Atomic force microscopy can be used to mechanically stimulate osteoblasts and evaluate cellular strain distributions

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Abstract

In this study, atomic force microscopy (AFM) was used to mechanically stimulate primary osteoblasts. In response to mechanical force applied by the AFM, the indented cell increased its intracellular calcium concentration. The material properties of the cell could be estimated and the membrane strains calculated. We proceeded to validate this technique experimentally and a 20% error was found between the predicted and the measured diameter of indentation. We also determined the strain distributions within the cell that result from AFM indentation using a simple finite element model. This enabled us to formulate hypotheses as to the mechanism through which cells may sense the applied mechanical strains. Finally, we report the effect of the Poisson ratio and the cell thickness on the strain distributions. Varying the Poisson ratio did not change the order of magnitude of the strains; whereas the cellular thickness dramatically changed the order of magnitude of the cellular strains. We conclude that AFM can be used for controlled mechanical stimulation of osteoblasts and that cellular strain distributions can be computed with a good accuracy when the cell is indented in its highest part. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: AFM; FEM; Osteoblasts; Biomechanics; Calcium; Mechanical force

1. Introduction

The atomic force microscope (AFM) was invented in 1986 by Binnig et al. [1] and has, since then, been used extensively in many different fields and increasingly in biology. It has served in particular to give biology a sense of space by enabling the three-dimensional visualisation of biological phenomena such as mitosis [2], vesicle trafficking or cytoskeletal rearrangement [3,4]. Furthermore, due to its capability of applying very small controlled forces (<10pN), the AFM can be used as an ultra-precise indentor to determine the material properties of soft membranes [5] and biological materials [6]. A number of different cell types have been examined using this approach [7–13]. More recently, AFM has enabled the quantitation of the effects of cytoskeleton disrupting drugs on cellular material properties [14]. The use of AFM enables the examination of cellular mechanics through the use of engineering techniques via the measurement of the cellular material properties and with a high degree of spatial precision.

Virtually all cell types have been reported to adapt to their mechanical environment [15].
Amongst these, bone cells are particularly interesting as bone is a dynamic material that continually adapts its structure in response to mechanical usage [16]. This adaptive process is driven and effected by three types of bone cells: osteoblasts that form bone, osteoclasts that resorb bone and osteocytes that are embedded within the bone matrix. How bone senses the mechanical stimuli on the tissue level is still unclear. However, there is growing evidence that either the osteocytes or their precursors, the osteoblasts may act as a sensing system in bone [17,18]. Both cell types have been shown to be mechanically responsive in vitro [19,20] and in vivo [21,16].

Direct sensing of strain by bone cells could be mediated by stretch activated channels, cytoskeletal deformation or integrin cell adhesion receptors coupled to the cytoskeleton. Indeed, stretch-activated ion channels have been reported in many different cell types including osteoblasts [15–24]. Integrins are linked to the cytoskeleton which could, in turn, activate intracellular secondary messengers [25,26]. The cytoskeleton is perturbed as the bone matrix is deformed and may serve to amplify small strains through a tensegrity structure [26]. Many different events are triggered by application of mechanical strain to osteoblasts; one of the very early and easily measurable events is a rise in intracellular calcium levels [19,27,28].

A number of different techniques have been used to apply strain to cells ranging from: poking with a micropipette [19,28], movement of magnetic microbeads in a magnetic field [27], optical tweezers, stretching of the substrate [29], fluid flow or hydrostatic pressure. However, none of these techniques enables both a precise control of the force applied and the measurement of the mechanical properties at the point of application.

The goal of this study was to determine whether or not AFM could be used to mechanically stimulate osteoblasts and to examine the different mechanical aspects resulting from this deformation. To assess whether or not the cells had sensed the stimulus, we chose to monitor changes in the intracellular calcium levels during AFM indentation. We checked the validity of our method for calculating indentation diameter and gave an estimate of the precision of the strain computation. As the thickness of the cell under the area of indentation was not known, we examined the effect of varying the thickness on the resulting strains. Because the Poisson ratio of living cells remains unknown, we also examined the effect of varying the Poisson ratio on the cellular strains.

2. Methods

2.1. Cell Culture

Osteoblasts were isolated from the long bones of neonatal rats and cultured for 72 h at 37°C in an atmosphere of 5% CO₂ in air in DMEM (Gibco Life Technologies, Paisley, UK) supplemented in 10% FCS, 2% Glutamine, 2% PS, 2% 1 M HEPES and acidified with 86 μl of 11 N HCl per 100 ml.

2.2. Intracellular calcium measurements

To assess intracellular calcium intensity, the cells were incubated in medium for 1 h with 6 μM of the cell permeant calcium sensitive dye Fluo3-AM (Molecular Probes, OR, USA). The cells were imaged in physiological buffer (127 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 0.5 mM Na₂HPO₄, 2 mM CaCl₂, 5 mM NaHCO₃, 2 mM CaCl₂, 5 mM NaHCO₃, 10 mM glucose, 10 mM HEPES, 0.1% BSA adjusted to pH = 7.4). Intracellular calcium levels were assessed via a confocal scanning laser microscope (Bio-Rad Radiance 2000, Bio-Rad Microscience, UK) fitted onto a Nikon TE300 inverted microscope via the microscope side port [30]. A Nikon Neoplan objective with long focal distance and a 20× magnification was used for imaging. The Bio-Rad time course software was used to capture images of the cells at intervals of 1 s for as long as desired. The temporal evolution of the fluorescence intensity at several locations could be assessed and was used to determine variations in the intracellular calcium concentration.

2.3. Atomic force microscopy

We used a Thermomicroscopes Explorer AFM (Thermomicroscopes, CA, USA) interfaced onto
the inverted microscope as previously described [31]. The cantilever spring constant was determined in air as described by Hutter and Bechhoefer [32]. Glass beads (nominal diameter between 10 and 20 \( \mu m \), Sigma, UK) were glued onto the cantilevers as previously described [31] to act as indentors. It was assumed that gluing the glass beads onto the cantilevers had no significant effect on the cantilever spring constant. The size of the glass beads was on the same order of magnitude as the cellular dimensions after 3 days of culture (typically 50 \( \mu m \times 50 \mu m \)). Prior to each experiment a sensor response was taken on the glass surface to obtain the conversion ratio between nA and nN and the diameter of the glass bead was measured via the confocal microscope.

2.4. Experimental procedure

The AFM cantilever was positioned above the chosen cell, the confocal microscope data collection was started and, after 30 s, the cantilever was brought into contact with the cell surface. After 20 s in contact with the cell, a force-distance curve was taken to enable post hoc material property determination. The AFM cantilever was then retracted from the surface and the data collection was continued for a further 30 s to monitor any stress relaxation reactions.

2.5. Measurement of material properties

The material properties were evaluated using the indentation of a Hertzian half space with a spherical indentor [33,8]. The indentation resulting from the force \( F \) applied by spherical indentor onto the surface of a cell with the elastic modulus \( E \) is

\[
\delta_{\text{spherical}} = \left( \frac{3}{4} \frac{F(1 - v^2)}{E} \frac{1}{\sqrt{R}} \right)^{2/3}
\]

with \( R \) the radius of the spherical tip and \( v \) the local Poisson ratio. The loading force \( F \) can be obtained from the spring constant of the cantilever \( k \) and the deflection \( d \).

Let \( z \) be the distance travelled by the AFM head and \( z_0 \) be the height at contact. One can express the change in height as a function of the deflection \( d \), the initial deflection \( d_0 \) and the indentation \( \delta : z - z_0 = d - d_0 + \delta \). The equation of the force–distance curve after contact for the spherical indentor can be rewritten as (adapted from Ref. [9])

\[
z - z_0 = d - d_0 + \left( \frac{3}{4} \frac{k(1 - v^2)}{E} \frac{1}{\sqrt{R}} \right)^{2/3} (d - d_0)^{2/3}.
\]

A good fit of this curve can be obtained by taking two points of the curve and solving for \( E \) and \( z_0 \) [9]. The equation for the spherical indentor was rewritten as a third degree equation and solved exactly using Cardano’s method (described in Ref. [34]). The elastic modulus obtained is a measure of the compound local elasticity over the area of indentation. To automate this process, a program was written under Pw-Wave (Visual Numerics, CO, USA). In our experiments, force distance curves were taken after the AFM cantilever had been in contact with the cell for 20 s in order to minimise viscoelastic effects in the estimation of the local cellular modulus using the force–distance curve.

2.6. Determining the surface strains

From the elastic modulus and the force applied, one can calculate the strains applied on the surface of the cell by a spherical indentor using the Hertzian theory of contact. The total force \( P \) applied by a spherical indentor with a radius \( R \) to a surface with the elastic modulus \( E \) (determined from the force–distance curve) creates an indentation with a radius \( a \) [33]

\[
a = \left( \frac{3}{4} \frac{PR(1 - v^2)}{E} \right)^{1/3}.
\]

The relationship between the maximum pressure \( p_0 \) applied and the total load \( P \) applied by the AFM is [33]

\[
P = \frac{2}{3} \pi p_0 a^2 \Leftrightarrow p_0 = \frac{3}{2} \frac{P}{\pi a^2}.
\]

Thereafter, one can determine the radial displacements \( \delta_r \) on the surface of the elastic half-plane [33] and from those the radial strains \( \varepsilon_{rr} \) at the surface can be calculated for all radii \( r \) from the
centre of indentation:
\[
\varepsilon(r) = \frac{\partial \pi(r)}{\partial r} = \frac{(1 - 2v)(1 + v)}{3E} \frac{a^2}{r^2} 
\]
\[
p_0 \left\{ 1 - \left( \frac{r^2}{a^2} \right)^{3/2} \right\} 
\]
\[
- \frac{(1 - 2v)(1 + v)}{E} p_0 \left( 1 - \frac{r^2}{a^2} \right)^{1/2}, \quad r \leq a
\]
\[
\varepsilon(r) = \frac{\partial \pi(r)}{\partial r} = \frac{(1 - 2v)(1 + v)}{3E} \frac{a^2}{r^2} p_0, \quad r > a.
\]

The strain distribution under the area of indentation had both a compressive and a tensile component (Fig. 2). Both components were calculated for each cell. To automate the whole process, a program was written under Pv-Wave. For the numerical calculations, the following values were chosen: \( E = 10 \text{ kPa}, \quad v = 0.3, \quad R = 15 \mu \text{m}, \quad F = 1 \text{ nN} \).

2.7. Experimental validation

An experimental validation of the proposed model was undertaken. Cells were cultured in the same conditions as previously described and loaded with the cell-permeant green-fluorescent dye calcein-AM (Molecular Probes, OR, USA). The optical slice of the confocal microscope was reduced to its minimum size. The AFM cantilever was brought into contact with the cell of interest and was left to rest for 20 s. The microscope focus was adjusted such that the cell surface was visible. A darkened area corresponding to the area of indentation was grabbed. A force–distance curve was taken at the area of indentation. Post hoc, the local cellular material properties were estimated from the force–distance curve. The diameter of the indented area was measured using the Bio-Rad post-processing software and was compared with the diameter calculated from the Hertzian half space theory using the estimated Young’s modulus. The error was defined as

\[
\text{%Error} = \frac{|d_{\text{th}} - d_{\text{m}}|}{d_{\text{m}}} 100
\]

with \( d_{\text{th}} \) the theoretical diameter and \( d_{\text{m}} \) the experimentally measured diameter.

2.8. Error analysis

There are several sources of error that can affect our experimental results. We grouped the errors into three separate groups: \( e \) the error committed in the measurement of distances using microscope images, \( \gamma \) the error stemming from the estimation of forces with the AFM, and \( \xi \) a compound error stemming from several sources but in particular from the mismatch between the theory and the experiment due to fact that the cell is not a homogenous half space. One can rewrite the estimated sphere radius \( R_e \), the estimated total load \( P_e \), the diameter of the indented zone predicted by the theoretical calculations \( a_e \) and the experimentally measured diameter of the indented zone \( a_m \) obtained from the experiments as

\[
R_e = R(1 + e),
\]
\[
P_e = P(1 + \gamma),
\]
\[
a_e = a(1 + \xi),
\]
\[
a_m = a(1 + e + \xi)
\]

with \( R, P \) and \( a \) the exact values. All of the other parameters of interest can be rewritten as a function of \( R_e, P_e \) and \( a_e \). We assumed that the errors \( e, \gamma, \) and \( \xi \) were small compared to 1. The expressions for \( R_e, P_e \) and \( a_e \) can then be substituted into the formulae for \( \delta, E, p_0 \) and the surface strains. Keeping only the first-order errors \( e, \gamma, \) and \( \xi \), one can then rewrite the formulae to obtain an estimate of the precision of the half space theory applied to living cells. Briefly, excluding the second-order error terms, we find:

\[
\delta_e = \frac{a_e^2}{R_e} \approx \delta(1 - e + 2\xi),
\]
\[
E_e = \frac{3P_e R_e (1 - v^2)}{4a_e^3} \approx E(1 + e + \gamma - 3\xi),
\]
\[
p_{0e} = \frac{3P_e R_e (1 - v^2)}{2\pi a_e^3} \approx p_0(1 + e + \gamma - 2\xi).
\]
For the radial strains with \( r \leq a \), ignoring the error on the terms in \( (1 - (r^2/a^2))^{3/2} \), we get

\[
\varepsilon_{\text{me}}(r) \approx \frac{(1 - 2v)(1 + v) a^2}{3E} \frac{r^2}{p_0} \left\{ 1 - \left( 1 - \frac{r^2}{a^2} \right)^{3/2} \right\} \\
(1 - \varepsilon + 3\xi) - \frac{(1 - 2v)(1 + v)}{E} p_0 \times \left( 1 - \frac{r^2}{a^2} \right)^{1/2} (1 - \varepsilon + \xi), \; r \leq a
\]

A conservative estimate for the error on radial strains within the indentation zone is \( \varepsilon + 3\xi \). From the experimental validation, one can obtain estimates for \( \varepsilon \) and \( \xi \). The error when measuring a length from an optically acquired image is a half pixel on either side, leading to a total error of one pixel. With a \( 20 \times \) objective, the width of one pixel is 1.22 \( \mu \text{m} \). Therefore, an estimate of the optical measurement error \( \varepsilon \) is \( \varepsilon \approx 1.22/d_x \). Since the total error on the experimentally measured diameter \( d_m \) is \( \varepsilon + \xi \), one can obtain an estimate of the theoretical mismatch error \( \xi \) knowing \( \varepsilon \).

2.9. FEM modelling

Because the theoretical solution giving the strain distribution within the thickness of the elastic half-space is not easily solvable and because these distributions may help to determine candidate mechanisms of transduction of the mechanical stimulus, a finite element model of the indented half-space was established. Taking the symmetries of the problem into account, only one quarter of the space was modelled. A box 15 \( \mu \text{m} \) in length in the \( x \)- and \( y \)-directions and with a thickness \( t \) was meshed with eight-noded parametric volumic elements with a higher density of elements in the area of indentation paying particular attention to the boundary between the indented and the non-indented regions. The model was constrained in displacement in the \( z \)-direction at its base and in the \( x \)- and \( y \)-directions, respectively on the \( y \)- and \( x \)-sides in which the indentation was performed [35]. The other sides were left free. The cellular material was assumed to be elastic linear isotropic and the modulus was chosen to be 10 kPa. The Poisson ratio \( v \) was varied between 0.2 and 0.5 to examine the effect of the Poisson ratio on the strain distributions. The thickness \( t \) was varied between 0.25 and 5 \( \mu \text{m} \) to examine the effect of cell height on the strain distribution.

All of the finite element calculations were carried out with CASTEM 2000, a general purpose finite element solver with an integrated pre- and post-processor (CASTEM2000, Commissariat à l’Energie Atomique, Saclay, France, http://www.castem.org:8001/HomePage.html) and was run on an SGI O2 workstation (SGI, CA, USA).

2.10. Statistics

All statistics were performed with a paired \( t \)-test on two populations. Results were deemed statistically significant when \( p < 0.001 \).

3. Results

3.1. Cellular reactions

Cellular reactions were observed in 25% of the cases \((n = 231)\). The mean strain induced in the reacting cells was on the order of 30000\(\mu\varepsilon\) for the tensile component of the strain distribution and –50000\(\mu\varepsilon\) for the compressive component, and the mean deformation induced in the non-reacting cells was on the order of 25000\(\mu\varepsilon\) and –35000\(\mu\varepsilon\). Both of these values were significantly different from the reacting population to the non-reacting population.

Fig. 1 shows a typical positive experiment. Fig. 1a shows the cells prior to stimulation. The cell about to be stimulated is shown by the white arrow (A). Fig. 1b shows the cells post stimulation. The stimulated cell (B) has a markedly increased intracellular calcium concentration in 1b when compared to 1a. Fig. 1c shows the time course of the experiment (TD: Touch down, FD: Force distance, LO: Lift off, A: image 1a corresponds to this time point, B: image 1b corresponds to this time point). In all experiments, the cells reacted immediately after being stimulated. In some of the experiments, the cells reacted as the pressure was removed.
3.2. Surface strains

Both compressive and tensile strains are present in the strain distribution. With the numerical values chosen, the indentation depth was 67.7 nm, the radius of contact was 1 μm, the maximal strain was 8100 με and the minimal strain was −12200 με (Fig. 2).

3.3. Experimental validation and error analysis

The predicted and the measured indentation diameters were closely related in the 23 cells examined. Fig. 3 shows a typical validation experiment. The darkened indented area on the top of the cell can clearly be seen. For this experiment, the force applied was 20 nN, the spring constant of the cantilever was 0.043 N m⁻¹, the radius of the indentor was 14.5 μm. The estimated elastic modulus was 2100 Pa. The estimated diameter was 9.1 μm and the measured diameter was 9.5 μm yielding an error of 4%. The predicted diameter underestimated the measured diameter by an average of 20% (n = 23).
measurement error $\varepsilon$ averaged 13% ($n = 23$). An estimate of the theoretical error $\dot{\varepsilon}$ was therefore 7%. Assuming $\gamma$ is 10%, we could use these numerical values to calculate an upper limit of the estimated errors on $\delta$, $E$, $p_0$ and the surface strains. These results are summarised in Table 1.

3.4. Strain distributions

The strain distributions calculated by FEM were in good agreement with the theory for strains at the elastic half-plane surface.

The maximal absolute value of all three strain parameters are on the same order of magnitude.

The maximal and minimal radial strains (Fig. 4a) are found on the surface and a large strain gradient is present at the interface between the indented region and the non-indented region. Tangential strains (Fig. 4b) are minimal on the cell surface and maximal just under the area of indentation. However, the maximal tangential strains are one order of magnitude smaller than the tangential minimal strains. This yielded a large variation in the $z$-direction. The vertical strains (Fig. 4c) are maximal just under the area of indentation. Fig. 4d shows the deformation of the surface after indentation is amplified 15 times.

3.5. Influence of the Poisson ratio and the cell thickness on the strain distribution

The Poisson ratio had relatively little influence on the order of magnitude of the strains applied to the cell (Fig. 5a). However, the effect was very different depending on the directions. The radial strains ($\epsilon_\tau$) changed only by 30% over the whole range. Tangential strains ($\epsilon_\tau$) changed drastically but at their maximum remained on the same order of magnitude as the radial strains. The minimal vertical strains ($\epsilon_z$) changed by only 12%.

The effect of cell thickness on the magnitude of strains was dramatic (Fig. 5b). For thicknesses smaller than 2 $\mu$m, the magnitude of all of the strains increases greatly. For thicknesses greater than 2 $\mu$m, there is little change in the magnitudes of the strains. This was to be expected as the half space approximation is valid for thicknesses greater than twice the radius of indentation [33]. The radial strains, however, stay relatively un-

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Table 1
Experimental estimation of the accuracy of the half space model applied to live cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Error</th>
<th>Numerical value of error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphere radius</td>
<td>$\varepsilon$</td>
<td>13</td>
</tr>
<tr>
<td>Force estimation</td>
<td>$\gamma$</td>
<td>10 (hypothesised)</td>
</tr>
<tr>
<td>Indentation diameter (experimentally measured)</td>
<td>$\varepsilon + \dot{\varepsilon}$</td>
<td>20</td>
</tr>
<tr>
<td>Indentation diameter (predicted)</td>
<td>$\dot{\varepsilon}$</td>
<td>7</td>
</tr>
<tr>
<td>Indentation depth</td>
<td>$\varepsilon + 2\dot{\varepsilon}$</td>
<td>27</td>
</tr>
<tr>
<td>Young’s modulus</td>
<td>$\gamma + \varepsilon + 3\dot{\varepsilon}$</td>
<td>24</td>
</tr>
<tr>
<td>Maximal pressure</td>
<td>$\gamma + \varepsilon + 2\dot{\varepsilon}$</td>
<td>17</td>
</tr>
<tr>
<td>Surface strain, $r \leq a$</td>
<td>$\varepsilon + 3\dot{\varepsilon}$</td>
<td>34</td>
</tr>
<tr>
<td>Surface strain, $r &gt; a$</td>
<td>$\varepsilon + 3\dot{\varepsilon}$</td>
<td>34</td>
</tr>
</tbody>
</table>
changed until a thickness equal to the radius of indentation (1 μm).

4. Discussion

In this study, AFM was used to apply a known force onto osteoblasts sufficient to elicit an intracellular calcium response. The elastic modulus of the cells was calculated and the strains on the cell surface and in the cellular thickness could be derived either using the Hertzian indentation theory or using finite element analysis. The technique used was experimentally validated and estimates of the errors were given. The effect of the Poisson ratio and the cellular thickness on the magnitude of strains were evaluated.
There have been different techniques employed to apply sufficient mechanical strain onto cells for them to increase their intracellular calcium levels. AFM, however, offers several advantages. Poking with a micropipette [19,28] offers little or no control on the force applied and may puncture the cell. Magnetic microbeads [27] and optical tweezers enable the application of a controlled force but not the measurement of the elastic modulus of the underlying cellular material and therefore one cannot evaluate the induced deformations. Stretching of the substrate [29], fluid flow or hydrostatic pressure offer little control on the deformations applied to the cell. AFM not only offers the possibility to apply a very precise controlled force sufficient to elicit an increase in intracellular calcium, but also enables the measurement of the underlying elastic modulus and, hence, the evaluation of the induced deformations. Furthermore, AFM indentation has been shown to be innocuous to cells [36,37].

The absolute value of the strains needed to elicit an intracellular calcium reaction was in the order of 40000 με and was significantly higher than that applied to the cells that did not react. The cells reacted immediately after being indented with the AFM tip and in some cases reacted after the AFM tip was lifted from the surface. There is a large body of literature concerning the straining of osteoblasts via the substrate; the strains applied vary widely (from 250 με to 240000 με) and have a variety of effects. Changes in intracellular calcium levels have only been reported for strains in excess of 5000 με [38,39]. However, there may a significant difference in the way that cells sense strain in substrate stretch experiments compared with AFM indentation. In the substrate stretch studies, strain is relayed to the cell interior via focal adhesions which are connected to the cellular cytoskeleton; in contrast in our experiments there is no attachment between the cell surface and the AFM probe. Hence, different mechanisms may be involved.

The precision of our technique was estimated and we found a good correlation between the predicted values for the radius of indentation and the experimentally measured ones. Furthermore, we were able to derive an estimate of the accuracy of the predictions of the elastic modulus and the surface strains. The difference between the predicted and the experimentally determined indentation diameter was 20%, the error on the elastic modulus was 24% and the inaccuracy on surface strains was 34%. However, one major source of error appears to be the actual optical measurement of lengths. This error could easily be reduced by effecting the measurements with a higher magnification objective. For example an objective with a 100x magnification would bring the measurement errors down to approximately 2%. A more in depth study should be carried out using this magnification. None the less, the estimated error due to the homogenous half-plane approximation underlines the necessity to find a more realistic model than the Hertzian half space model in order.
to describe living cells. Several limitations can be pointed out. Firstly, the cell membrane is a complex structure; it is best described as a fluid bilayer that is not uniformly tethered to the cytoskeleton and can equilibrate independently of the cytosol. Secondly, the cell is by no means homogeneous and isotropic, but possesses a complex cytoskeleton comprising of a highly interconnected network of fibres. Furthermore, living cells exhibit viscoelastic properties.

The strain distributions could be determined throughout the cell thickness using finite element modelling and were in good agreement with the theory. This enables us to formulate a number of hypotheses regarding the way in which the cells sense the applied strains. The maximal and minimal radial strains were located on the cell surface and there was a high strain gradient at the interface between the region where the AFM tip was in contact with the cell and the region where there was no contact. This could suggest a detection mechanism mediated by the cell membrane. Stretch-activated calcium channels that open in response to membrane strain, and let extracellular calcium into the cell, are prime candidates. Furthermore, as there is a high gradient at the interface between the region of indentation and the unindented region, the strain varies greatly in amplitude over a short distance possibly exerting sufficient strain to open the small stretch activated ion channels. The vertical strain distribution shows a large compressive strain just under the area of indentation. In the cell, the submembranous area is a prime area of connection between the cell membrane and the cellular cytoskeleton [40]. Therefore, the signal may be relayed through a direct deformation of cellular cytoskeleton, which may in turn activate mechanisms that increase intracellular calcium levels.

The effect of the Poisson ratio and the cellular thickness on the magnitude of strains was examined as both of these variables remain unknown. To date there has been no definite measurement of the Poisson ratio of living cells. As cells are mainly composed of water, it may be argued that, on the time scale of the experiments, they are mainly incompressible and therefore have a Poisson ratio of 0.5. However, Maniotis et al. [41] reported a cytoplasmic Poisson ratio of 0.25 to 0.3 in micromanipulation experiments. Guilak [42] visualised changes in volume in chondrocytes located within an hemi-cylinder of cartilage being compressed, and suggested that the Poisson ratio is not 0.5 for living cells. The Poisson ratio only had a marginal effect on the magnitude of the highest deformations yielding changes of up to 30%. Therefore, knowledge of its exact value may not be crucial in our case. The thickness of the cell at the point of indentation cannot be examined as we lack a reference point to measure it. This is unfortunate as this information is crucial: the strains elicited by the indentation increase greatly for thicknesses that are comparable to the radius of indentation. As a general rule, in order to obtain meaningful results, one must try to indent the cells on their highest parts in order to have an adequate precision in the evaluation of the cellular strain distributions, when using the elastic half-space approximation.

In summary, using AFM we were able to elicit intracellular calcium responses in primary osteoblasts and evaluate the magnitude of the strains elicited both on the cell membrane and in the cellular material. The determination of the strain distributions suggested two possible detection mechanisms. One based on the detection of radial strains via stretch activated channels and the other based on the detection of vertical strains by the cytoskeleton. Also, in order to obtain a reasonable accuracy in the evaluation of the cellular strains, we established that the cells should be indented at their highest parts. Future studies will concentrate on determining the regulatory pathway that mediates the increase in intracellular calcium, as well as modulating the cellular sensitivity to mechanical forces.

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