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Monitoring of hormonal drug effect in a single breast cancer cell using an estrogen responsive GFP reporter vector delivered by a nanoneedle

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ABSTRACT

In this study, we have evaluated a sensor system for a hormonal drug effect in a single cell level using a novel low invasive single cell DNA delivery technology using a nanoneedle. An estrogen responsive GFP reporter vector (pEREGFP9) was constructed and its estrogenic response activity was confirmed in breast cancer cells (MCF-7) using lipofection as the means of transferring the vector to the cells. The pEREGFP9 vector was delivered to a single MCF-7 using a nanoneedle and the effect of ICI 182,780, which is an antagonist of estrogen, was observed using the GFP expression level. By ICI 182,780 treatment, the fluorescence intensity of the GFP was decreased by 30–50% within 24 h. This technology is the very first trial of single cell diagnosis and we are looking forward to applying it to precious single cell diagnosis in medical fields.

1. Introduction

Nanotechnological devices for manipulations and biosensors have great potential to enable diagnosis at the single cell and molecular levels. Many research groups have incorporated these in current molecular diagnostic methods, such as biochips (Konry et al., 2007; Polte et al., 2007; Wu et al., 2007), nanoparticles (Kang et al., 2007; Obermajer et al., 2007) and various other nanotechnological devices. All of these methods are promising for potential clinical applications.

Single cell diagnosis based on the application of cell arrays (Cheng et al., 2006; Dohler et al., 2008), microinjection (Yano et al., 1997) or patch clamp (Fischer and Montal, 2007) are desirable because the behaviour and changes of single cells or molecules can be monitored in real time with these methods. However, current methods have some limitations, for example invasiveness of target cells and delivery of relatively extremely large amounts of objective molecules to the target cells.

Development of the human breast cancer cell line MCF-7 is promoted by estrogen stimulation (Chau et al., 1998; Klinge et al., 2001). Estrogen binds to its receptor (ER) inside the nucleus and the estrogen–ER complex interacts directly with estrogen response elements (ERE) (Gruber et al., 2004; Klein-hitpab et al., 1986). ER also regulates transcription factors such as Sp1, c-Jun, and activator protein-1 (McDonnell and Norris, 2002). The activated ER recruits multiproteins such as Ciz1 or p160CoA (den Hollander et al., 2006; Weldon et al., 2004) by dependence upon ER concentration, resulting in gene expression and cell proliferation.

The antiestrogens such as ICI 182,780 and tamoxifen, can control the expression of estrogen response genes and down-regulate through the formation of transcriptionally active ER dimmers (DeFriend et al., 1994; Dudley et al., 2000). Therefore, antiestrogens are usually utilized as hormone therapy. However, drug resistance can develop, or the treatments have no effect or the toxicity of drugs gives adverse reactions in some patients (Macgregor and Jordan, 1998).

If the effects on patients were determined early a proper prescription could be determined. For this drug challenge test, cells isolated from the patient's body are used; these isolated cells should be as intact as possible. However, the number of cells from a tissue isolated from patient's body by biopsy is very few and current techniques for drug challenge testing, for example lipofection or microinjection, cannot be performed on such few cells because large numbers of cells die during or after the manipulation.

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Single cell manipulation technology using a nanoneedle and AFM is most adequate for this kind of accurate cell manipulation because the invasiveness is low enough and the cell never dies after manipulation (Han et al., 2005a,b). The judgment of successful insertion is possible in every trial from the information of the resulting force–distance curve and almost 100% insertion is possible (Obataya et al., 2005a,b) and very high efficiency DNA delivery (Han et al., 2008) can be achieved. In this study, we have attempted to evaluate the hormonal drugs effect in a single MCF-7 cell from the expression level of an estrogen responsive GFP reporter vector delivered by a nanoneedle.

2. Materials and methods

2.1. Cell culture

MCF-7 cells were grown under routine maintenance in RPMI 1640 supplemented with 10% FBS and antibiotics/antimyocytics (PSA, Cascade Biologics, Portland, U.S.A.) and maintained in a humidified incubator under 5% CO_2 at 37 °C. Flasks containing cells were treated with trypsin and then centrifuged to form a pellet. The cell pellet was dispersed and the cells were plated on fibronectin coated 35 mm tissue culture dishes (BioCoat™ Fibronectin, BD, NJ, U.S.A.) at 2×10^4 cells/dish.

2.2. Apparatus

MFP-3D™-Bio Systems (Asylum Research, Santa Barbara, U.S.A.) were used for needle manipulations and force measurements. In order to observe cells while operating the MFP, the probe was placed on the stage of an inverted fluorescence microscope and top view module. This combination allowed us to observe cells on the culture dish under the head unit of the MFP. We obtained images of cells from a highly sensitive CCD camera fitted to the microscope (DP70/IX71, Olympus, Tokyo, Japan). A heater and a CO_2 regulator were used to maintain cell conditions. This apparatus was covered with a sound-proofing hood to reduce interference by acoustic noise.

2.3. Construction of estrogen responsive GFP reporter vector

Estrogen responsive GFP reporter vector pEREGFP9 was generated from pERE-TA-SEAP and pEGFP-C1 (Clontech, CA, U.S.A.). The DNA fragment containing the estrogen response elements and TA promoter region were amplified by PCR. Then, the PCR fragment and pEGFP-C1 vector were digested using *Age* I and *Aat* II (New England Biolabs Ltd., Herts, United Kingdom). Finally, these DNA fragments were ligated using DNA Ligation Kit ver. 2.1 (TaKaRa Bio Inc., Shiga, Japan) and transformed into competent DH5α cells. The direction of the inserted fragment was analyzed by the colony direct PCR method and DNA sequence was analyzed using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems, CA, U.S.A.). Gene expression was confirmed by a lipofection method using Lipofectamine 2000™ (Invitrogen, Carlsbad, U.S.A.). The protocol of this method is in the product manual. Colony direct PCR was achieved using the primers for the ERE site and TA promoter region for forward 5′-GGGGACGTCCTTTCCCCGTCAAGCTCTAA-3′, and for reverse 5′-GGGACCGGTAGCAGCAGCATGGTGGGCGA-3′. The primers for sequence analysis were forward 5′-CGGGGTCATTAGTTCATAGCC-3′ and reverse 5′-TCGAAGCTTCCATTATATACCC-3′.

2.4. Lipofection and treatment of ICI 182,780

A cultured dish was treated with the conjugation of Lipofectamine 2000™ and pEREGFP9, and incubated for 24 h. RPMI 1640 medium containing FBS was used for the proliferation of MCF-7 cells before and after lipofection. We pre-tested charcoal stripped RPMI 1640 medium for the proliferation and evaluation of estrogenic responsiveness of pEREGFP9 to MCF-7 cells. However, the proliferation and DNA transfer were not successful and we concluded that the charcoal stripped medium is inadequate for the proliferation of MCF-7 cells. The treated dish was cultured in RPMI 1640 with 10 μM 17β-estradiol (E2, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The phrGFP DNA (Stratagene, North Torrey, U.S.A.) (Levy et al., 1996) was used for the control in lipofection. The fluorescence observation was achieved every 2 h under 5% CO_2 at 37 °C using a DP30/IX71 (Olympus, Tokyo, Japan) fitted with a NIBA filter (ex 470-490/em 515-550). The exposure time was controlled to be the same for each observation. Then, medium was exchanged to RPMI 1640 with 10 μM ICI 182,780 (Wako) which is a pure antagonist of E2. During treatments of ICI 182,780, fluorescence images were obtained in the same manner. The images were analyzed using the software Image Pro (Media Cybernetics, Maryland, U.S.A.). The fluorescence intensity of GFP expressing cells was obtained by converting to gray-scaled images and the results were calculated from the density of fluorescence intensity.

2.5. Fabrication of nanoneedle and adsorption of pEREGFP9 onto nanoneedle

We prepared nanoneedles from pyramidal Si AFM tips that had spring constants of approximately 0.2 N/m (ATEC-CONT, Nanosensors, Neuchatel, Switzerland) using a focused ion beam (FIB, SMI9200, Seiko Instruments Inc., Chiba, Japan). The nanoneedles were fabricated with 200 nm diameters. The nanoneedle fabrication was achieved as previously described (Han et al., 2005a,b). The surface of the nanoneedle was cleaned and oxidized with an ozone cleaner and then treated with 2% 3-mercaptopropyltrimethoxysilane (Sigma–Aldrich, St. Louis, U.S.A.) and 2% MQ water in ethanol for 30 min. The nanoneedle was further treated with 100 μM *N*-(6-maleimidocaproyloxy)succinimide (EMCS, Dojindo Laboratories, Kumamoto, Japan) for 30 min. The succinimidyl nanoneedle was soaked in a 10 μM poly-L-lysine solution of 10 mM HEPES buffer (pH 7.4). Then, 100 ng/μL of the pEREGFP9 DNA in TE buffer was dropped onto the cantilever so as to adsorb the DNA on the positively charged surface of the nanoneedle by electrostatic interaction. The pEREGFP9 DNA-adsorbed nanoneedle was inserted into an MCF-7 cell as previous described (Han et al., 2008).

2.6. Drug challenge test to a single MCF-7 cell using nanoneedle

pEREGFP9 were adsorbed onto the surface of nanoneedle by the method described above. First, culture medium was changed to opti-MEM (Invitrogen). Then, DNA-adsorbed nanoneedle was inserted into a single MCF-7 and insertion was kept for 3 min. Finally, the nanoneedle was evacuated from the cell, and cells were cultured in RPMI 1640 with 10 μM E2.

The fluorescence observation after 24 h was achieved using the DP30/IX71. Then, medium was exchanged to RPMI 1640 with 10 μM ICI 182,780 (Wako). During treatments of ICI 182,780, fluorescence images were obtained every 6 h. Fluorescence images were analyzed by Image Pro.

3. Results and discussion

3.1. The evaluation of estrogenic responsiveness of pEREGFP9

Fig. 1 shows the schematic diagram of single cell drug challenge test. The constructed pEREGFP9 which is an estrogen responsive