ARTICLE

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Reproducible Fashion of the HSP70B' Promoter-Induced Cytotoxic Response on a Live Cell-Based Biosensor by Cell Cycle Synchronization

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ABSTRACT: Live cell-based sensors potentially provide functional information about the cytotoxic effect of reagents on various signaling cascades. Cells transfected with a reporter vector derived from a cytotoxic response promoter can be used as intelligent cytotoxicity sensors (i.e., sensor cells). We have combined sensor cells and a microfluidic cell culture system that can achieve several laminar flows, resulting in a reliable high-throughput cytotoxicity detection system. These sensor cells can also be applied to single cell arrays. However, it is difficult to detect a cellular response in a single cell array, due to the heterogeneous response of sensor cells. The objective of this study was cell homogenization with cell cycle synchronization to enhance the response of cell-based biosensors. Our previously established stable sensor cells were brought into cell cycle synchronization under serum-starved conditions and we then investigated the cadmium chloride-induced cytotoxic response at the single cell level. The GFP positive rate of synchronized cells was approximately twice as high as that of the control cells, suggesting that cell homogenization is an important step when using cell-based biosensors with microdevices, such as a single cell array.

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KEYWORDS: cell-based biosensor; cytotoxic response; HSP 70B'; cell cycle; serum starvation

Introduction

Cell-based biosensors provide functional information about the efficacy of chemicals in cellular signaling cascades (Bousse, 1996; Haruyama, 2003; Keusgen, 2002; Pancrazio et al., 1999; Wang et al., 2005). The reporter gene system is the most useful method for monitoring gene expression within cells. Many types of reporters for various applications have been described in live cells, such as fluorescent proteins (Carroll et al., 2003; Li et al., 2001, 2002), luciferase (Nguyen et al., 1988), beta-galactosidase (Rose et al., 1981), and secreted alkaline phosphates (SEAP) (Berger et al., 1988). Previously, we developed cell-based biosensors using the firefly luciferase reporter or green fluorescent protein (GFP) reporter (i.e., sensor cells). These detect cytoplasmic protein denaturation-induced cytotoxicity, such as heavy metal-induced cytotoxity, using sensor cells containing an engineered HSP70B' promoter (Wada et al., 2005, 2007a,b) or using DNA damage-detecting sensors containing an engineered BTG2 promoter (Wada et al., 2009).

Recently, cell-based biosensors were combined with microfabrication technology for high throughput analysis of multiple cellular functions, and numerous microdevices for cell-based biosensors have been developed and reported (Bashir, 2004; El-Ali et al., 2006). For instance, molecular patterning techniques were used to fabricate cell-based microarrays (Anderson et al., 2004; Flaim et al., 2005; Mousses et al., 2003; Revzin et al., 2004; Ziauddin and Sabatini, 2001), and microfluidic channels that were treated to grow the cells then provided step-wise dilution of the sample to the cells (Thompson et al., 2004; Wada et al., 2008). The combination of GFP reporter cells and microdevices is useful for monitoring multiple cellular functions with high throughput and small sample volumes. These biosensors are attractive as a tool for real-time monitoring of gene expression profiling in a small population of cells rather than the current gene expression profiling methods (e.g., single dish or multi-well format). However, it is

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difficult to detect a cellular response in a single cell array, due to the heterogeneous response of cells. In the case of stable sensor cells using the HSP70B' promoter, only about 40% of cells respond to cadmium chloride cytotoxity.

The objective of this study was cell homogenization with cell cycle synchronization to enhance the response of the cell-based biosensors. Our previously established sensor cells (Wada et al., 2008) were brought into cell cycle synchronization under serum-starved conditions and then we investigated the cadmium chloride-induced cytotoxic response at the single cell level by flow cytometry. The results suggest that the cell cycle is associated with the cytotoxic response of sensor cells and that cell homogenization is an important step when using cell-based biosensors with microdevices, such as a single cell array.

Materials and Methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), Dulbecco's modified phosphate buffer saline (DPBS), and RNase were purchased from Sigma (St. Louis, MO). Cadmium chloride and G418 were purchased from Wako Pure Chemical Ltd (Osaka, Japan). Propium iodide (PI) was purchased from Dojin Chemical (Kumamoto, Japan).

Cell Culture

The cytotoxicity sensor cells, which were a GFP reporter cell line containing an engineered HSP70B' promoter were generated by stably transfecting NIH/3T3 cells with the pd2EGFP-1 (Clontech Laboratories, Inc., Terra Bella Ave., CA) inserted the DNA segment containing the region from −287 to +112 bp of the HSP70B' gene (Wada et al., 2008). These cells were cultured in DMEM containing 4,500 mg/L glucose and supplemented with 10% fetal bovine serum (FBS) and 800 μg/mL G418. Cultured cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Cell Cycle Synchronization

To synchronize the cell cycle, cells were cultured under serum-starved conditions of DMEM containing 0.5% FBS for 24 and 48 h. Following that, the cell cycle was analyzed by flow cytometry (BD Bioscience, Bedford, MA). Cells were seeded at 4×10^4 cells/cm^2 for serum starvation and at 3×10^5 cells/cm^2 for full confluency.

Cell Cycle Analysis

The cells were recovered into a test tube, washed in PBS, fixed in 70% ethanol, and then stained in 50 μg/mL PI solution containing 0.25 mg/mL RNase. Cell cycle analysis was performed using FACSCaliber (BD Bioscience). Histogram plots of DNA content were created using Cell Quest software (BD Bioscience). The populations of cells in each phase were calculated from the histograms using cell cycle analysis software (ModiFit LT; BD Bioscience).

Evaluation of Positive Rate by GFP Fluorescence

Cadmium chloride (Wako, Osaka, Japan) was solubilized in deionized water and diluted in culture medium immediately before exposure to the cells. The cells were washed in PBS, fixed in 4% pararformaldhyde for 20 min at room temperature and the GFP-positive rate was determined using flow cytometry using FACSCaliber (BD Bioscience). First, threshold point was decided on the histogram including approximately 99.5% Cd^{2+} non-exposed cells (it means intrinsic fluorescence). Then, GFP positive rate was calculated from Cd^{2+} testing data reference with threshold point.

Results and Discussion

To synchronize the cell cycle, the sensor cells were treated by serum starvation. The cell cycle distributions of the cells were calculated from their DNA contents by flow cytometry. Figure 1 shows the cell cycle distribution following culturing in DMEM containing 0.5% FBS for 0, 48, and 72 h, and following culturing of 3×10^5 cells/cm^2 in DMEM containing 10% FBS to induce contact inhibition by full confluency. The distribution of the control cells among the three phases of the cell cycle (G0/G1, S, and G2/M) was 52.70%, 39.75%, and 7.54%, respectively. The distribution of the synchronized cells among G0/G1, S, and G2/M was 53.05%, 35.53%,

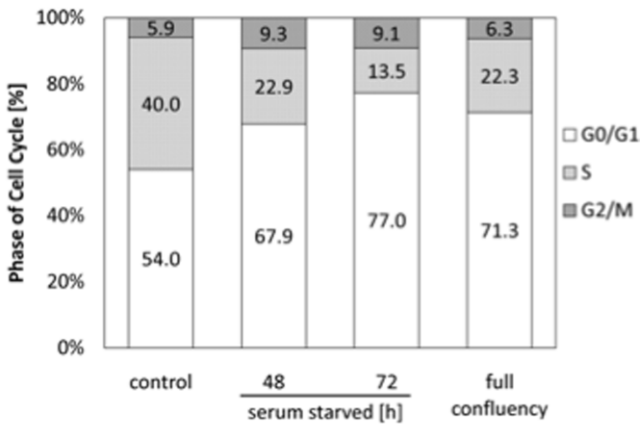

Figure 1. Cell cycle distributions of sensor cells in response to serum starvation or contact inhibition. After staining the nuclei with PI solution, the cell cycle was analyzed using flow cytometry. The population of cells in each phase was calculated from the cell cycle histogram using ModiFit software. The data represent the average of three experiments.

and 11.59%, respectively, for the 24 h incubation, 87.86%, 22.86%, and 9.30%, respectively, for the 48 h incubation, and 76.98%, 13.52%, and 9.13%, respectively, for the 72 h incubation. The distribution of fully confluent cells among G0/G1, S, and G2/M was 53.05%, 35.53%, and 11.59%, respectively. The cell cycle distribution of cells cultured under serum-starved conditions for 24 h was similar to that of the control cells (data not shown). For the serum-starved cells cultured for 48 and 72 h, the percentage of cells in G0/G1 phase was increased compared to control cells, and this increase was time dependant.

Figure 2 shows the typical histograms of distribution of fluorescent intensity of the cadmium chloride exposed sensor cells. The cadmium chloride-induced GFP fluorescent intensity was drastically shifted. Most of the cells cultured under serum-starved condition were responded, on the other hand, non-treated control cells were responded about half of serum-starved cells.

Figure 3 shows the response to cadmium chloride of cells cultured under serum-starved conditions for 0, 48, and 72 h, and those cultured to full confluency. Following starvation or growth, the cells were exposed to 8 μg/mL cadmium chloride at 37°C for 8 h and the percentage of GFP-positive cells was then analyzed using flow cytometry. The cadmium chloride-induced GFP-positive rate of 48 h synchronized cells (78.46%) was approximately twice as high as that of the unsynchronized (0 h, 43.87%) cells. For the 72 h synchronized cells, the number of GFP-positive cells was markedly lower than that of the unsynchronized cells. On the other hand, approximately 70% of confluent cells produced a GFP fluorescence signal. Cells cultured under serum-starved conditions for 72 h showed higher synchronization in G0/G1 than the cells starved for 48 h. However, fewer of 72 h serum-starved cells showed a response to cadmium chloride. Although serum starvation is the conventional method for cell cycle synchronization, it often reduces cell survival and increases DNA fragmentation (Peng et al., 1998). Thus, holding the culture overlong under serum-starved conditions has a deleterious effect on detecting a cytotoxic response.

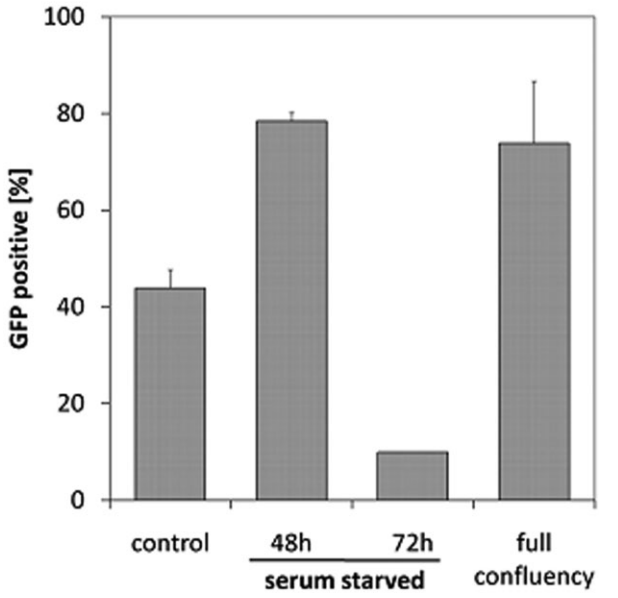

Figure 3. GFP-positive rate of sensor cells exposed to $CdCl_2$. After exposure to 8 μg/mL of $CdCl_2$ for 8 h, the GFP-positive cells were analyzed by flow cytometry. The data represent the mean ± SD of three experiments.

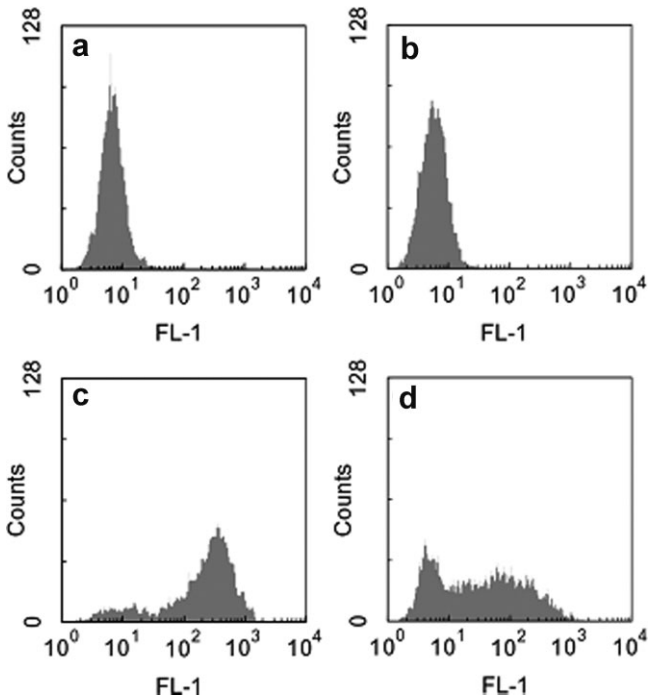

Figure 2. Distribution of fluorescent intensity of $CdCl_2$ exposed sensor cells cultured under the serum-starved condition. After exposure to $CdCl_2$ for 8 h, the GFP-positive cells were analyzed by flow cytometry. (**a**) Without $CdCl_2$ for 48 h serum-starved cells, (**b**) without $CdCl_2$ and non-treated control cells, (**c**) 8 μg/mL $CdCl_2$ exposed 48 h serum-starved cells, and (**d**) 8 μg/mL $CdCl_2$ exposed non-treated control cells.

Figure 4 shows optical microscopic and fluorescence microscopic images of cell cycle unsynchronized cells and 48 h synchronized cells. In the case of 0 μg/mL cadmium chloride exposure, neither cell type showed a GFP fluorescence signal. When the 48 h synchronized cells were exposed to 4 μg/mL cadmium chloride, most showed a GFP fluorescence signal. On the other hand, unsynchronized cells showed hardly any GFP fluorescence signal in response to 4 μg/mL cadmium chloride. Both unsynchronized cells and 48 h synchronized cells showed a noticeable GFP fluorescence signal in response to 8 μg/mL cadmium chloride (data not shown). GFP positivity appears to be markedly increased by cell cycle synchronization by serum starvation.

Figure 5 shows the percentage of GFP-positive cells among unsynchronized and 48 h synchronized cells when they were exposed to 0, 2, 4, 6, 8, and 10 μg/mL of cadmium

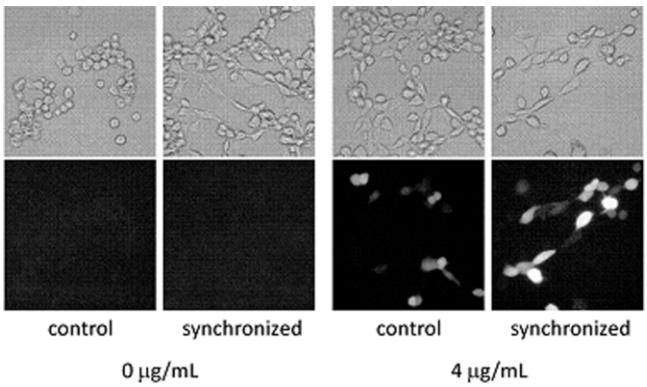

Figure 4. Optical microscopic and fluorescence microscopic images of sensor cells exposed to $CdCl_2$. The cells were exposed to 0 (left panel) and 4 μg/mL (right panel) $CdCl_2$ for 8 h.

chloride at 37°C for 8 h. The GFP-positive rate of 48 h synchronized cells exposed to cadmium chloride was 0.36%, 19.0%, 71.1%, 81.7%, 78.5%, and 76.0% for 0, 1, 2, 3, 4, 6, 8, and 10 μg/mL cadmium chloride, respectively. The data eventually plateaued when cells were exposed to ≥4 μg/mL cadmium chloride. On the other hand, the GFP-positive rate of unsynchronized cells exposed to cadmium chloride was 0.38%, 2.69%, 19.5%, 27.6%, 43.9%, and 53.0% for 0, 1, 2, 3, 4, 6, 8, and 10 μg/mL cadmium chloride, respectively.

In order to increase the cadmium chloride-induced cytotoxic response of the sensor cells, the cell cycles were synchronized by culture under serum-starved conditions for 48 h before analysis of the cellular response. We found that the response of the synchronized 48 h serum-starved cells was twice as high as that of the unsynchronized control cells. These results suggest that the G0/G1 phase is associated with cadmium chloride-induced cytotoxicity. The data of cell cycle distribution and GFP-positive rate from the fully confluent cells support this relationship between cells in G0/G1 and cytotoxic response. These findings suggest that cell homogenization is an important step when using cell-based biosensors with microdevices, such as a single cell array.

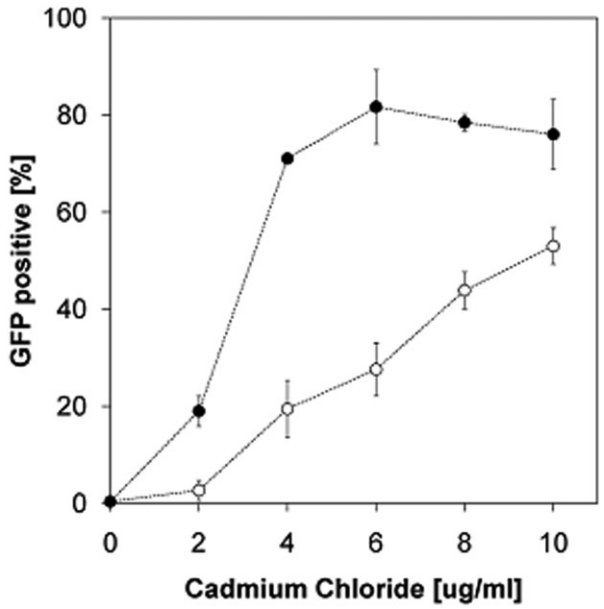

Figure 5. Dose response of the sensor cells to $CdCl_2$ exposure. After exposure to $CdCl_2$ for 8 h, the proportion of GFP-positive cells was analyzed by flow cytometry. Open circles and filled circles represent the data of the control and 48 h serum-starved cells. The data represent the mean ± SD of three experiments.

References

Anderson DG, Levenberg S, Langer R. 2004. Nanoliter-scale synthesis of arrayed biomaterials and application to human embryonic stem cells. Nat Biotechnol 22:863–866.

Bashir R. 2004. BioMEMS: State-of-the-art in detection, opportunities and prospects. Adv Drug Deliv Rev 56:1565–1586.

Berger J, Hauber J, Hauber R, Geiger R, Cullen BR. 1988. Secreted placental alkaline phosphatase, a powerful new quantitative indicator of gene expression in eukaryotic cells. Gene 66:1–10.

Bousse L. 1996. Whole cell biosensors. Sens Actuators B Chem 34:270–273.

Carroll JA, Stewart PE, Rosa P, Elias AF, Garon CF. 2003. An enhanced GFP reporter system to monitor gene expression in Borrelia burgdorferi. Microbiology 149:1819–1828.

El-Ali J, Sorger PK, Jensen KF. 2006. Cells on chips. Nature 442:403–411.

Flaim CJ, Chien S, Bhatia S. 2005. An extracellular matrix microarray for probing cellular differentiation. Nat Methods 2:119–125.

Haruyama T. 2003. Micro- and nanobiotechnology for biosensing cellular responses. Adv Drug Deliv Rev 55:393–401.

Keusgen M. 2002. Biosensors: New approaches in drug discovery. Naturwissenschaften 89:433–444.

Li HY, Ng EKO, Lee SMY, Kotaka M, Tsui SKW, Lee CY, Fung KP, Waye MMY. 2001. Protein-protein interaction of FHL3 with FHL2 and visualization of their interaction by green fluorescent proteins (GFP) two-fusion fluorescence resonance energy transfer (FRET). J Cell Biochem 80:293–303.

Li J, Xu H, Herber WK, Bentley WE, Rao G. 2002. Integrated bioprocessing in Saccharomyces cerevisiae using green fluorescent protein as a fusion partner. Biotechnol Bioeng 79:682–693.

Mousses S, Caplen NJ, Cornelison R, Weaver D, Basik M, Hautaniemi S, Elkahloun AG, Lotufo RA, Choudary A, Dougherty ER, Suh E, Kallioniemi O. 2003. RNAi microarray Analysis in cultured mammalian cells. Genome Res 13:2341–2347.

Nguyen VT, Morange M, Bensaude O. 1988. Firefly luciferase luminescence assays using scintillation counters for quantitation in transfected mammalian cells. Anal Biochem 171:404–408.

Pancrazio JJ, Whelan JP, Borkholder DA, Ma W, Stenger DA. 1999. Development and application of cell-based biosensors. Annals Biomed Eng 27:697–711.

Peng X, Maruo T, Matsuo H, Takekida S, Deguchi J. 1998. Serum deprivation-induced apoptosis in cultured porcine granulosa cells is characterized by increased expression of p53 protein, Fas antigen and Fas ligand and by decreased expression of PCNA. Endocr J 45:247–253.

Revzin A, Rajagopalan P, Tilles AW, Berthiaume F, Yarmush ML, Toner M. 2004. Designing a hepatocellular microenvironment with protein microarraying and poly(ethylene glycol) photolithography. Langmuir 20:2999–3005.

Rose M, Casadaban MJ, Botstein D. 1981. Yeast genes fused to beta-galactosidase in Escherichia coli can be expressed normally in yeast. Proc Natl Acad Sci 78:2460–2464.

Thompson DM, King KR, Wieder KJ, Toner M, Yarmashu LY, Jayaraman A. 2004. Dynamic gene expression profiling using a microfabricated living cell array. Anal Chem 76:4098–4103.

Wada KI, Taniguchi A, Xu L, Okano T. 2005. Rapid and highly sensitive detection of cadmium chloride induced cytotoxicity using the HSP70B' promoter in live cells. Biotechnol Bioeng 92:410–415.

Wada KI, Taniguchi A, Okano T. 2007a. Highly sensitive detection of cytotoxicity using a modified HSP70B' promoter. Biotechnol Bioeng 97:871–876.

Wada K-I, Okuda-Shimazaki J, Taniguchi A. 2007b. Improvement in sensitivity of live cell-based sensor cells by co-transfection of a reporter gene driven by a modified HSP70B' and HSF1 expression vector. Open Biotechnol J 1:21–25.

Wada KI, Taniguchi A, Kobayashi J, Yamato M, Okano T. 2008. Live cells-based cytotoxic sensorchip fabricated in a microfluidic system. 99: 1513–1517.

Wada KI, Hamaguchi Y, Furukawa K, Taniguchi A. 2009. DNA damage sensible engineered promoter for cellular biosensing of cytotoxicity. Biotechnol Bioeng 102:1460–1465.

Wang P, Xu G, Qin L, Xu Y, Li Y, Li R. 2005. Cell-based biosensors and its application in biomedicine. Sens Actuators B Chem 108:576–584.

Ziauddin J, Sabatini DM. 2001. Microarrays of cells expressing defined cDNAs. Nature 411:107–110.