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Use of live imaging analysis for evaluation of cytotoxic chemicals that induce apoptotic cell death

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ABSTRACT

We carried out live imaging of PC12 cells expressing SCAT3, a caspase-3 cleavage peptide sequence linking two fluorescent proteins, ECFP and Venus, which function respectively as the donor and acceptor for FRET. Live imaging of SCAT3-expressing cells was performed from 60 to 300 min after exposure to sodium arsenite ($NaAsO_2$: 0, 1, 5, or 10 μM) was initiated. We then measured the emission ratio of ECFP to Venus to monitor the activity of caspase-3 and found that the ratio was temporally and dose-dependently increased by $NaAsO_2$. The mean ECFP/Venus emission ratio between 200 and 300 min after exposure to $NaAsO_2$ at a dose of 5 or 10 μM, but not at 1 μM, was significantly higher than that in the control group. We showed by other methods that $NaAsO_2$ significantly increased the amount and activity of mature caspase-3 and the amount of nucleosomes generated from DNA fragmentation, and decreased cell viability. However, methods other than live imaging required a longer time and higher doses of $NaAsO_2$ than did live imaging to detect significant effects. This result suggests that live imaging using SCAT3 is a useful method for the screening of chemical toxicities and for improving the efficiency of toxicity evaluation.

1. Introduction

Apoptosis is a phenomenon that controls cell fate and determines cell survival and cell death. Cell death by apoptosis often, although not always, occurs if the cells are exposed to toxic chemicals. Heavy metal compounds, which are capable of causing numerous acute or chronic pathologies, are known to induce apoptotic cell death (Rana, 2008). For example, the inorganic arsenic compounds, sodium arsenite ($NaAsO_2$) and arsenic trioxide, exhibit cytotoxicity accompanied by the induction of apoptotic cell death in a variety of cells, including immune cells (Bustamante et al., 1997; Hossain et al., 2000), neuronal cells (Namgung and Xia, 2001; Wong et al., 2005; Chattopadhyay et al., 2002), a gastric cancer cell line (Jiang et al., 2001), a leukemia cell line (Iwama et al., 2001), and a neuroblastoma cell line (Watcharasit et al., 2008). In addition, cell death by apoptosis occurs when cells are exposed to other heavy metal compounds, such as lead acetate (Shabani and Rabbani, 2000; Columbano et al., 1985), lead nitrate (Pachauri et al., 2009), hexavalent chromium (Hill et al., 2008; Carlisle et al., 2000; Wang et al., 2000; Azad et al., 2008; Gambelunghe et al., 2006; Rudolf and Cervinka, 2006), methyl mercury (Homma-Takeda et al., 2001; Close et al., 1999; Shenker et al., 1999; Toimela and Tahti, 2004), mercuric chloride (Araragi et al., 2003), and cadmium chloride (Dong et al., 2001; Choi et al., 2002; Gennari et al., 2003; Habeebu et al., 1998). Caspase-3 is an executor proteinase of apoptosis, whose enzymatic activity is stimulated by death signals. It has been reported that the caspase-3 of cells cultured *in vitro* is activated by lead acetate (Dong et al., 2009), hexavalent chromium (Hill et al., 2008; Russo et al., 2005), methyl mercury (Toimela and Tahti, 2004), arsenic trioxide (Jiang et al., 2001; Tse et al., 2008, 2009; Piga et al., 2007) or $NaAsO_2$ (Watcharasit et al., 2008). The combined data suggest that activation of caspase-3 may be a major irreversible step towards apoptotic cell death following exposure to such heavy metal compounds. Thus, the activity of caspase-3 is a suitable end-point for evaluation of the cytotoxicity of chemicals.

Abbreviations: ECFP, enhanced cyan fluorescent protein; FRET, fluorescence resonance energy transfer; $NaAsO_2$, sodium arsenite; NGF, nerve growth factor; PBS, phosphate-buffered saline; PEI, polyethyleneimine; pNA, *p*-nitroanilide; SDS, sodium dodecyl sulfate; Z-VAD-FMK, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (broad-spectrum caspase inhibitor).

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The dynamics and temporal patterns of caspase activation can be monitored by fluorescence resonance energy transfer (FRET) methods. In a single living cell undergoing apoptosis, temporal changes in the activity of caspase-3 are profiled by measurement of the extent of FRET within a recombinant substrate composed of enhanced cyan fluorescent protein (ECFP) linked by the caspase-3 cleavage sequence, Asp-Glu-Val-Asp (DEVD), to enhanced yellow fluorescent protein (EYFP) (Tyas et al., 2000; Luo et al., 2001; Rehm et al., 2002). In addition, Takemoto et al. improved such a recombinant substrate by replacing EYFP with a variant of EFYP named Venus (Nagai et al., 2002) and called the improved indicator for caspase-3 activation "SCAT3" (Takemoto et al., 2003). The usefulness of FRET technology using SCAT3 for monitoring caspase-3 activation in living cells both *in vitro* and *in vivo* has been well documented (Takemoto et al., 2003; Kanuka et al., 2005; Kuranaga et al., 2006; Takemoto et al., 2007).

In the present study, we applied live cell imaging analysis using SCAT3 for determination of cytotoxic effects of chemicals on the PC12 cell line, which is a cell line derived from a pheochromocytoma of rat adrenal medulla and which has the potential to differentiate into neuron-like cells. By live imaging of SCAT3-expressing PC12 cells, we profiled the temporal changes in caspase-3 activity after exposure of the cells to $NaAsO_2$. In addition, we examined the effects of $NaAsO_2$ on apoptosis induced by other methods to determine whether the sensitivity and efficiency of detection of toxicity is improved by live imaging analysis using SCAT3. In this study, we performed western blotting analysis for determination of the protein expression of the cleaved, active form of caspase-3, measured the enzymatic activity of caspase-3, the amount of nucleosomes generated from apoptotic DNA fragmentation, and the viability of PC12 cells after they were exposed to $NaAsO_2$.

2. Materials and methods

2.1. Cell culture and reagent preparation

PC12 cells (Collection No. IFO50278; the Health Science Research Resources Bank, Kobe, Japan) were used to test the usefulness of the SCAT3 system for detection of $NaAsO_2$ toxicity. The effects of $NaAsO_2$ on the protein expression and enzymatic activity of caspase-3, on DNA fragmentation, and on cell viability were assayed. PC12 cells were maintained in RPMI 1640 medium (Gibco-Invitrogen, Carlsbad, CA, USA) containing 10% horse serum, 5% fetal bovine serum, 0.1 mM Non-Essential Amino Acids Solution (Invitrogen, Carlsbad, CA, USA), a mixture of penicillin and streptomycin (100 units/ml and 100 μg/ml each; Invitrogen), and 100 μg/ml normocin (InvivoGen, San Diego, CA, USA) in 75-cm^2 flasks coated with poly-D-lysine (Becton Dickinson and Co., Oxford, UK) in an atmosphere containing 5% CO_2 at 37 °C. PC12 cells at passage 7–12 were used for the following experiments.

For each experiment, PC12 cells were cultured in medium that was exclusively used for experiments and not for cell maintenance. This medium hereafter referred to as experimental medium consisted of RPMI 1640 medium (Gibco-Invitrogen) containing 0.01 mM Non-Essential Amino Acids Solution (Invitrogen), a mixture of penicillin and streptomycin (10 units/ml and 10 μg/ml each; Invitrogen) and 10 μg/ml normocin (InvivoGen), with or without supplementation with 1% horse serum and 0.5% fetal bovine serum. PC12 cells cultured in this medium were simultaneously treated with nerve growth factor (NGF; 50 ng/ml) for neuron-like cell differentiation and were exposed to $NaAsO_2$ (Wako Pure Chemical Industries, Osaka, Japan) at a dose of 0, 1, 5, or 10 μM. Crystalline $NaAsO_2$ was dissolved in sterile-filtered water (Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 100 mM. $NaAsO_2$ solution (100 mM) was diluted with experimental medium with or without serum supplementation to generate the indicated concentrations.

2.2. Transfection and imaging analysis of SCAT3

PC12 cells (10^6 cells) were transfected with 2.0 μg of the SCAT3 expression vector pcDNA-SCAT3, provided by Dr. M. Miura (University of Tokyo, Tokyo, Japan) (Takemoto et al., 2003), using Nucleofector™ II (Lonza Cologne AG, Cologne, Germany). PC12 cells (10^4 cells/well) were then seeded onto 8-well chamber slides (LAB-TEK™ Chambered Coverglass; Nalge Nunc International, Rochester, NY, USA) coated with 0.25% polyethyleneimine (PEI; Sigma–Aldrich) and were cultured in the above-described subculture medium (300 μl) under an atmosphere containing 5% CO_2 at 37 °C.

One or two days after transfection, live imaging of SCAT3-expressing PC12 cells was performed using a fluorescent time-lapse microscope BioZero 8100 (Keyence Co., Osaka, Japan) controlled by a computer with a BZ Viewer version 1.0 software installed (Keyence). SCAT3-expressing PC12 cells were simultaneously treated with NGF (50 ng/ml) and exposed to $NaAsO_2$ (0 or 10 μM) in 300 μl of experimental medium with or without serum supplementation. Live imaging of such cells was then carried out in five independent experiments. In some experiments, SCAT3-expressing PC12 cells were simultaneously treated with NGF (50 ng/ml) and exposed to $NaAsO_2$ at a dose of 0, 1, 5, or 10 μM in serum supplemented experimental medium. Live imaging of these cells was then performed in five independent experiments.

SCAT3-expressing PC12 cells were placed in an incubation chamber (Microscope Incubation System INU-KI-F1, Tokai Hit, Shizuoka, Japan) whose temperature was controlled at 37 °C and within which the gas concentration was maintained at 5% CO_2 and 95% air. Fluorescent time-lapse imaging was started at 60 min and ended at 300 min after initiation of $NaAsO_2$ exposure. A 440AF21 excitation filter, a 455DRLP dichroic mirror, and two emission filters 480AF30 for ECFP and 535AF25 for Venus (Opto Science Inc., Tokyo, Japan) were used for imaging of SCAT3-expressing cells. The excitation intensity was attenuated to 40% of the maximum power of a light source with a neutral density filter. Fluorescent images of SCAT3 were captured every 20 min using an objective lens (Plan Fluor ELWD DM 20xC, NA 0.45; Nikon, Tokyo, Japan) and a CCD camera in the KEYENCE BioZero 8100.

After live imaging was completed for each experiment, the intensity of ECFP and Venus emissions of SCAT3-expressing PC12 cells was measured using the digital image data. The region of interest corresponded to from 1.12 to 1.69 mm of the cultured slides (the mean number of SCAT3-expressing PC12 cells: approx. 150). The RGB digital images of ECFP (*cyan blue*) and Venus (*yellow green*) taken from SCAT3-expressing PC12 cells were converted into monochromatic color images (*red* for ECFP; *green* for Venus). These images were merged at each time point in order to determine the intensities of ECFP and Venus of the same region, which were obtained by measuring the brightness of *red* and *green* colors, respectively with the aid of a computer and the KEYENCE BZ Viewer version 1.0 software (Keyence). After the brightness values of *red* and *green* colors in the same region of each merged image were measured, the ECFP/Venus emission ratio was calculated by dividing the brightness value of the *red* color by that of the *green* color. The ECFP/Venus emission ratio at each time point was calibrated using the ratio of the same area at 60 min after $NaAsO_2$ exposure, which was set at a value of 1.

2.3. Western blot analysis of caspase-3

PC12 cells, seeded on 0.25% PEI-coated 12-well plates, (10^5 cells/well) were treated with NGF (50 ng/ml) and exposed to