E is the Young’s

modulus of the cell, θ is the half-opening angle of the conical tip, R is the radius of spherical tip. The indentation depth δ was computed by subtracting the cantilever deflection from the vertical movement of the probe according to the contact point visually determined in the force curve [19]. The software for extracting the Young's modulus from the force curves was programmed by us using Matlab. By fitting the force curves with formula (1) or (2), we obtained the cellular Young's modulus E . A two-tailed Student's t test was applied to determine the significant difference of the cellular Young's modulus.

The generalized Maxwell viscoelastic model (second-order) was applied to the stress-relaxation curves to extract the cellular relaxation time [20]:

$$F(t) = A_0 + A_1 \exp\left(\frac{-(t-t_0)}{\tau_1}\right) + A_2 \exp\left(\frac{-(t-t_0)}{\tau_2}\right) \quad (3)$$

where F is the applied loading force of the AFM probe, A_0 is the instantaneous (purely elastic) response, A_1 and A_2 are the force amplitudes, τ_1 and τ_2 are the relaxation times. By fitting the normalized stress-relaxation curves with formula (3), we obtained the relaxation times τ_1 and τ_2 . The fitting was performed with the use of finite element software Abaqus 6.13 (Dassault Systems Simulia Corp., RI, USA), as shown in Fig. 3. The stress-relaxation curves recorded by oscilloscope were normalized by Matlab. Then the normalized stress-relaxation curves were inputted to Abaqus (Fig. 3a) which can then automatically fit the curves with the generalized Maxwell model (Fig. 3b). A two-tailed Student's t test was applied to determine the significant difference of cellular relaxation times.

3 Results and discussion

Figure 4 shows the effects of methotrexate on the proliferation of C2C12 cells observed by optical and fluorescence microscopy. Figure 4a, d are the C2C12 cells from the control group (without methotrexate) cultured for 24 h. Figure 4b, e show the C2C12 cells cultured with 25 $\mu\text{g}/\text{ml}$ methotrexate for 24 h and Fig. 4c, f show the C2C12 cells cultured with 50 $\mu\text{g}/\text{ml}$

Fig. 3 User interface of fitting the stress-relaxation curve by Abaqus. After inputting the normalized stress-relaxation data into the software (a), the relaxation times were obtained by the module of material evaluation (b)

Fig. 4 The effects of methotrexate on the growth of C2C12 cells observed by optical and fluorescence microscopy. **a–c** Bright field images. **d–f** Corresponding PI fluorescence images. **a, d** Control group (without methotrexate) with incubation time 24 h. **b, e** Stimulated by 25 $\mu\text{g/ml}$ methotrexate for 24 h. **c, f** Stimulated by 50 $\mu\text{g/ml}$ methotrexate for 24 h. **g** Bright field image of C2C12 cells stimulated by 25 $\mu\text{g/ml}$ methotrexate for 48 h. **h** Bright field image of C2C12 cells from control group (without methotrexate) with incubation time 48 h

methotrexate for 24 h. Figure 4a, b, c show the optical bright field images and Fig. 4e, f, g show the corresponding PI fluorescence images. We can observe that the growth density of C2C12 cells significantly decreased after the 24-h stimulation of methotrexate (Fig. 4b, c). Besides, methotrexate could directly cause cytotoxic effects on some C2C12 cells (denoted by the red fluorescence in Fig. 4e, f). When the stimulation time was increased to 48 h (Fig. 4g), no adherent and spreading cells were observed (cells became round), meaning that the 48-h stimulation of methotrexate could fully inhibit the proliferation of cells. In contrast from the control group cultured for 48 h (Fig. 4h), we can see that C2C12 cells adhered and spread on the Petri dish. The results (Fig. 4) showed that methotrexate had inhibitory and cytotoxic effects on the proliferation of C2C12 cells. Methotrexate acts as a cancer chemotherapeutic agent by inhibiting dihydrofolate reductase with high affinity, resulting in the depletion of tetrahydrofolates that are required for the synthesis of purines and thymidylate [15]. Subsequently, the synthesis of DNA, RNA and protein are interrupted [16], which ultimately results in the inhibitory and cytotoxic effects on the proliferation of C2C12 cells. We also performed the same optical and fluorescence microscopy experiments on L929 cells, A549 cells and HEK 293 cells (data not shown) and observed the similar results: methotrexate significantly inhibited the growth of cells and had cytotoxic effects on minor cells.

Figure 5 shows applying AFM to measure the viscoelastic properties of living C2C12 cells with the conical tip. Figure 5a is the optical image of indenting living C2C12 cells using conical tip. The position of the tip on the cantilever was recognized by the upright bright image of

Fig. 5 Measuring the viscoelastic properties (Young's modulus, relaxation time) of C2C12 cells by AFM with conical tip. **a** Optical image of indenting cells with conical tip. The *inset* shows an upright bright optical image of the tip. **b** A typical force curve obtained on C2C12 cells. The approach curve is converted into indentation curve according to the contact point. **c** Fitting the indentation curve with Hertz–Sneddon model to extract cellular Young's modulus. **d** A typical stress-relaxation curve and the corresponding **(e)** vertical distance curve of AFM tip recorded on C2C12 cells. **f** Fitting the normalized stress-relaxation curve with second-order Maxwell model to extract cellular relaxation times

the probe (inset in Fig. 5a). For one approach-reside-retract cycle, we obtained a force curve (Fig. 5b), a stress-relaxation curve (Fig. 5d), and a vertical distance curve (Fig. 5e) simultaneously. Figure 5b is a typical force curve obtained on living C2C12 cells during one approach-reside-retract cycle. We can see that the end point of the approach curve did not coincide with the start point of the retract curve. This is due to the cellular relaxation in the resident stage between the approach and retract processes. The approach curve was converted into an indentation curve according to the contact point in the approach curve. The contact point was determined visually (Fig. 5b). The approach curve was flat before the tip contacted the cell, and it became bent after the tip contacted the cell. Figure 5c shows the contrast of the indentation curve and the Hertz–Sneddon fitting curve. We can see that the indentation curve was well fitted with the Hertz–Sneddon model. The result of Hertz–Sneddon fitting indicated that the cellular Young's modulus extracted from the approach curve (Fig. 5b) was 3.1 kPa. Figure 5d is a typical stress-relaxation curve during the approach-reside-retract cycle and Fig. 5e is the corresponding vertical distance curve. The two dashed lines in Fig. 5d, e denote the relaxation process. We can see that during the relaxation process the vertical position of the AFM tip was unchanged (Fig. 5e) while the force decayed (Fig. 5d). Figure 5e shows the contrast of normalized stress-relaxation curve and generalized Maxwell fitting. We can see that the stress-relaxation curve could be well fitted with a second-order Maxwell model. The fitting result indicated that the relaxation times extracted from the stress-relaxation curve were 0.04233 s (τ_1) and 0.43629 s (τ_2).

Current AFM single-cell mechanical assays have mainly measured the Young's modulus of cells, which reflects the elastic properties of cells [12], whereas cells are essentially viscoelastic due to cytoplasm [21]. However, the information about the role of cellular viscoelasticity during cellular physiological activities (such as cancer-related changes) is so far still scarce [22]. Investigating cellular viscoelastic properties can undoubtedly improve our understanding of cell behavior. Hence, in this work we simultaneously measured the Young's modulus and relaxation time of cells to explore the dynamics of cellular viscoelasticity during the action of methotrexate. The viscoelastic properties of cells are mainly related to the cytoplasm, which is

comprised of different compositions, including cytosol, organelle, cytoskeleton, and inclusion. We can see that the cytoplasm is highly heterogeneous. These different compositions have variable relaxation characteristics, and as a result the first-order Maxwell element model often cannot fit the relaxation curve well [23]. For living cells, the second-order Maxwell model is often appropriate [20].

In order to examine the effects of loading force on the measured cellular relaxation time, we obtained stress-relaxation curves on cells under different loading forces. Figure 6 shows three stress-relaxation curves obtained on a living C2C12 cell under three different loading forces (1 nN, 3 nN, and 5 nN) using a conical tip. When the loading force was 1 nN, the cellular relaxation times were 0.03294 s (τ_1) and 0.48982 s (τ_2). When the loading force was 3 nN, the cellular relaxation times were 0.04410 s (τ_1) and 0.55721 s (τ_2). When the loading force was 5 nN, the cellular relaxation times were 0.04348 s (τ_1) and 0.54444 s (τ_2). We can see that the cellular relaxation time was variable when the loading force changed (the cellular relaxation times at 1 nN loading force were much less than the cellular relaxation times at 3 nN and 5 nN). In order to obtain results with statistical significance, we then recorded stress-relaxation curves on another four living C2C12 cells to measure cellular relaxation times, and the results are shown in Table 1. From Table 1, we observe that, on the whole, the cell relaxation time increased as the loading force increased. When the loading force increased, the AFM tip indented deeper into the cell, making it more difficult for the cell to relax to the original state. Studies about measuring cellular Young's modulus have comprehensively shown that several factors influence the Young's modulus measured by AFM, such as loading rate, indentation depth, substrate, tip shape, temperature, cell areas being probed, and working medium [24, 25]. For cellular relaxation behavior, a recent theoretical and experimental study by Nguyen et al. on chondrocytes has shown that cellular relaxation parameters depended on the strain-rates [26]. Here, our results showed that the increased loading force resulted in the increase of cellular relaxation times. Hence, in order to make measurements comparable, in the flowing experiments (measuring the cellular Young's modulus and relaxation times after the stimulation of methotrexate), experimental curves (force curves and stress-relaxation curves) were recorded under the identical conditions: the loading rate was 12 $\mu\text{m/s}$, the loading force was 5 nN for the conical tip and 20 nN for the spherical tip.

Fig. 6 Stress-relaxation curves recorded on a living C2C12 cell under different loading forces using a conical tip. Two-order Maxwell fitting was performed to extract cellular relaxation times. **a** Loading force 1 nN. **b** Loading force 3 nN. **c** Loading force 5 nN

Table 1 Measuring cellular relaxation times under different loading forces on four living C2C12 cells

Cell no.	Loading force: 1 nN		Loading force: 3 nN		Loading force: 5 nN	
	τ_1 (s)	τ_2 (s)	τ_1 (s)	τ_2 (s)	τ_1 (s)	τ_2 (s)
Cell 1	0.03471	0.33136	0.05156	0.61430	0.05134	0.61828
Cell 2	0.03338	0.54501	0.04764	0.56887	0.05047	0.62849
Cell 3	0.03500	0.60691	0.04368	0.60932	0.04494	0.58661
Cell 4	0.02700	0.49557	0.04386	0.62938	0.04719	0.44980

Figure 7 shows measurement the Young's modulus and relaxation times of C2C12 cells using a spherical tip. Figure 7a is the optical image of indenting living C2C12 cells with a spherical tip. The position of the tip on the cantilever was recognized by the upright bright image of probe (the inset in Fig. 7a). Figure 7b is a typical force curve obtained on C2C12 cells. The Hertz–Sneddon fitting (Fig. 7c) indicated that the cellular Young's modulus extracted from the force curve in Fig. 7a was 0.32 kPa. We can see that the cellular Young's modulus measured by spherical tip (0.32 kPa in Fig. 7c) was significantly smaller than the cellular Young's modulus measured by the conical tip (3.1 kPa in Fig. 5c). This is because the interaction areas between the spherical tip (the diameter of sphere used here was about 20 μm) and the cell were much larger than the interaction areas between conical tip (the tip curvature of conical tip used here was about 20 nm) and cell, resulting in the smaller pressure on the spherical tip and thus the smaller cellular Young's modulus measured by a spherical tip. The advantage of a spherical tip is that it can better characterize the viscoelastic properties of the whole cell [10, 21], while the advantage of a conical tip is that it can probe the viscoelastic properties of local areas on the cell. With the conical tip, we can get the elastic maps by obtaining arrays of force curves on the cell surface [27, 28], which is useful for us in understanding the elasticity of different areas on cells

Fig. 7 Measuring the Young's modulus and relaxation times of C2C12 cells using a spherical tip. The loading force was 20 nN. **a** Optical image of indenting cells with spherical tip. The inset is the upright optical bright image of the tip. The tip position is denoted by a dashed circle. **b** A typical force curve. **c** Hertz–Sneddon fitting of the indentation curve. **d** A typical stress-relaxation curve. **e** Fitting the stress-relaxation curve with second-order Maxwell model

and for establishing the relationship between cell mechanics and cell structures [29]. Figure 7d is a typical stress-relaxation curve obtained on C2C12 cells with a spherical tip and Fig. 7e shows second-order Maxwell model fitting. We can see that the stress-relaxation curve was well matched with Maxwell fitting. The fitting result indicated that the relaxation times were 0.03687 s (τ_1) and 0.43926 s (τ_2). We can observe that the cellular relaxation times measured by a spherical tip (Fig. 7) were comparable to the cellular relaxation times measured by a conical tip (Fig. 6). Okajima et al. [30] have used both a conical tip and a spherical tip to probe the relaxation time of fibroblasts and their results showed that the relaxation time of fibroblasts probed by a conical tip (0.176 ± 0.1 s) was similar to that probed by a spherical tip (0.182 ± 0.07 s), consistent with our results here. The results showed that the measured cellular relaxation times by AFM were independent of the tip shape.

Figure 8 shows the changes of cellular Young's modulus of the four types of cells (C2C12 cells, L929 cells, A549 cells, and HEK 293 cells) after 24-h stimulation with methotrexate. Figure 8a is the contrast of cellular Young's modulus of C2C12 cells from the control group (without methotrexate) and from the drug group (with methotrexate) measured by using both the conical tip and the spherical tip. The detailed Young's modulus values in each bar in Fig. 8a are shown in Table 2. Force curves were obtained on ten cells from the control group and ten cells from the drug group to obtain the changes of cellular Young's modulus with statistical significance. From Fig. 8a and Table 2, we can see that the Young's modulus of C2C12 cells from the control group was 2.9714 ± 0.1879 kPa for the conical tip and 0.7751 ± 0.0552 kPa for the spherical tip. After the 24-h stimulation with methotrexate, the Young's modulus of C2C12 cells became 2.1666 ± 0.2915 kPa for the conical tip and 0.3591 ± 0.0970 kPa for the spherical tip. We can clearly see that the stimulation of methotrexate resulted in the significant decrease

Fig. 8 Changes of cellular Young's modulus after 24-h stimulation with methotrexate. **a** C2C12 cell. **b** L929 cell. **c** A549 cell. **d** HEK 293 cell (mean $\pm$ SEM; * p < 0.05; ** p < 0.01; *** p < 0.001; n = 50 for each value)

Table 2 Changes of cellular Young's modulus and relaxation times of C2C12 cells during the actions of methotrexate

Conditions	Young's modulus (kPa)		Relaxation time τ_1 (s)		Relaxation time τ_2 (s)	
	Conical tip	Spherical tip	Conical tip	Spherical tip	Conical tip	Spherical tip
Control	2.9714 ± 0.1879	0.7751 ± 0.0552	0.0452 ± 0.0016	0.0435 ± 0.0009	0.5771 ± 0.0249	0.5578 ± 0.0125
With methotrexate	2.1666 ± 0.2915	0.3591 ± 0.0970	0.0409 ± 0.0007	0.0346 ± 0.0019	0.5093 ± 0.0136	0.5080 ± 0.0296

of cellular Young's modulus and the changes of cellular Young's modulus, which could be probed by both conical and spherical tips. For L929 cells (Fig. 8b), A549 cells (Fig. 8c), and HEK 293 cells (Fig. 8d), we can observe the same results: the stimulation of methotrexate caused the softening of cells. The results in Fig. 8 also show the consistency of the conical tip and spherical tip in probing the dynamics of cellular Young's modulus after drug stimulation. Traditional biological methods investigate the killing effects of methotrexate by counting the viability of cells after stimulation by methotrexate [31, 32]. The dynamics of cell mechanics during the actions of methotrexate are so far unclear. Here, the results obtained by AFM show that the stimulation of methotrexate results in the decrease of cellular Young's modulus, improving our understanding of the killing effects of methotrexate from the perspective of cell mechanics.

Figure 9 shows the changes of cellular relaxation times of the four types of cells after 24-h stimulation with methotrexate. Figure 9a, b show the changes of relaxation time τ_1 (Fig. 9a)

Fig. 9 Changes of cellular relaxation time after 24-h stimulation with methotrexate. **a, b** C2C12 cells. **c, d** L929 cells. **e, f** A549 cells. **g, h** HEK 293 cells. **a, c, e, g** τ_1 . **b, d, f, h** τ_2 (mean $\pm$ SEM; * p < 0.05; ** p < 0.01; *** p < 0.001; n = 50 for each value)

and τ_2 (Fig. 9b) of C2C12 cells measured by using both a conical tip and a spherical tip. The detailed relaxation time values in each bar in Fig. 9a, b are shown in Table 2. The relaxation time τ_1 of C2C12 cells was 0.0452 ± 0.0016 s for conical tip and 0.0435 ± 0.0009 s for spherical tip. After 24-h stimulation with methotrexate, the relaxation time τ_1 became 0.0409 ± 0.0007 s for conical tip and 0.0346 ± 0.0019 s for spherical tip. We can see that the stimulation of methotrexate resulted in the significant decrease of relaxation time τ_1 . For relaxation time τ_2 (Fig. 9b), τ_2 decreased from 0.5771 ± 0.0249 s to 0.5093 ± 0.0136 s after the stimulation of methotrexate using conical tip and τ_2 decreased from 0.5578 ± 0.0125 s to 0.5080 ± 0.0296 s using spherical tip. For C2C12 cells, both relaxation time τ_1 and τ_2 decreased after the stimulation of methotrexate. For L929 cells (Fig. 9c, d), both relaxation times τ_1 and τ_2 evidently decreased after the stimulation of methotrexate when a conical tip was used. The relaxation times τ_1 and τ_2 decreased slightly when a spherical tip was used. For A549 cells (Fig. 9e, f), we can see that there was a significant decrease in relaxation time τ_1 probed by spherical tip and relaxation time τ_2 probed by conical tip after the stimulation of methotrexate. For HEK 293 cell (Fig. 9g, h), significant changes were observed in relaxation time τ_1 probed by conical tip and relaxation time τ_2 probed by spherical tip after the stimulation of methotrexate. As observed in Figs. 6 and 7, the cellular relaxation times measured by AFM were independent of tip shape. Thus, we can use either the conical tip or spherical tip to probe the cellular relaxation time. In some situations (such as the spherical tip for L929 cells), the changes of cellular relaxation time were not significant. This may be because the cell quantity being probed was not sufficient (here we probed ten cells for each situation). Probing more cells may improve the quality of the results. On the whole, the results of Fig. 9 show that cellular relaxation times decreased after the stimulation of methotrexate. Figure 8 shows that methotrexate resulted in the decrease of cellular Young's modulus, meaning the changes of cellular elasticity. Figure 9 shows that methotrexate also resulted in the decrease of cellular relaxation times, meaning the changes of cellular viscosity. Previous studies of AFM single-cell mechanical assay commonly measure the single mechanical properties of cells, such as elasticity [5, 9] and viscosity [20]. Here, by applying AFM indenting technique in approach-reside-retract mode to measure Young's modulus and relaxation time, the changes of cellular viscoelastic properties during the actions of methotrexate were investigated, offering a new way to simultaneously quantify the elastic and viscous properties of living cells.

In order to explore what causes the changes of cellular viscoelastic properties during the actions of methotrexate, AFM imaging was applied to visualize the morphological changes of C2C12 cells, as shown in Fig. 10. Figure 10a, d shows the AFM images of living C2C12 cells from the control group (without methotrexate). Figure 10b, e shows the AFM images of living C2C12 cells that were cultured with methotrexate for 24 h. We can clearly see the well-defined filamentous structures [33] in the C2C12 cells from the control group, while the fibrous structures were unapparent in C2C12 cells stimulated by methotrexate, meaning that the addition of methotrexate could cause the structural changes in C2C12 cells. In the experiments, some C2C12 cells became rounded after the stimulation of methotrexate, as shown in Fig. 10c, f. Because it was difficult to image the living rounded cells, AFM images of Fig. 10c, f were obtained on chemically fixed C2C12 cells in PBS. From the section curves (Fig. 10g–i), we can clearly see that the cell height increased from ~ 3 μm to

Fig. 10 Morphological changes of C2C12 cells after 24-h stimulation with methotrexate. **a, d, g** A C2C12 cell from the control group (without methotrexate) cultured for 24 h. **b, e, h** A C2C12 cell stimulated by methotrexate for 24 h. **c, f, i** Another C2C12 cell stimulated by methotrexate for 24 h. **a–c** AFM height images, **d–f** corresponding deflection images, and **g–i** section curves

$\sim 7 \mu\text{m}$ as the cell rounded. Cell shape emerges from the interaction of many constituent elements, such as the cytoskeleton, the cell membrane, and cell–substrate adhesions [34]. Cells adhere and spread on the substrate via forming focal adhesions between cell and substrate [35]. When a cell rounds, stress fibers disassemble and most proteins disengage from focal adhesions, which shrink down to small anchoring sites [36]. Stewart et al. [37] have shown that actin cytoskeletons generate the rounding pressure during mitotic cell rounding. Hence, when spreading cells round, cellular structures such as the cytoskeleton change significantly. The stimulation of methotrexate could cause the rounding of cells (Fig. 10c), which was accompanied by cytoskeletal changes. Studies have shown that the cytoskeleton directly determines cell mechanics (such as Young's modulus [38, 39]). Hence, the cytoskeletal changes induced by methotrexate eventually caused the decrease of cellular Young's modulus as observed in Fig. 8. On the other hand, as the cells rounded after the stimulation of methotrexate, the cellular cytoplasm also changed, which caused a decrease of cellular relaxation times, as observed in Fig. 9. Comparing Fig. 8 with Fig. 9, we can see that significant changes of cellular Young's modulus could be observed using either the conical tip or the spherical tip after the 24-h stimulation of methotrexate, while significant changes of cellular relaxation times could be only observed in certain situations, meaning that the cellular Young's modulus was more sensitive to the

changes of cell states than cellular relaxation time. Young's modulus corresponds to the purely elastic properties of cells, while relaxation time corresponds to the viscous properties of cells. We know cellular physiological activities are very complex so that Young's modulus alone may fail to universally indicate the changes of cell states. A recent study by Park and Lee [40] has shown that mechanical compliance alone cannot serve as an effective indicator for metastatic progression, while utilizing the dual cellular mechanical properties (mechanical compliance and cell–substrate adhesion) can provide critical phenomenological information for a critical step of metastasis for different types of cancer (prostate cancer and breast cancer). Hence, investigating cell behaviors from multiple mechanical facets (such as measuring both elastic properties and viscous properties) can help us to better understand cellular physiological activities. In recent years, researchers have applied AFM to probe the relaxation time of diverse cells, such as MCF-7 cells [20], HeLa cells [21], chondrocytes [41], fibroblasts [30], and HepG2 cells [42]. However, to our knowledge, so far, dynamically detecting the changes of cellular relaxation time after drug actions or during cellular physiological activities has not been reported. Here, we examined the effects of methotrexate on the relaxation time of four different types of cells. The results (Fig. 9) show that the stimulation of methotrexate resulted in the decrease of cellular relaxation time, providing novel insights into the actions of methotrexate and also demonstrating the feasibility of developing new label-free biomarkers based on cellular relaxation time.

It is increasingly evident that cell mechanics plays a crucial role in modulating the physiological activities of cells [43, 44]. Physiological or pathological changes within cells are accompanied by chemical and mechanical [45, 46]. For example, cancer cells not only have genetic mutations and acquire new functions to proliferate unlimitedly (chemical alteration) [47], but also are softer than their normal counterparts (mechanical alteration) [5]. For detecting the chemical properties of cells, traditional methods (such as Western blot, immunohistochemistry, polymerase chain reaction, electron microscopy, and X-ray crystallography) require the pretreatments of cells (such as lysis, fixation, labeling, and staining), which destroy the structures and activities of cells [48, 49]. Hence, the measurements are off-line and cannot reveal the dynamic changes of cells. On the contrary, detecting cellular mechanical properties by AFM can be performed directly on single living cells [50] and thus allows us to monitor the dynamic changes of cellular mechanical properties during physiological activities (such as after the stimulation of therapeutic agents) of a single cell, which can yield new knowledge about cell behaviors and drug actions. This knowledge can potentially be used in the coming era of precision medicine where personalized treatment has become the therapeutic trend [51]. For example, after establishing the relationship between cellular mechanical properties and drug efficacies *in vitro*, we can trap cancer cells from the clinical patient (such as via micro-fluidics [52, 53]) and then examine the mechanical properties of cancer cells. The measurement results can help us to select adequate drugs for the patients. Current AFM single-cell mechanical tests are commonly focused on the elastic properties of cells, while the responsiveness of cytoplasm to deformation (viscous properties of cells) is connected to the fundamental mechanisms of cell behaviors [54]. Here, an AFM-based method, which can simultaneously measure the elastic properties and viscous properties of cells, was established. In the future, we would like to apply the established method to primary tumor cells

prepared from clinical cancer patients to investigate the role of cell mechanics in cancer development. The studies directly performed on patient tumor cells are closer to the clinical environment and thus can provide invaluable insight of cell behaviors and drug actions.

4 Conclusions

In this work, AFM was applied to quantitatively investigate the effects of methotrexate on the viscoelastic properties of four types of cells (C2C12 cells, L929 cells, A549 cells, and HEK 293 cells) by simultaneously measuring the dynamic changes of cellular Young's modulus and cellular relaxation times. Optical and fluorescence microscopy showed that the addition of methotrexate remarkably inhibited the growth of cells. Cellular Young's modulus and relaxation times were measured by simultaneously acquiring force curves and stress-relaxation curves on cells using both a conical tip and a spherical tip. Experimental curves (force curves and stress-relaxation curves) recorded on cells were well consistent with the theoretical mechanical models, showing that the cellular relaxation times probed by AFM increased as the loading force increased. The cellular relaxation times measured using a conical tip approximated those found using a spherical tip. The statistical measurements showed that the stimulation of methotrexate could significantly result in the decrease of both cellular Young's modulus and relaxation times. AFM imaging clearly revealed the structural changes of cells (filamentous structures vanished, cell rounded, cell height increased) after the stimulation of methotrexate, which caused changes of cellular viscoelastic properties. The results demonstrate the effectiveness of cellular viscoelastic properties in quantifying drug actions at the level of a single cell. In future studies, we plan to investigate the viscoelastic properties of primary tumor cells prepared from clinical cancer patients after the stimulation of drugs. These studies will be particularly useful for us to explore the role of cell mechanics in cancer development, which is of potential significance for us to develop novel methods for cancer diagnosis and treatment in the era of personalized medicine.

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