Chapter 25

Noninvasive Imaging of Natural Killer Cell-Mediated Apoptosis in a Mouse Tumor Model

Thoudam Debraj Singh, Jaetae Lee, and Yong Hyun Jeon

Abstract

Natural killer (NK) cells are cytotoxic lymphocytes that induce apoptosis in cancer cells infected with viruses and bacteria through a caspase-3-dependent pathway. Effective NK cell-based immunotherapy requires highly sensitive imaging tools for in vivo monitoring of the dynamic events involved in apoptosis. Here, we describe a noninvasive bioluminescence imaging approach to determine the antitumor effects of NK cell-based therapy by serial imaging of caspase-3-dependent apoptosis in a mouse model of human glioma.

Key words Natural killer cells, Caspase-3 biosensor, Apoptosis, Immunotherapy, Reporter gene, *Renilla* luciferase

1 Introduction

Natural killer (NK) cells are cytotoxic lymphocytes that are the critical effectors of innate immunity [1, 2]. The killing effect of NK cells is regulated by activating receptors that identify ligands on target cancer cells or by inhibitory receptors that recognize major histocompatibility complex class I molecules on cancer cells. NK cells induce caspase-3-dependent apoptosis of cancer cells by exocytosis of cytotoxic granules containing perforins and granzymes and by aggregation of proteins belonging to tumor necrosis factor (TNF) superfamily (FasL/TNF) and their corresponding ligands (Fas/TNFR) [3–5].

The therapeutic effect of NK cell-based immunotherapy depends on the ability of these cells to maintain a balance between their activating and inhibitory receptors and their susceptibility to cancer cells [5–7]. Although many studies have examined the efficacy of NK cells in tumor immunotherapy, their distinct clinical benefits have been not demonstrated because of several factors such as alteration of their functions and unfavorable tumor microenvironments [8]. Another problem is the lack of reliable tools for the in vivo monitoring of apoptosis induced by NK cell therapy in a cancer model. Therefore,

Srinivas S. Somanchi (ed.), *Natural Killer Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 1441,
DOI 10.1007/978-1-4939-3684-7_25,

effective, practical, and noninvasive imaging tools are required to evaluate the efficacy of NK cell-based therapy, to monitor the progression of apoptosis in tumors accurately, and to understand the mechanisms underlying the failure of NK cell therapy.

A specialized molecular imaging system that can demonstrate the dynamic processes involved in apoptosis will provide a comprehensive understanding of apoptosis-related biochemical cascades. This system will also facilitate the evaluation of various important factors for NK cell-based therapy, such as dose, frequency, and injection route. In a recent study, apoptosis induced in a human glioma model by chemotherapeutic drugs was successfully monitored by performing noninvasive imaging with a reporter of caspase-3 proteolysis [9]. This apoptosis biosensor contained split luciferase domains fused with interacting peptides pepA and pepB and an intervening caspase-3 cleavage motif DEVD. Upon induction of apoptosis, this biosensor was proteolytically cleaved by caspase-3 at the DEVD motif. The cleavage induced the reconstitution of the luciferase activity through an interaction between NLuc and CLuc (Fig. 1). This system enabled real-time monitoring of the progression of apoptosis in living subjects in a repetitive and noninvasive manner. These advantages of the apoptosis biosensor suggest that it can be used to monitor the killing activity of NK cells both in vitro and in vivo.

In this chapter, we have emphasized on the application of this caspase-3 biosensor for monitoring the effects of NK cell-based immunotherapy in vivo. For this, we established human glioma cells expressing the caspase-3 biosensor as a surrogate marker for caspase-3 activation and *Renilla* luciferase (Rluc) as a surrogate marker for cell viability. Human NK92 cells were used as effector cells for NK cell immunotherapy. Activation of apoptosis by NK cell therapy was serially monitored in a glioma model by using the caspase-3 biosensor, and its therapeutic outcomes were evaluated using Rluc.

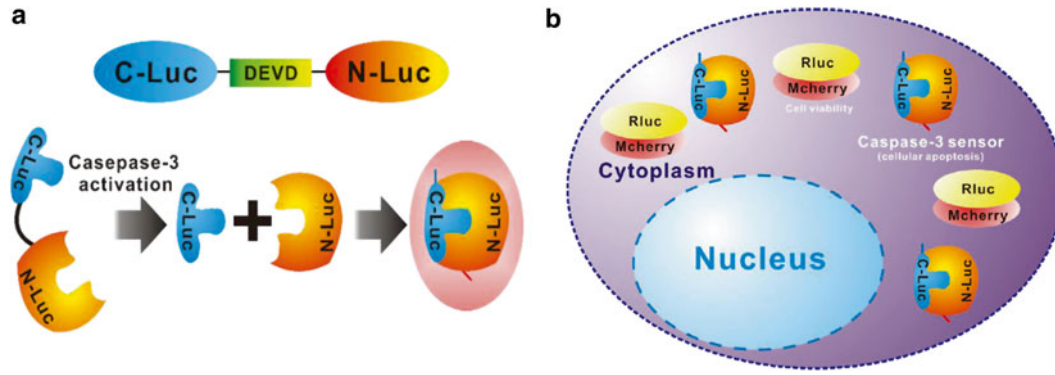

Fig. 1 Schematic representation of (**a**) Caspase-3 sensor (**b**) Stable glioma cancer D54 cells coexpressing caspase-3 sensor, R*luc*, and mCherry genes

2 Materials

2.1 Cell Lines and Culture Medium

1. Human IL-2-dependent NK92 cells line (ATCC).
2. Tumor cell line of choice: For the purposes of this chapter, we are describing the method using human glioma cell line D54 (provided by Dr. Alnawaz Rehemtulla, University of Michigan).
3. NK cell culture medium: Alpha-minimum essential medium without ribonucleosides and deoxyribonucleosides (HyClone), 2 mM L-glutamine (Sigma), 1.5 g/L sodium bicarbonate (Sigma), 0.2 mM inositol (Sigma), 0.1 mM 2-mercaptoethanol (Invitrogen), 0.02 mM folic acid (Sigma), 12.5% horse serum (HyClone), 12.5% fetal bovine serum (HyClone), 1% antibiotics (penicillin/streptomycin; Gibco), 1000 IU/mL IL-2 (Roche).
4. Commercial NK cell medium (optional): (ALyS505NK-EX) supplemented with 10% human serum, 1000 IU/mL IL-2, and 1% penicillin/streptomycin (this media can also be used instead of NK cell culture medium Subheading 2.1, **item 3**).
5. D54 cell culture medium: RPMI 1640 medium (HyClone), 10% FBS (HyClone), 1× Penicillin/Streptomycin (Gibco), 1× GlutaMAX (Gibco).
6. 96-Well black plates with a clear bottom (Corning).
7. T-75 Flasks (BD Falcon).
8. 6-Well plates (Corning).

2.2 Establishment of Caspase-3 Biosensor and Rluc Cell Line

1. Geneticin (G418; Gibco).
2. Puromycin (Sigma).
3. X-tremeGENE 9 transfection reagent (Roche).
4. Lentiviral vector coexpressing genes encoding Rluc, mCherry, and puromycin under the control of CMV promoter (GeneCopoeia).
5. Caspase-3 Biosensor plasmid [9] was provided by Dr. Alnawaz Rehemtulla (University of Michigan). This caspase-3 sensor is constructed into the vector of pEF that includes the neomycin resistance gene. Thus, it can be selected with neomycin (G418) after transfection of caspase-3 biosensor.
6. FACSAria II (BD Biosciences).

2.3 In Vivo Experiments: Mice, Imaging Reagents, and Instruments

1. Six-week-old pathogen-free female BALB/c nude mice.
2. D-Luciferin (DV Medical Research Innovations): Prepare fresh stock solution of 30 mg/mL D-luciferin in DPBS. Filter the solution using a 0.2-μm filter.

3. Coelenterazine h: For in vitro experiment, dilute commercial RediJect Coelenterazine h (120 μg/mL stock concentration; PerkinElmer) in respective culture media to 1 μg/mL. For in vivo experiment, dilute commercial Coelenterazine h (NanoLight Technology) in absolute ethanol (Sigma) to prepare 2 mg/mL stock solution.
4. GloSensor™ reagent (Promega): Prepare HEPES buffer (10 mM) in deionized water; adjusted pH to 7.5 by using KOH. Add 817 μL HEPES buffer to a reagent vial containing 25 mg of GloSensor™ reagent.
5. Isoflurane: Keep at room temperature, away from light.
6. IVIS Lumina III (PerkinElmer).

3 Methods

3.1 NK92 Cell Culture

1. Seed NK92 cells at a density of 1×10^5 cells/mL and culture in a T-75 flask containing NK cell medium.
2. To passage NK92 cell, collect a large, healthy colony of cells (as observed under a microscope) transfer to a conical tube and resuspend in 5–10 mL fresh NK cell medium to obtain a single cell suspension (*see* **Note 1**).
3. Centrifuge the cells at $300 \times g$ (*see* **Note 2**).
4. Reseed the cells in a T-75 flask in NK cell medium at a density of 1×10^5 cells/mL for subculture.

3.2 Expression of Caspase-3 Biosensor, Rluc, mCherry in Tumor Cells

Different cancer cell lines can be transfected with the caspase-3 sensor construct using X-tremeGENE 9 transfection reagent, and stable clones can be selected with geneticin (Gibco) as described below. The established stable cell lines expressing the caspase-3 sensor should be transducted with lenti-Rluc-mCherry vector and selected for stable clones by FACS sorting to get tumor cells coexpressing caspase-3 sensor, Rluc-mCherry. But for the purposes of this chapter, we are describing this method using D54 cell line. It should be noted that Caspase-3 biosensor can also be applicable to another human cancer cell lines. In our experiences, we successfully monitored the caspase-3 activation in thyroid cancer, colon cancer, and breast cancer cells with caspase-3 biosensor after treatment of Natural killer cells such as NK92 (IL-2 independent), NK92MI (IL-2 independent), and primary human NK cells derived from peripheral blood.

1. Transfect 5×10^5 cells D54 glioma cells in a 6-well plate with the caspase-3 biosensor construct using X-tremeGENE 9 transfection reagent as per manufacturer's protocol (*see* **Note 3**).

2. Culture the transfected D54 cells in RPMI complete medium supplemented with geneticin for 2 weeks to select for stable expression of caspase-3 biosensor (*see* **Note 4**).
3. Transduce (5 MOI) lentiviral vector coexpressing Rluc, mCherry, and puromycin genes to 1×10^6 cells D54 caspase-3 biosensor stable cells in presence 10 μg/mL polybrene. Replace with RPMI complete media after 24 h containing puromycin.
4. Culture transduced cells in RPMI culture medium supplemented with puromycin for 2 weeks to select for stable expression of Rluc and mCherry genes (*see* **Note 5**).
5. Sort using the FACSAria II cell sorter to isolate a pure population of cells coexpressing Rluc and mCherry (*see* **Note 6**).
6. Maintain established cell line stably coexpressing the caspase-3 biosensor, Rluc, and mCherry (labeled in this case as D54/CR) in culture under geneticin and puromycin selection (Fig. 1).

3.3 In Vitro Bioluminescence Imaging

In order to validate engineered reporter cells as a caspase-3 biosensor, it is essential to perform an in vitro assay in live cells (without cell lysis) for detection of caspase-3 activation and viability upon treatment of NK92 cell. This assay will provide a readout for a time- and dose-dependent increase in bioluminescence activity of the caspase-3 biosensor, which will indicate activation of apoptotic pathway in the tumor cells following NK cell mediated lysis.

1. Seed the gene-modified tumor cell line—D54/CR—cells at a density of 1×10^4 cells in 100 μL/well in a 96-well black plate with a clear bottom.
2. After 24 h, replace old culture medium with 100 μL NK cell medium supplemented with 1 % GloSensor™ and equilibrate for at least 1–2 h until a steady-state basal bioluminescence signal is obtained at 37 °C in a 5 % CO_2 atmosphere. Measure the basal luciferase activity in D54/CR cells using IVIS Lumina III (*see* **Note 7**).
3. Add 10 μL NK92 cells at different target to effector ratios (1:1, 1:2.5, 1:5, and 1:10) in 100 μL of NK cell medium (99 μL) supplemented with 1 % GloSensor™ (1 μL) and incubated at 37 °C in a 5 % CO_2 atmosphere, and measure the bioluminescence activity of the caspase-3 biosensor after 2, 4, and 6 h of incubation, using IVIS Lumina III (Fig. 2) (*see* **Note 8**).
4. Measure the luciferase activity of Rluc by adding 1 μg/mL Coelenterazine h and determine viability of tumor cells—D54/CR—cells using IVIS Lumina III.

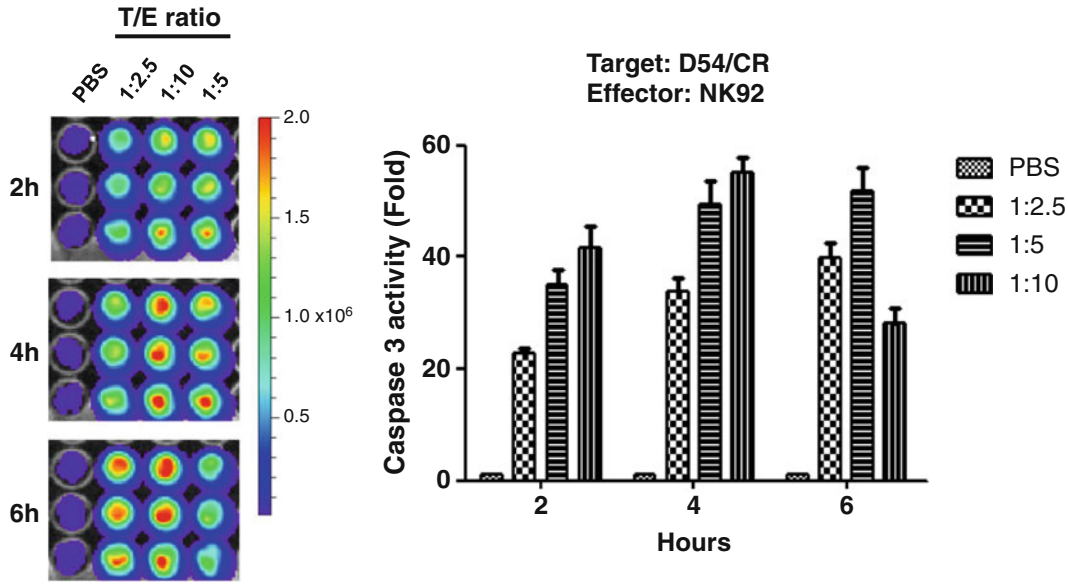

Fig. 2 In vitro monitoring of caspase-3 activity after NK92 treatment in D54/CR cells

3.4 Noninvasive In Vivo Imaging of Caspase-3 Activation

The in vivo imaging study is described to demonstrate the feasibility of caspase-3 biosensor for evaluation of caspase-3-dependent apoptosis event noninvasively and repetitively in living mice with D54/CR tumor. The dosages of NK cells to be used in mouse model need to be titrated when using a different cell line.

1. Inject 5×10^6 D54/CR cells subcutaneously into the right hind flank region of mice ($n = 30$ mice) (*see* **Note 9**).
2. Monitor the mice for tumor formation by inspection and palpation. Normally, the tumors are detectable within 2–3 weeks (*see* **Note 10**).
3. Determine basal bioluminescence activity of the caspase-3 biosensor and Rluc D54/CR tumors after 3 weeks.
4. Randomized into three groups ($n = 10$ mice/group) with equivalent Rluc mean bioluminescence value.
5. *Group 1*: Administer 100 μL PBS (control group) via tail vein.
6. *Group 2*: Administer 2×10^6 NK92 cells resuspended in 100 μL of PBS via the tail vein (*see* **Note 11**).
7. *Group 3*: Administer 1×10^7 NK92 cells resuspended in 100 μL of PBS via the tail vein.
8. For in vivo BLI, administer each tumor-bearing mouse with a single dose of the substrate of each reporter protein as follows: (1) To determine viability of tumor cells through the luciferase activity of Rluc: Anesthetize the mice and then inject 2 mg/kg of Coelenterazine h carefully injected through the tail vein.

Place the mice in the imaging chamber of IVIS Lumina III immediately after injecting the substrates, and acquire bioluminescent images. (2) To determine the bioluminescence activity of the caspase-3 biosensor: Inject 150 mg/kg of D-luciferin intraperitoneal, and anesthetize the mice using 1%–2% isoflurane/air mixture within 5 min after injecting D-luciferin. Obtain bioluminescent images of mice using IVIS Lumina III.

9. BLI with the caspase-3 biosensor and Rluc was performed using IVIS Lumina III at designated times points (12, 24, and 48 h) after treatment with NK92 cells (*see* **Note 12**).
10. Acquire the images of caspase-3 activation using the sequence imaging acquisition mode (Fig. 3a, black arrow) and by changing respective parameters such as field of view (FOV), image acquisition time (from second to minute), and delay time. Same FOV should be used at each imaging time point (Fig. 4, blue arrow) because different FOVs cannot be used to compare the changes in bioluminescence activity at different time points (*see* **Note 13**). Analyze Region of interest (ROI) on the mice images to determine the peak activity of the caspase-3 biosensor and Rluc, using Living Image software. Representative

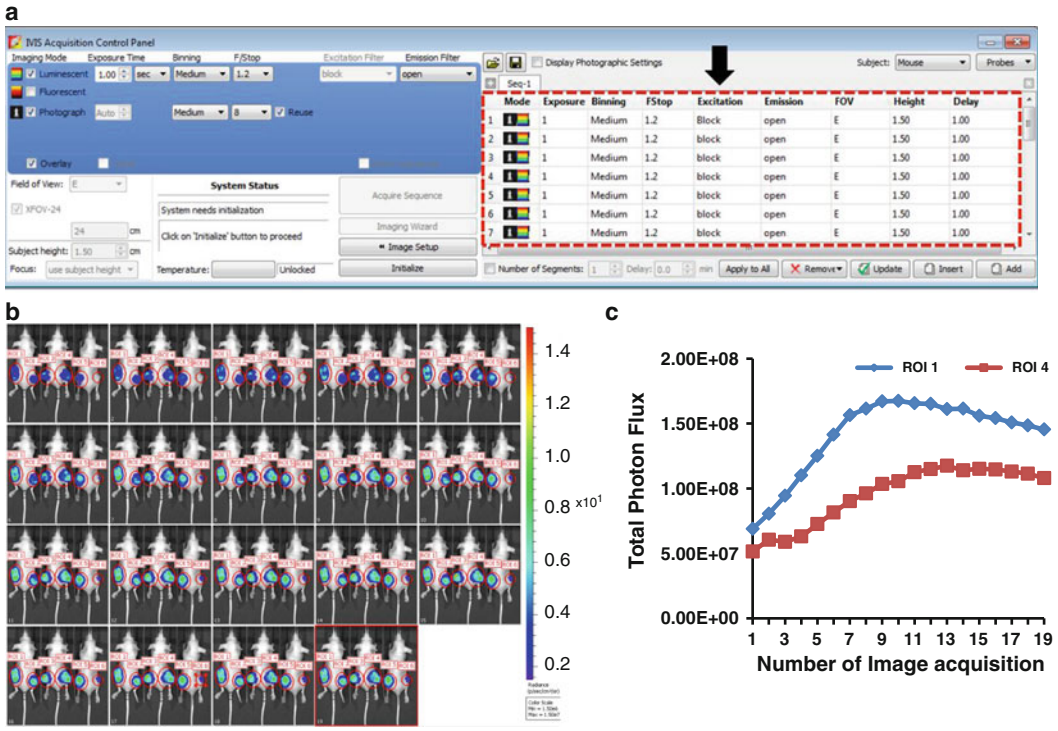

Fig. 3 (**a**) Schematic image for visualization of caspase-3 activation by sequential imaging acquisition mode, (**b**) Representative data of sequential imaging acquisition and (**c**) ROI analysis

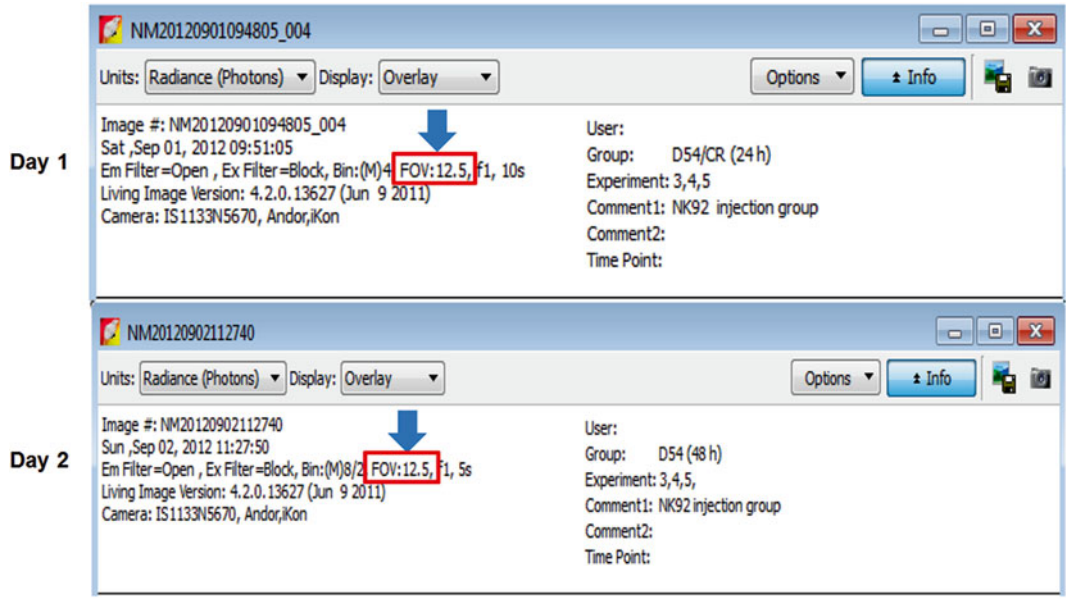

Fig. 4 Template showing sequential imaging acquisition parameters

Table 1
Representative example of calculation of relative caspase-3 activity

	Day 0	Day 1	Day 2	Day 3
Caspase-3 activity (photon flux)	6.84×10^7	2.08×10^8	3.62×10^8	2.33×10^8
Rluc activity (photon flux)	1.07×10^8	9.07×10^7	8.05×10^7	6.08×10^7
Relative Caspase-3 activity (Caspase-3 activity/Rluc activity)	0.638	2.294	4.495	3.837

sequential images obtained for caspase-3 biosensor, and a graph depicting 2 ROIs from these sequential images are shown in Fig. 3b, c.

11. Finally, calculate the relative increase in the bioluminescence activity of the caspase-3 biosensor by dividing the bioluminescence activity of the caspase-3 biosensor (cellular apoptosis) with the bioluminescence activity of Rluc (cell viability) at each time point (*see* Table 1).

4 Notes

1. The condition of NK92 cells was examined carefully. If a colony was small, the cells were incubated for an additional day to obtain larger colonies.

2. Viability of cells was >80% (determined by trypan blue dye exclusion test). After centrifugation, the conditioned medium of NK92 cells was retained and was used for culturing fresh NK92 cells to enhance their proliferation.
3. Prepare transfection complex by incubating 2 μg plasmid DNA and 6 μL transfection reagent in 100 μL of opti-MEM medium for 15 min at room temperature. Transfect D54 cells by adding 100 μL transfection complex in each well containing 2 mL complete media. Replace the media after 24 h. However, titration of DNA and transfection reagent is necessary to get maximum transfection efficiency in different cell lines.
4. Optimal lowest concentration (kill curve) of geneticin to kill 100% nontransfected cells was determined by treating D54 cells plated in 6-well plates with different concentrations of geneticin ranging from 100 to 1000 μg/mL. We used 200–400 μg/mL geneticin for selecting stable clones expressing the caspase-3 biosensor.
5. The optimal lowest concentration (kill curve) of puromycin to kill 100% nontransfected cells was determined by treating D54 cells plated in 6-well plates with different concentrations of puromycin ranging from 1 to 10 ng/mL. We used 6 ng/mL puromycin for selecting stable clones.
6. The lentiviral vector used in this study contains the internal ribosome entry site (IRES) flanked by two multiple cloning sites under the control CMV (cytomegalovirus) promoter. Rluc and mCherry genes located in upstream and downstream of IRES, respectively. Thus, mCherry fluorescent protein can be used for surrogate for Rluc genes and we can expect that all mCherry positive cells are also Rluc positive cells as Rluc and mCherry are expressed in a single construct. We measure the Rluc activity from live cells plated in 96-well black plate with clear bottom in the presences of its substrate without cell lysis using a Luminometer or IVIS machine.
7. The use of the 1% GloSensor™ reagent allowed the serial monitoring of apoptosis events in intact cells expressing the caspase-3 biosensor for at least 7 days without adding D-luciferin at each time point.
8. For negative control, add 5 μL PBS in 100 μL of NK cell medium (99 μL) supplemented with 1% GloSensor™ (1 μL) in first column of D54/CR cells.
9. The number of mice to be used in the study should be determined based on statistical considerations for each experiment.
10. Since the duration of tumor formation may vary for different cell lines, monitoring of tumor growth is necessary for at least twice a week using optical imaging or palpation and inspection.

11. PBS or cell suspension containing 5×10^6 NK92 cells was carefully injected into the tail vein of tumor-bearing mice (100 or 200 μL of NK cell suspension is usually used for in vivo therapy. Please note that NK cells should be injected slowly because rapid injection of NK cells may result in the death of mice).
12. Rluc signal decreased rapidly within 1 min after injecting its substrate. Therefore, BLI by using Rluc should be carried out before performing BLI for the caspase-3 biosensor.
13. Sequential images of BLI of the caspase-3 biosensor with 5 or 10 s (image acquisition time) were captured for 30–40 min at 1 min interval (delay time). Bioluminescence activity of the caspase-3 biosensor peaked within 30 min after injecting its substrate and remained at that level for approximately 0.5–1 h.

Acknowledgments

This work was supported by a grant from the National Research Foundation (NRF) of Korea funded by the Korean government (MEST, 2009-0078234); the National Nuclear R&D Program of the NRF funded by the Ministry of Education, Science and Technology (No. 2012M2A2A7014020); a grant from the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A111345); a grant (RTI04-01-01) from the Regional Technology Innovation Program of the Ministry of Knowledge Economy and the Kyungpook National University Research Fund (2013); a grant from the Medical Cluster R&D Support Project of Daegu-Gyeongbuk Medical Innovation Foundation, Republic of Korea; and a grant from the NRF of Korea funded by the Korean Government (MSIP, 2014R1A1A1003323).

References

1. Cerwenka A, Lanier LL (2001) Natural killer cells, viruses and cancer. Nat Rev Immunol 1:41–49
2. Lanier LL (2008) Evolutionary struggles between NK cells and viruses. Nat Rev Immunol 8:259–268
3. Rosen D, Li JH, Keidar S et al (2000) Tumor immunity in perforin-deficient mice: a role for CD95 (Fas/APO-1). J Immunol 164:3229–3235
4. Trapani JA, Smyth MJ (2002) Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2:735–747
5. Screpanti V, Wallin RP, Grandien A et al (2005) Impact of FASL-induced apoptosis in the elimination of tumor cells by NK cells. Mol Immunol 42:495–499
6. McQueen KL, Parham P (2002) Variable receptors controlling activation and inhibition of NK cells. Curr Opin Immunol 14:615–621
7. Shi J, Tricot GJ, Garg TK et al (2008) Bortezomib down-regulates the cell-surface expression of HLA class I and enhances natural killer cell-mediated lysis of myeloma. Blood 111:1309–1317
8. Zamai L, Ponti C, Mirandola P et al (2007) NK cells and cancer. J Immunol 178:4011–4016
9. Coppola JM, Ross BD, Rehemtulla A (2008) Noninvasive imaging of apoptosis and its application in cancer therapeutics. Clin Cancer Res 14:2492–2501