Real-time monitoring of B16 melanoma cell viability by the firefly luciferase-based bioluminescence

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Abstract

Various cytotoxicity assays have been primarily established by parameters associated with plasma membrane permeability. However, current methods are not suitable for cell viability measurement in mixed cell cultures, for example, in an analysis of cell-mediated cytotoxicity between responder cells (such as cytotoxic T cells) and the target cells (tumor cells). In this study, we attempted to establish an experimental system using firefly luciferase bioluminescence, which is useful for quantification and real-time monitoring of target cell viability. We transfected B16F10 melanoma cells with the expression plasmid containing the firefly luciferase gene and monitored bioluminescent signals associated with apoptotic cell death induced by calcium ionophore A23187 or an aminonucleoside antibiotic, puromycin. With A23187 or puromycin treatment, we observed a remarkable, concentration- and time-dependent decrease in the luminescence value. When B16F10 melanoma cells were co-cultured with stimulated splenocytes, the luminescence value gradually decreased, whereas no splenocytes, the luminescence value gradually increased for about 30 h. These results imply that the activated responder cells affected the target B16F10 viability, by inducing apoptotic cell death. We propose that the firefly luciferase-based reporter is a useful experimental system for quantifying cell-mediated cytotoxicity between the responder and the target cells.

Keywords: Firefly luciferase, melanoma, cytotoxicity, lymphocytes

Introduction

To date, various cell cytotoxicity assays are used to measure dead/live cells in *in vitro* cell culture [1]. A frequently used method is dye exclusion assay using a trypan blue dye, which is normally excluded from viable cells. Another assay is based on the measurement of the fluorescence signal produced by compromised cells using fluorescent dyes preferentially bind to DNA. Annexin V staining is used to detect early apoptotic cells, due to its calcium-dependent affinity for phosphatidylserine residues, which is exposed to the cell surface in damaged cells. Alternatively, for quantifying cell toxicity, an assay based on the activity of cytoplasmic enzymes is commonly used. Lactate dehydrogenase (LDH) is selectively released out of dead cells when they have lost their membrane integrity. However, LDH is affected by fetal calf serum and ingredients in culture media that interfere and cause high background, therefore experiments are conducted under limited conditions, serum-free or low-serum culture for a minimum time scale that does not induce cell death under normal growth conditions.

Although the currently established *in vitro* cytotoxic assays are valid for measuring dead/live cells when cultured cells are with uniform properties, they are not suitable to measure cell viability in mixed cell culture, for example, in an analysis of cell-mediated cytotoxicity between responder cytotoxic T cells (CTLs), and target cells (tumor cells).

In cancer immunity, anti-tumor CTLs in the draining lymph nodes are differentiated from naive lymphocytes by interaction with antigen-presenting cells that have phagocytosed tumor antigens. They migrate from the lymph nodes to blood vessels, infiltrate into the tumor and specifically attack target cancer cells expressing the same antigen [2]. CTLs have a short life span, therefore it is necessary to distinguish between targets and CTLs to accurately evaluate the targets' cell viability. Conventionally, chromium release assay is used for measuring target-derived cell death *in vitro* [3]. The method can directly detect CTL or Natural Killer cell-induced target cell death by a simple experimental procedure with excellent sensitivity and reproducibility. However, the major drawbacks of this assay are the use of radioactive substances, which cause handling problems due to environmental safety concerns, and the short half-life of isotopes. Therefore, a sensitive and non-radioactive cytotoxicity assay is required.

Bioluminescent assays have been widely applied to monitoring various biological activities *in vitro* and *in vivo*, including gene expression, protein-protein interaction, and signal transduction [4, 5]. The firefly luciferase is commonly used for cell-based assays, which require low-background and highly sensitive

measurements. Given that a fluorescent reporter causes cell damage due to excitation, bioluminescent assays overcome this problem and provide us with a better way to track cell viability in real-time. In this study, we used the firefly luciferase, which evaluates the viability of mouse B16F10 melanoma cells from a decrease in luminescence value. We examined whether apoptosis in B16F10 can be monitored after treatment with calcium ionophore A23187 and antibiotic puromycin, and a co-culturing with stimulated splenocytes from melanoma-carrying mice.

Materials and Methods

Cell cultures

A murine melanoma cell line, B16F10 was purchased from the American Type Culture Collection. The cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 5% (v/v) FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 0.1 mM non-essential amino acids, and 10 mM HEPES. B16F10 seeded at 1 x 10^5 cell/well in a 6-well plate was transfected with 3 μg/well pGL3-control vector (Promega) by the PEI-MAX (Polysciences) according to the manufacturer's instructions.

Preparation of mouse splenocytes

C57BL/6N mice (Japan SLC) at six weeks of age were subcutaneously injected with 1 x 10^6 B16F10 cells. Two weeks after injection, the tumor-bearing mice were euthanized and the spleen was harvested. The cells were mechanically released from the tissue and then collected by centrifugation at 1,300 rpm for 5 min at 4°C. After lysing erythrocytes with a lysis buffer (0.83% NH_4Cl, 0.17 M Tris-Cl pH 7.65), splenocytes were resuspended with RPMI1640 supplemented with 10% FCS. All mice were housed in a temperature- and humidity-controlled facility on a 12-hour light-dark cycle. All animal experiments followed protocols approved by the ethics review committee for animal experimentation of Kindai University.

Generation of B16F10 reactive splenocytes in vitro

The B16F10 cells were seeded at 2 x 10^6 cells/60-mm cell culture dish and incubated overnight. The cells were treated with 10 μg/ml mitomycin C (Wako) for 3 h, and then seeded at 5 x 10^4 cells per well in a 24-well plate. After overnight, 4 x 10^5 splenocytes prepared as described above were added to each well, and co-cultured with mitomycin C-treated B16F10 cells in RPMI1640 supplemented with 10% FCS and 100 U/ml IL-2 (Invitrogen) for three days at 37°C in a CO_2 incubator.

Bioluminescence measuring

The B16F10 cells transfected with pGL3-control vector were seeded at 3 x 10^5 cells/35-mm cell culture dish and cultured overnight at 37°C in a CO_2 incubator. The transfected cells (fLuc-B16F10) were treated with A23187 or puromycin in a phenol red-free culture medium supplemented with 5% FCS and 150 μg/ml D-luciferin (VivoGlo Luciferin, Promega), and subjected to bioluminescent measurement using a Kronos instrument (Kronos Dio, Atto). For mixed cell culture, 1 x 10^7 splenocytes were added to each 35-mm culture dish containing 1 x 10^5 fLuc-B16F10 cells.

Flow cytometry

The splenocytes prepared as described above were pretreated with the hybridoma HB-197 supernatant containing anti-CD16/32 antibody, collected by centrifugation, resuspended in the FACS buffer (phosphate-buffered saline containing 0.1% bovine serum albumin, 100 U/ml penicillin, and 100 μg/ml streptomycin). The cells were then stained with 2 μg/ml FITC-conjugated anti-mouse CD62L (MEL-14, BioLegend), 1 μg/ml PE-conjugated anti-human/mouse CD44 (IM7, eBioscience), and 2 μg/ml biotin-conjugated anti-mouse CD8α (53-6.7, Tonbo Bioscience) antibodies for 30 min on ice. The cells were subsequently stained with 1 μg/ml DyLight 649-conjugated streptavidin (Vector Laboratories), rinsed with the FACS buffer, spun down at 1,200 rpm for 5 min at 4°C, and resuspended in the FACS buffer. Flow cytometric analysis was performed on a flow cytometer (FACS-BD LSR Fortessa, BD Biosciences).

Results and Discussion

The fLuc-B16F10 cells, which transiently express the firefly luciferase gene, showed higher emission value than control B16F10 cells immediately after the start of measurement (Fig. 1). The fLuc-B16F10 cells emitted 5,000 times higher bioluminescence levels compared to a control B16F10. The signal was continuously detected for more than 12 h.

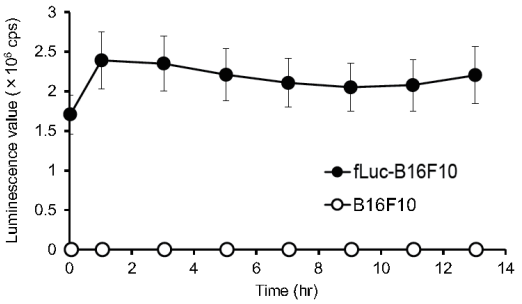

Fig. 1 Time-lapse measurement of luminescence value of B16F10 expressing firefly luciferase

The luminescence value of 2 x 10^5 fLuc-B16F10 was measured. Representative data of six independent experiments is shown. Each data point represents the mean (SD) of triplicate.

To confirm that the luminescence value of fLuc-B16F10 reflects the survival rate, we analyzed the temporal changes in the signal value accompanied by cell death after treatment with calcium ionophore A23187 and the antibiotic puromycin. Regarding A23187-induced cell death, there was no significant decrease in

luminescence value in cells treated with 0.3 μM compared to the untreated control group, whereas those with 3 μM and 15 μM, a decrease in bioluminescence signal was observed 1 h after the start of measurement (Fig. 2). The signal in cells treated with 15 μM A23187 was decreased more rapidly than those with 3 μM. About 30% of the maximum luminescence value was detected with 3 μM at 1.5 h, whereas luminescence was hardly detected with 15 μM.

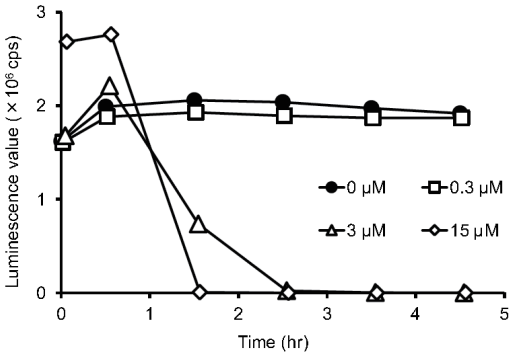

Fig. 2 Induction of cell death by calcium ionophore
The luminescence value of fLuc-B16F10 cells were measured after addition of the indicated concentrations of A23187. Representative data of three independent experiments is shown.

Our data reflect the previous study using the trypan blue dye exclusion, where the survival rate 1 h after A23187 treatment was 100% with 3 μM, about 60% with 5 μM, and 5% with 15 μM [6]. Regarding puromycin-induced cell death, there was a slight decrease in luminescence value with 1 μg/ml treatment or the untreated group, whereas with 3 and 6 μg/ml, a decrease in a time-dependent manner and almost no signal after 14 h (Fig. 3). These results suggest that we have successfully measured the rate of cell death by changes in bioluminescence signal.

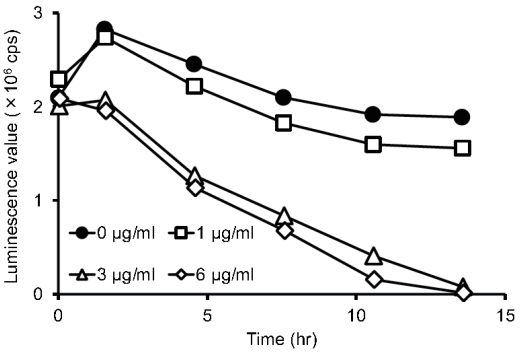

Fig. 3 Induction of cell death by puromycin
The luminescence value of fLuc-B16F10 cells were measured after addition of the indicated concentrations of puromycin. Representative data of four independent experiments is shown.

We next attempted the selective detection of target cell killing by activated lymphocytes in mixed cultures. To induce melanoma-specific CTLs, mouse splenocytes were harvested from B16F10 tumor-bearing mice and co-cultured with the mitomycin-treated target B16F10 cells. As shown in Fig. 4, the proportion of CTL (activated CD8 T cells) present about 25% before co-culture increased to about 50%, suggesting that some proportion of co-cultured splenocytes have CTL properties.

When we monitored the viability of fLuc-B16F10 in response to the responder splenocytes, there was no significant change between the responder-added wells and untreated control in 10 h, and both showed the maximum luminescence value approximately 5 h after the start of measurement (Fig. 5). In contrast, the signal was gradually decreased in the

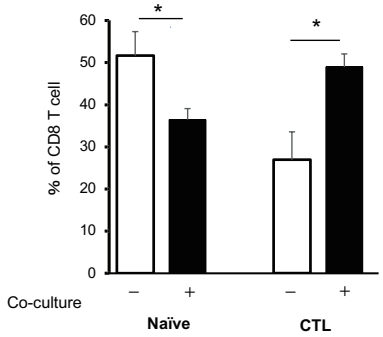

Fig. 4 *In vitro* simulation of splenocytes

Splenocytes from B16F10-carrying mice were co-cultured with B16F10 cells *in vitro*, and the percentages of naïve ($CD44^-/CD62L^+$) and activated ($CD44^+/CD62L^-$) cells of CD8 cells were analyzed by flow cytometry. Mean (SD) of triplicate samples analyzed by the Student's *t* test was shown. *:$p<0.05$

responder-added wells, whereas it continued to increase until about 30 h in the control. This result suggests that cells with CTL properties in the responder splenocytes induced fLuc-B16F10 cell death to some extent.

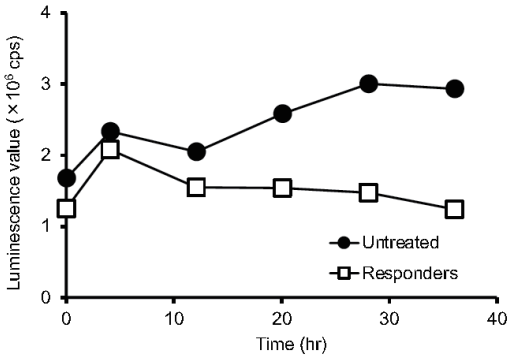

Fig. 5 B16F10 viability with or without stimulated splenocytes

The luminescence value of 1 x 10^5 fLuc-B16F10 was measured with or without stimulated responder splenocytes (1 x 10^7 cells / well).

Luminescence and fluorescence imaging are used for specifically detecting target cell death induced by CTLs. A previous report has evaluated the antigen-specific cytotoxicity of CTLs against mouse mammary tumor cells by measuring cell-associated luciferase activity. In the report, 72 h co-culturing of antigen-specific CTLs with the target cell in a 10:1 ratio reduced luciferase activity by 1/15 compared to control [7]. When the ratio was 20:1, the signal decreased by 1/100. In another study using fluorescence time-lapse imaging of B16 cell survival, co-culturing CTLs with B16 cells in a 25:1 ratio induced rapid B16 cell death in 10 min and the B16 viability decreased to 1/10 after 4 h [8]. In this study, the luminescence value increased gradually in the splenocyte-free group, whereas decreased with stimulated splenocytes, suggesting that fLuc-B16F10 cell death has been induced by the cells with CTL properties in stimulated splenocytes, although the antigen-specific CTL activity, if any, is low. The low CTL activity might be due to a low proportion of antigen-specific CTL in the stimulated splenocytes used for co-culture experiments, which is insufficient to induce efficient target cell death. It will be necessary to use CTLs enriched by cell sorting and confirm the specificity of target cell killing.

Conclusions

In this study, we have established a method for detecting cell death using a bioluminescence reporter. In this method, the cell viability can be measured using a low-background and high sensitivity with minimum damage. In addition, cell death of the target cells can be specifically detected, even in mixed cell cultures. We expect that the method contributes to evaluating the effect of reagents and cells on cell viability in various biological aspects.

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