Detecting Cancer Cells in Normal Tissue by Scanning Force Modulation Microscopy

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Abstract

We introduce a new application of scanning force modulation microscopy (SFMM) which we show can be used to differentiate transformed from normal live cells simply by measuring their nanomechanical properties. This technique relies on the fact that cellular elasticity changes drastically when cells become cancerous. Hence a single cancerous cell amid a culture of normal cells can be observed. This technique can also measure the mechanical properties of the underlying substrate, or the extracellular matrix (ECM), which is also known to have differences in mechanical properties between the proteins secreted by normal and cancer cells. Hence we have the ability to correlate the state of the cell in reference to that of its underlying ECM.

Keywords

scanning force modulation microscopy, confocal microscopy, mechanical properties, keratinocytes, squamous cell carcinoma, dermal fibroblasts, extracellular matrix

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INTRODUCTION

Recently Guck et al. [1] and Beil et al. [2] have shown that increased cellular elasticity can be used as a marker of cancer. Both groups used optical deformation techniques that require the isolation of single cells. Even though these methods accurately measure mechanical response, they require precisely controlled deformations and are not practical for detecting the behavior of individual cancer cells embedded in otherwise healthy tissue.

Here we introduce another method - scanning force modulation microscopy (SFMM) - which uses very low forces and hence is minimally invasive, at the same time is able to probe mechanical properties with nanoscale precision. We show how this technique can detect the relative elastic moduli of individual cells in culture and unambiguously identify the cancerous or transformed ones based on their mechanical properties.

The advantage of this method is is that it is robust and uses only a minor mechanical disturbance, which does not perturb the cells from their environment. Because SFMM accurately detects differences between normal and transformed cells without the need for analyzing single cell in suspension this technique allows us to detect differences between normal and cancer cells in contact with their extracellular matrices. This approach should more accurately reflect the properties of the cells in situ. Using this same technique, we also measured the mechanical response from individual extracellular matrix (ECM) fibers produced by normal and transformed cells.

MATERIALS AND METHODS

Cell Culture

Epithelial Cells: Human keratinocytes strain D033 and squamous cell carcinoma cell line SCC13 [3] grown with a feeder layer of 3T3 cells [4], were maintained and serially passaged according to the methods described in reference [5]. The standard medium contained a 3:1 (v/v) mixture of Dulbecco’s Minimal Essential Medium (DMEM) and Ham’s F12 medium containing adenine (1.8 x 10^4 M), 0.4 µg ml^-1 hydrocortisone, 5 µg ml^-1 insulin, 10 ng ml^-1 EGF, and 1.2 x 10^-10 M chlorella toxin and was supplemented with 5% fetal bovine serum (HyClone, Utah). 3T3 cells for use in generating feeder layers were maintained in DMEM with 10% bovine calf serum. Prior to use as feeders, confluent cultures of 3T3 cells were harvested with Ca and Mg-free phosphate buffered saline, pH 7.2-7.4 (PBS), containing 0.5 mM EDTA, resuspended in DMEM medium containing adenine (1.8 x 10^4 M), 0.4 µg ml^-1 hydrocortisone, 5 µg ml^-1 insulin, 10 ng ml^-1 EGF, and 1.2 x 10^-10 M chlorella toxin and was supplemented with 5% fetal bovine serum (HyClone, Utah). 3T3 cells for use in generating feeder layers were maintained in DMEM with 10% bovine calf serum. Prior to use as feeders, confluent cultures of 3T3 cells were harvested with Ca and Mg-free phosphate buffered saline, pH 7.2-7.4 (PBS), containing 0.5 mM EDTA, resuspended in DMEM medium containing adenine (1.8 x 10^4 M), 0.4 µg ml^-1 hydrocortisone, 5 µg ml^-1 insulin, 10 ng ml^-1 EGF, and 1.2 x 10^-10 M chlorella toxin and was supplemented with 5% fetal bovine serum (HyClone, Utah).

Fixation and Staining

Cells: Cells were fixed with 3.7% formaldehyde and then permeabilized with 0.4% Triton X-100.

Fibroblasts: Normal and transformed human dermal fibroblasts were purchased from ATCC (CRL 7761 and CRL 7762). Fibroblasts were grown in Dulbecco’s modified Eagle medium in 4% L-glutamine adjusted to contain 1.5 g L^-1 sodium bicarbonate and 4.5 g L^-1 glucose with 10% fetal bovine serum (Cionetics). The cells were fed every two days with their respective media/serum solutions.

Cell Plating: The cells were harvested, counted and then plated either on tissue culture polystyrene dishes, either on polybutadie (PB) films at a density of 2,000 cells cm^-2 in their respective media/serum and incubated in a 5% CO_2 incubator. In order to observe the extracellular matrix production by normal and transformed cells, cells were plated in serum-free media for one day on a sulfonated polystyrene surface and then the ECM fibres were imaged by AFM.

Resolution of Mechanical Properties

There are several important factors that may affect the resolution of mechanical properties.

1. Surface roughness: The surface roughness of the sample can affect the resolution of mechanical properties. A rough surface can result in a larger effective contact area, which can lead to a decrease in the resolution of mechanical properties.

2. Sample thickness: The thickness of the sample can also affect the resolution of mechanical properties. A thin sample will have a larger effective contact area, which can lead to a decrease in the resolution of mechanical properties.

3. Imaging conditions: The imaging conditions, such as the indentation depth, can also affect the resolution of mechanical properties. A larger indentation depth will result in a larger effective contact area, which can lead to a decrease in the resolution of mechanical properties.

Figure 1: Schematic showing the measurement of mechanical properties with an AFM tip. This technique is called scanning force modulation microscopy (SFMM) and follows equation 1.
ton. Cells were then stained for actin using Alexa Fluor phalloidin-488 (Molecular Probes). Nuclei were visualized using DAPI (Molecular Probes) staining. After this process was completed, the confocal microscope was utilized for imaging.

**Surface Preparations**

One-side polished single-crystal silicon wafers (Wafer World Corporation, FL) were used as substrates. These wafers had a roughness no larger than 1 nm. They were cleaned and treated to be hydrophobic. Polybutadiene (PB) stock solution (Exxon Mobil) was used to create a polybutadiene layer 250 nm thick. These PB-coated Si wafers were then annealed in a vacuum oven set at 170°C for 12 hours in order for the polymer to react with the Si and to sterilize the samples.

**Atomic Force Microscopy**

A Dimension 3000 atomic force microscope, (Nanoscope from Digital Instruments) was used to image cells and ECM fibers, as well as to measure the mechanical response from these cells or ECM fibers. Samples with cells or/and with ECM fibers were placed in a liquid cell and all the experiments were made in media/serum by using the AFM in contact mode. The mechanical properties of normal, transformed and cancer cells were assessed through lateral force modulation.

In order to measure the modulus of a material, we have developed a new technique using atomic force microscopy as described in reference [6]. A modulation was applied to the AFM tip at a frequency of 1400 Hertz and the response amplitude is followed. The applied normal force (25 nN) of the instrument was constant in order to maintain contact between the tip and the sample. The torsion of the tip in the sample is a function of the penetration depth and the adhesion of the tip to the surface, as shown in Figure 1. Here h is the depth to which the tip has penetrated. The deeper the penetration, the softer the material is. In the Hertz model, we assume that $\Delta X$ is proportional to the penetration distance since the deeper the indentation, the larger the deflection for a given lateral force.

To calculate the modulus we used this equation (equation 1):

$$\Delta X \propto h = \left(\frac{3}{4} \frac{\delta}{E} \frac{F}{R^2}\right)^{2/3}$$

which is derived from a classical mechanics model known as the Hertz model [6]. This equation can be rewritten where the terms corresponding to the tip and substrate are separated from the term that describes the modulus. The modulus depends then on the tip parameters and it is very important to use a known substrate for calibration or to keep the same tip to conduct the experiments, hence the parameters for the tip become constant. On a given sample, the tip terms are always constant for the same tip. Therefore, we are able to simplify the equation to: $\Delta X \propto h = \Delta X_{\text{tip}} \propto E^{2/3}$. In order to find the shear modulus E we measured the tip deflection $\Delta X$.

Figure 2:

Normal (A, C) and cancerous (B, D) keratinocytes plated on polybutadiene for 2 days. (A) and (B) are optical images of keratinocytes imaged by AFM. The triangular shade is the AFM tip holder. (C) and (D) are confocal microscopy images where cells were specifically stained for actin (green, phalloidin-alexa fluor 488).

Figure 3:

Mechanical response from normal and transformed keratinocytes measured by SFMM. (A) Amplitude response from different keratinocytes (normal or cancerous) as a function of the tip driving amplitude in order to measure the cell modulus by using the scanning force modulation microscopy technique (SFMM). Each line corresponds to a different cell. (B) Average relative modulus for normal and cancer keratinocytes plotted in histogram form (N: normal; T: transformed). (C) Amplitude responses measured by SFMM on 7 cells cultured as a 50:50 mixture of normal and cancerous keratinocytes. (D) Relative modulus plotted in a histogram form for these 7 cells and for the normal N and transformed T standards.
as a function of driving force. A linear relationship is indicative of the tip driving the modulation of the surface. If the response is not linear, then the material is unyielding or the tip is slipping. The relationship between the deflection of the tip and the hardness of the material can be obtained from mechanical theory. According to the Hertz theory of linear elasticity, the deflection is proportional to the penetration of the tip into the sample, which in turn is related to the modulus of the sample. This relationship is given in Equation 1. Therefore, from the slope of the curves we can obtain the modulus of the sample. In order to determine a quantitative number, we need to know the tip diameter, \( d \), a value which is intrinsic to each individual tip. Therefore, here, we calculated relative numbers so that we could divide these units and remove these factors. The relative moduli are derived from Equation 1.

This technique can be used in the scanning mode, where images of areas with differences in relative moduli are observed, or in the localized mode, where data are acquired from a single area.

Confocal microscopy
Cells that were fluorescently stained for actin were imaged using a Leica SP confocal microscope with a 40X oil immersion objective.

RESULTS AND DISCUSSION
Keratinocytes isolated from normal adult epidermis (strain DO33) and from a squamous cell carcinoma (SCC13) were grown for 48 hours on a polybutadiene (PB) rubber surface in keratinocyte media with single cell precision. We tested this hypothesis by comparative measurements of the moduli in normal keratinocytes and previously passaged neonatal foreskin keratinocytes. The experiment showed no differences in either parameter.

In order to determine if this difference was sufficient to detect transformed cells among otherwise normal ones, we mixed both cell types in equal numbers and cultured them together. We randomly selected seven cells from this culture and measured their mechanical response. The curves for each cell are plotted in Figure 3c and show a clear bimodal distribution. In Figure 3d we present the calculated relative moduli for each of the cells. The relative magnitudes of the cell moduli correspond to either the normal or transformed cell moduli previously measured in the pure cell cultures (Figure 3b), suggesting that this technique can be used to easily identify transformed cells.

We also detected differences between normal and transformed dermal fibroblasts, when cultured on standard tissue-culture plastic for 48 hours in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were then stained for F-actin with phalloidin conjugated with Alexa fluor (green) and DNA stained with DAPI (blue). Confocal images are shown in Figures 4 a, b, and c for the normal and transformed fibroblasts cultured separately and together. Morphological differences between these cells were minimal. Both types of cells have well formed actin structures and occupy similar

![Figure 4: Normal human dermal fibroblasts compared to transformed human dermal fibroblasts. Confocal microscopy images of human dermal fibroblasts cultured from (A) normal, (B) transformed and (C) a mixture of the two cell lines, where actin fibers appear green and nuclei blue. In-situ AFM topography images are shown in (D) and (F) and lateral modulation AFM images in (E) and (G). Scan size: 80 x 80 µm; arrows indicate the imaged cell.](image)
In conclusion we have shown that one can measure the moduli of individual cells and their corresponding extracellular matrix. This technique works well on cultured dermal fibroblasts and epidermal keratinocytes. The results show large differences in the modulus between normal cells and transformed cells and their corresponding extracellular matrices and provide a powerful tool to study the effects of mechanics in triggering neoplasia.

REFERENCES