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Structural responses of cells to intracellular magnetic force induced by superparamagnetic iron oxide nanoparticles

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Abstract

In this paper, we study the effects of intracellular force on human umbilical vein endothelial cells. We generated intracellular force on endothelial cells under different magnetic fields using the cell uptake of superparamagnetic iron oxide nanoparticles. Cell responses to intracellular force were observed using fluorescent microscopy. Our results indicated that nanoparticles were taken up by the cell by endocytosis and were deposited in lysosomes. Nanoparticles and lysosomes inside the cell could be relocated by the application of a magnetic force. The intracellular magnetic force could also be used to accelerate cell migration by adjusting the magnetic fields and giving the cell free culture space. No cytotoxicity of nanoparticles was found in our experiments. By comparing intracellular relocation with migration of the whole cell, we obtained a better understanding of the self-defence mechanisms of cells based on their mechanical properties. Based on the promising mechanical properties and low cytotoxicity of our magnetic nanoparticles, their potential applications in cytomechanics and cell patterning are discussed.

1. Introduction

It is well known that nanoparticles (NPs) can be taken up by cells. In recent years, studies have focused on the effects of NPs with different sizes, uptake amounts and surface modifications.^{1–6} Cells take up NPs in the size range 10–200 nm.⁷ Cells can take up NPs at concentrations measured in pg iron per cell,^{2,8,9} which can be controlled by changing NP density in the culture medium and the co-culture time. NPs have been successfully coated in different types of lipids and polymers for use in cellular studies.^{6,10,11} Most studies analyze cell effects after NP uptake by measuring cell viability and topology, such as shape and size.

The cellular uptake of specific NPs can be used in medical applications. Magnetic nanoparticles (MNPs) have been widely used in cellular and organism studies^{8,12} and have been applied successfully, for instance, in *in vivo* tumor targeting^{13,14} and drug/modified-cell delivery.^{7,14,15} MNPs or cells containing MNPs can be precisely guided to target

locations in animal models using an additional magnetic field, generating a high local density of MNPs and eliminating non-specific effects. The release of MNPs or MNP-containing cells can be achieved quickly by changing the magnetic field. MNPs have therefore become a popular new biomaterial in research and applications.¹⁶ A magnetic field can be applied to MNPs inside cells, generating an intracellular force. Most studies consider the cell as a whole system^{2,17} and little attention has been paid to the forces between MNPs and intracellular structures. How mechanically sensitive cells, such as endothelial cells, respond to intracellular forces is an interesting topic.

Endothelial cells are mechanical-force-sensitive cells that line the inside surface of vessels. They sense hemodynamic force and align in a specific direction along the shear flow. Normal functions and pathological changes in endothelial cells are influenced by extracellular force in their micro-environment.^{18–20} Mechanotransduction of endothelial cells has been intensively studied for many years. Mechanical force has been applied to cells both *in vivo* and *in vitro* in mechanotransduction studies. Forces applied to the outside surface of the cell membrane trigger a series of responses which form the mechanical signal transduction pathway and explain how the cell senses mechanical force. Mechanotransduction responses have been observed and measured at different levels, including gene expression,²¹ protein expression,^{21,22} protein conformation,²³ cytoskeleton and cell alignment.^{20,21,24} Although the effect of extracellular mechanical force on endothelial cells has been extensively studied, the effect of intracellular mechanical force on endothelial cells is still poorly understood.

The effect, cytotoxicity and potential applications of intra-cellular mechanical force on endothelial cells are therefore an open area for research. In this paper, we studied the effects of intracellular force on Human Umbilical Vein Endothelial Cells (Huvec) and its potential applications. The subcellular location of superparamagnetic iron oxide nanoparticles (SPIOs) was verified. Cell responses on the cellular and subcellular levels, including intracellular relocation and cell migration, were observed and are discussed. We present an improved understanding of cells' mechanical properties and self-defense ability and discuss the potential applications of MNPs in cytomechanics and cell patterning.

2. Experimental methods

2.1 SPIO staining

SPIO nanoparticle synthesis and coating methods were described in previous publications.^{25,26} Nanoparticles were characterized by electron microscopy and light-scattering. The size of SPIOs was 15 nm before coating and 30 nm after coating. SPIO cores were coated with DSPE-PEG2000. The magnetization of SPIO cores was measured by using a SQUID magnetometer. The saturation magnetization was $\sim 80 \text{ emu g}^{-1}$. For fluorescence imaging, SPIOs were stained with a hydrophobic red dye DiI (D-282, Life Technologies, USA). As a hydrophobic dye, DiI will get into the lipid coating layer on SPIO and the staining is stable in an aqueous solution. $100 \mu\text{g ml}^{-1}$ coated SPIOs and $5 \mu\text{g ml}^{-1}$ DiI were co-incubated overnight at room temperature with shaking and shading from light. To remove free dye from the solution, a $0.2 \mu\text{m}$ Tuffryn membrane filter (Pall Life Sciences, USA) was used. Free dye adheres to the Tuffryn membrane by hydrophobic interactions, whereas dye

in the nanoparticle coating layer passes through the filter. The SPIO–DiI sample was filtered five times to remove the free dye. Stained SPIO was stored at 4 °C.

2.2 Quantification of SPIO uptake and cell viability

Cells were cultured in a 1-well-chamber until confluence. The SPIOs were then diluted in cell culture medium to a final concentration of 25 $\mu\text{g ml}^{-1}$ and added to the cell chamber. The cell chamber was then incubated in a cell incubator for 24 hours on a magnetic plate (MF14000, OZ biosciences). The cell chamber was moved several times on the magnetic plate during incubation for a more uniform uptake over the whole chamber. Cells were then detached from the cell chamber using a trypsin–EDTA solution. To quantify SPIO uptake, the cell density was determined using an automated cell counter (TC10, BioRad, USA). Cells were collected by centrifugation and supernatant removal. Cell samples were then placed in a vacuum oven overnight and dried out. The quantity of iron in the dried cell samples was determined using 0.1% ferrozine solution as previously described.^{25,26} Cells were placed in a 24-well-plate with the cell density for 50% confluence to determine cell viability. Cell viability was quantified using MTT (V13154, Life Technologies, USA) after uptake and one day post-culture. Native cells were used as a negative control.

2.3 Magnetic force generation and cell migration

To generate a transverse magnetic force on SPIO inside cells, a pair of antiparallel magnet bars (BY044, K&J Magnetics, Jamison, PA) (Fig. 3(a)) was placed under the cell culture chamber after inducing SPIO uptake. Cells were incubated for 1 day under the magnetic field in a cell incubator, followed by staining and imaging. To study cell migration, cells were cultured in a 1-well-chamber as before. After incubation to confluence, SPIO uptake was performed only on cells above the magnet interface, as shown in Fig. 4. After SPIO uptake, half of the cells were removed as shown in Fig. 4(c). A magnet block (B884, K&J Magnetics, Jamison, PA) was then placed under the cell culture chamber (Fig. 4(c)), creating a magnetic field gradient. Cells were imaged and analyzed after 1 day incubation.

2.4 Cell staining and imaging

For micro-scale observations, subcellular structures were stained green. Lysosomes were stained using CellLight® Lysosomes-GFP (C10507, Life Technologies, USA) using the manufacturer's protocol. The dye was diluted in a cell culture medium and incubated with cells overnight. Cells were then washed in fresh medium and the protocol for uptake of stained SPIO followed. Actin filaments and microtubules were stained green with Alexa Fluor® 488 Phalloidin (A12379, Life Technologies, USA) and Tubulin Trackert™ Green (T34075, Life Technologies, USA) respectively after SPIO uptake, following the manufacturer's protocol. To stain actin filaments, cells were fixed and stained for 20 min at room temperature. To stain microtubules, live cells were stained for 30 min in a cell incubator after SPIO uptake. Fluorescence images were obtained using a DeltaVision microscope (Olympus, Japan) and a confocal microscope (LSM 510 UV Confocal, Zeiss, Germany). For macro-scale observations, cells were stained with methylene blue. Large-scale images were taken by camera. All fluorescent image processing was performed using the software ImageJ.

3. Results and discussion

3.1 SPIO uptake pathway and intracellular location

The quantity of SPIO taken up by cells was calculated using cell density and iron concentration. There are approximately 23 000 MNPs per cell, which is roughly 0.63 pg ferrous oxide per cell. The cytotoxicity values measured immediately after uptake and one day post-culture were similar to the negative control, indicating that SPIO uptake in the pg range was safe for Huvec. Although the MNP uptake was much lower than that in previous studies,² the magnetic force generated was sufficient for use in the following studies.

The SPIO uptake pathway was verified by fluorescent imaging. The outermost PEG layer of the DSPE-PEG2000 coating results in rare interactions between the cell membrane and the surface of the MNPs. Cell endocytosis was hypothesized to facilitate SPIO uptake, based on the properties of the SPIO coating and previous studies.^{27,28} SPIOs were therefore hypothesized to localize in the lysosomes, as they are the end-point of cell endocytosis. Lysosomes and SPIO were stained green and red respectively for fluorescent imaging to test this hypothesis, as shown in Fig. 1. Green channel signals from the lysosomes were localized around the cell nucleus, as expected. Red channel signals from SPIO appear to localize in the same place. The merged image shows the colocalization of the two channels. As shown in the enlarged image (Fig. 1(d)), SPIO (red dots) are contained inside the lysosomes (green circles), verifying that SPIOs were taken up *via* the cell endocytosis. Red fluorescent background signals are weak, which indicates that the majority of the SPIOs were taken up *via* this pathway and it is rare for SPIOs to adhere to the cell surface or to be trapped in the cell membrane.

3.2 Lysosome relocation is controlled by magnetic force

SPIO-containing cells were placed under a magnetic field to show the effects of a transverse magnetic force. Fig. 2(c) shows the native state of the microtubules (green) in a cell without SPIOs. The black holes surrounding the cell nucleus are lysosomes. When SPIOs are taken up, the black holes are filled with red SPIO dots (Fig. 2(b1) and (b2)), which is consistent with Fig. 1. There is no difference in the size, shape and location of lysosomes before and after SPIO uptake.

After a magnetic force was exerted (Fig. 2(a1) and (a2)), the lysosomes and SPIOs were dragged to one side of the cell along the direction of magnetic force. In the area above the interface of the magnet pair (Fig. 3(a)), we observed the intracellular location of SPIOs at different distances from the interface, as shown in Fig. 3(c)–(i). In Fig. 3(d) and (e), which are 0.5 and 0.25 mm to the left of the interface respectively, SPIO MNPs and lysosomes were dragged to the right side of the cell, where they gathered at high density. The opposite was observed on the right side of the interface (Fig. 3(g) and (h)). In Fig. 3(f), which is directly above the interface, transverse magnetic forces are balanced. As a result, lysosomes are aligned across the whole cell in the direction of the transverse force. As the distance from the interface increases, the lysosome displacement decreases, until lysosomes surround the cell nucleus as in the native condition (a distance of 1.75 mm, Fig. 3(c) and (i)). Fig. 3(j)

summarizes the distance dependence of SPIO–lysosome relocation. A nanoscale SPIO can be considered a magnetic dipole. The magnetic force on a magnetic dipole is proportional to the gradient of the applied magnetic field. Fig. 3(b) shows the magnetic field of the imaging area, in which the decrease of gradient with the increase of distance is very clear. The magnetic force vanishes within 1.75 mm, which is consistent with calculations and previous publications²⁹ on nanoscale magnetic force.

The mechanical properties of SPIOs make them attractive for use in cytomechanics. Cytomechanics has been an area of research for the last thirty years. Researchers have focused on the mechanical properties of the cell, how cells sense force and how mechanical signals are transferred inside the cell. Many methods have been used to apply mechanical forces to the cell membrane, such as shear flow,^{18,19} optical tweezers^{30,31} and AFM.^{32,33} Although there are many methods for applying force to the outside of a cell, applying a force to a component inside the cell requires more elaborate mechanical methods. The use of MNPs can overcome the limitations of traditional methods. The mechanical properties and responses to intracellular localized force of a certain organelle or cell structure details can be studied. We use DSPE-PEG2000 coated SPIO MNPs, which are taken up by the cell through endocytosis and are stored in lysosomes. Antibodies and other biological molecules can be conjugated onto the coating layer, targeting the MNPs to other organelles or intracellular structures. Thus, the magnetic MNP method has potential as a general method to apply a localized intracellular force on different cell components to study the cell's responses to distinguishable intracellular force.

3.3 Cell migration is controlled by magnetic force

The studies mentioned above used cells incubated to confluence with little free space, which cannot easily move or change shape. We did not observe obvious changes in cell or actin filament alignment, unlike previous shear flow studies.^{18,20,34} Shear flow is performed on the whole cell culture surface, whereas here, magnetic force was only localized in the area above the magnet interface. When force is applied to the whole cell culture surface, the cells' response is coincident. However, there will be hindrance from neighboring cells if some cells try to make changes as part of a localized effect. To confirm this, we investigated the effect of giving cells free culture space.

After SPIO uptake, half of the cells were removed to give free space. A magnet block under the cell culture chamber (Fig. 4(c)) created a magnetic field (Fig. 5(b)) gradient, which exerted a magnetic force on the SPIOs. After 1 day of incubation, the cells were imaged and analyzed as shown in Fig. 5. A small bump formed over the magnets. In the absence of a magnetic force, cells tend to migrate to a less crowded place. Thus, the bump indicates that the magnetic force promoted cell migration in certain directions. Different points of the bump showed different alignment directions. Points 1–3 in Fig. 5(a) were observed by white light (Fig. 5(c)) and fluorescent imaging (Fig. 5(d)). Large numbers of cells were observed at point 2 (Fig. 6). As shown in Fig. 5(c), migrating cells had a long shape and pointed in the direction of the magnetic force. The shape change is especially obvious close to the magnets, as shown in Fig. 6(b) and (c). This implies that cell migration in the bump was induced by the magnetic force and the migration direction was the same as the force

direction. Fluorescent images of the actin filament structure are shown in Fig. 5(d) and 6(a). Obvious actin filament alignment in the direction of the magnetic force was observed. Actin filaments align uniformly during cell migration under a localized magnetic force, which is similar to the effect of shear flow. The results infer that actin filaments in the cytoskeleton not only sense extracellular forces like shear flow, but also sense the force generated inside the cell. As a mechanical structure of the cell, the cytoskeleton maintains the mechanical properties of the cell and protects its inside against forces both outside and inside the cell.

Relocalization of SPIOs–lysosomes was observed in confluent cultured cells; however, it was not observed in the cell migration study. As shown in Fig. 6(a), SPIOs (red) still surrounded the cell nucleus of migrating cells. We estimated the magnitude of the force in the relocalization and migration studies and found that the gradients of the magnetic fields were similar (Fig. 3(b) and 5(b)). The difference between the two studies is due to something other than force magnitude and is probably related to the mechanical property of the cell. When cells are not allowed to move freely, SPIOs and lysosomes are displaced under the force. When the cells are allowed to move freely, the force exerted on SPIOs causes the entire cell to move, rather than any intracellular displacement. Cells appear to prefer moving as an entirety rather than undergoing intracellular displacement, which is also a self-defense method to maintain the mechanical structure of the cell as a system.

Protein expression, protein–protein binding and protein–ligand binding are all involved in cell migration under shear stress.^{35,36} A force exerted on the outside surface of the cell membrane causes a series of molecular responses and results in cell “rolling” and “slipping.” Protein expression related to cell migration is affected by the uptake of a large quantity of MNPs. Here, the force exerted on the inside surface of the cell membrane guided and accelerated cell migration. Compared to forces on the outside of the cell membrane, forces inside the cell apply more specifically to a certain cell and can guide cell migration in a specific direction.

In this study, the movement of attached cells was controlled by magnetic force. Compared to attached cells, cells in suspension should be easier to control and trap. Thus, intracellular MNPs can also be used to trap cells at a certain location for cell patterning. Early studies on magnetic cell patterning have been promising.^{9,37} Nanomagnetic cell patterning has less limitations on cell type and patterning scale than traditional cell patterning methods based on microfabrication and biochemistry.^{38,39} Most anchorage-dependent cells can easily be induced to take up MNPs, which has potential applications in cell co-culture. Nanomagnetic cell patterning can reach the single-cell level of resolution and is also competent for large scale patterning. Drug-conjugated MNPs have been used for disease treatment *in vivo* by guiding MNPs to the target site using a magnetic field.⁴⁰ MNPs can be used as an *in vitro* model for early stage drug testing, which is less time and money consuming than *in vivo* models. Drugs or other compounds can be treated locally in different areas of the cell culture surface. Treated and untreated cells can be studied in parallel. Examples of possible applications are growth and migration rate differences, the interactions between treated and untreated cells, and drug release and delivery on the cell culture surface. By combining cell patterning co-culture and localized treatment, a complicated cell model can be formed *in vitro* for tissue engineering and biomedical engineering.

4. Conclusion

MNPs have promising advantages for biomedical research and application. MNPs can be used to apply mechanical force inside the cell. We used PEG-coated SPIO MNPs to study the effects of intracellular force on the endothelial cell Huvec. In the pg per cell SPIO uptake range, MNPs showed little cytotoxicity and were contained in lysosomes surrounding the cell nucleus. The cell's responses to the application of a magnetic force were observed, including lysosome relocation, actin filament alignment and cell migration. The cell's mechanical responses under intracellular force have potential applications. MNPs can generate force inside the cell at a specific location and in a specific direction. This is a novel way to study cytomechanics on distinguishable intracellular forces. The use of MNPs in cell patterning offers high resolution and flexible patterning size and can cover a number of cell types. The use of MNPs in traditional technologies will take advantage of MNPs' outstanding properties and overcome some of the limitations of other methods.

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Fig. 1.

Fluorescent image of lysosomes and SPIO inside cells after SPIO uptake. (a) The fluorescent image from the green channel, showing lysosomes stained with Cell Light. (b) The fluorescent image from the red channel, showing SPIO stained with DiI. (c) Images from the green and red channel are overlaid, showing lysosomes (green) and SPIO (red). (d) Enlarged version of the overlaid image, showing lysosomes (green) and SPIO (red).

Fig. 2.

Confocal image of microtubules and SPIO inside cells. (a1) and (a2) show cells after SPIO uptake and magnetic force exertion, (a1) shows microtubules stained with Tubulin Tracker (green) and (a2) shows micro-tubules (green) and SPIO stained with DiI (red), by merging images. (b1) and (b2) show cells after SPIO uptake, without magnetic force exertion, (b1) shows microtubules stained with Tubulin Tracker (green) and (b2) shows microtubules (green) and SPIO stained with DiI (red), by merging images. (c) shows cells without SPIO.

Fig. 3.

Intracellular relocation of SPIO and lysosomes under magnetic force. (a) Sketch map of a magnet pair placed under the cell culture chamber. (b) The magnetic field profile of a magnet pair placed in the cell chamber. The field positions are 0.172 mm above the magnets, which is the z -position inside cells. (c)–(i) Cell images at different distances above the magnet pair. The distances from the magnet interface are shown at the bottom. (j) Sketch map of SPIO intracellular relocation.

Fig. 4. Sketch map of cell migration. (a) Cells were incubated confluent. (b) A pair of antiparallel magnet bars was then placed under the cell chamber. (c) SPIO uptake was induced. (d) Half of the cells were then removed and a magnet block was placed under the cell chamber. (e) Cell migration was observed after 1 day incubation.

Fig. 5.

Results of cell migration. (a) Cells stained with methylene blue. The migration area is enlarged in the image on the right. (b) The magnetic field profile of a magnet block placed under the cell chamber. The field positions are 0.172 mm above the magnets, which is the z-position inside cells. Indicated positions were imaged as shown in (c) and (d). (c) White light cell images at different positions of the migration area. (d) The fluorescent image of the Alexa Fluor® 488 stained actin filament and the DiI stained SPIO. The arrows at the bottom show the direction of the magnetic force.

Fig. 6. Actin filaments and cell alignment at point 2 in Fig. 5(a). (a) The fluorescent image of the Alexa Fluor® 488 stained actin filament and the DiI stained SPIO. (b) and (c) the white light cell image.