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Full Length Research Paper

A flow cytometric assay for simultaneously measuring the proliferation and cytotoxicity of cytokine induced killer cells in combination with carboxyfluorescein succinimidyl ester (CFSE) labeling

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This research objective was to exploit a novel method for measuring the proliferation, cytotoxicity of cytokine-induced killer (CIK) cells using carboxyfluorescein succinimidyl ester/proliferation index (CFSE/PI) and flow cytometric assay. As cells divide, CFSE is apportioned equally between the two daughter cells, leading to a characteristic flow cytometric profile where a number of peaks of progressively halving CFSE fluorescence intensity were observed. The test principle of cytotoxicity is based on target cell labeling with CFSE and subsequent DNA-labeling with PI for the identification of target cells with compromised cell membranes. Results of CFSE fluorescence profile of the entire cell population showed that these cells underwent up to 6 divisions after 21 days. The percentage of total dividing cell population was 97.12%, of which 17.28% cells went through 6 rounds and 6.84% cells entered the seventh generation. The cytotoxicity of CIK cells showed a significant and positive correlation with effector: target (E:T) ratio ranging from 50:1 to 6.25:1. CFSE labeling combined with flow cytometry can therefore be used to monitor the proliferation of CIK cells. Moreover, it presents an advantageous method to measure cytotoxicity of CIK cells by avoiding radioactive substance, increasing sensitivity at low E:T ratio, analyzing on a cell-to-cell basis and allowing for long-term incubation.

Key words: Flow cytometry, CFSE, cytokine-induced killer cell, proliferation, cytotoxicity.

INTRODUCTION

Cytokine-induced killer (CIK) cells are a group of heterogeneous cell populations derived from the human peripheral blood mononuclear cells (PBMCs) co-cultured in the presence of a variety of cytokines such as interleukin-2 (IL-2), interleukin-1 (IL-1α) and interferon-gamma (IFNγ), as well as monoclonal antibody against CD3 (anti-CD3mAb). CIK cells are endowed with both a powerful anti-tumor T-lymphocyte activity and non-major histocompatibility complex-restricted in target cell recognition without any impact on bone marrow hemato-poietic system (Schmidt-Wolf et al., 1997; Linn and Hui, 2003). Therefore, CIK cells are considered to be the most potent and promising adoptive immunotherapy (Kim et al., 2009a, b, 2007a, b).

Actually, CIK cells are a unique population of cytotoxic T lymphocytes (CTL) with the characteristic CD3+CD56+ phenotype (Alvarnas et al., 2001). Although, the number of CD3+CD56+ lymphocytes is rare in human peripheral blood lymphocytes, these cells demonstrate dramatic

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Table 1. CFSE labeling conditions for the different target cells.

Cell line	K562	HL60	HeLa	A375	MCF7
Cell concentration	1×10^9	1×10^9	1×10^9	1×10^9	1×10^9
Dye concentration (μM)	2.5	2.5	5	5	1 0
Incubation time (min)	8	8	8	8	8
Incubation temperature (℃)	37	37	37	37	37

proliferation capacity and cytolytic activities when stimulated by various types of cytokines. The antitumor efficiency of CIKs is promoted by increasing proliferation, inhibiting apoptosis and enhancing cytotoxicity. The expansion efficiency of CIK cells depends on its ability of division and differentiation, and the proliferation ability of CIK cells is closely related to their antitumor activity (Yu et al., 2011; Li et al., 2010; Wang et al., 2010). Information on CIK cells proliferation and cytotoxicity is required in the clinical application of adoptive immunotherapy. Thus, it is particularly important to develop a suitable method to determine the proliferation, phenotype change and the cytotoxicity of CIK cells, to evaluate their characteristic biological properties and anticancer function. In this study, we exploited a novel flow cytometric strategy to measure the proliferation and cytotoxicity of CIK cells in combination with the application of vital dye carboxyfluorescein diacetate succinimidyl ester (CFDA-SE).

MATERIALS AND METHODS

Cell lines and cell culture

Human erythroleukemic cell line (K562), human promyelocytic leukemia cell line (HL-60), human cervical cancer cell line (HeLa), human melanoma cell line (A375) and human breast cancer cell line (MCF-7), were chosen as target cells. These cells were cultured in 5% CO_2 at 37℃ in Iscove's modified Dulbecco's medium (IMDM, Gibco) supplemented with 3024 mg/L $NaHCO_3$, 2 mM *L*-glutamine, 25 mMHEPES (Gibco, UK), 100 I.U. penicillin, 100 mg/L streptomycin and 10% fetal calf serum (FCS, Gibco), referred to as complete medium (CM). All cells were passaged the day prior to the experiment to ensure log phase growth.

Isolation and preparation of PBMCs

Heparinized peripheral blood obtained from healthy adult volunteers was layered on an equal volume Ficoll Paque™ plus (Institute of Biology Engineering, Chinese Academy of Medical Sciences, China), then spun at 300 *g* for 20 min at room temperature. The PBMCs in the interphase were removed and washed twice with PBS at 200 *g* for 5 min. These cells were counted to ensure the percentage of viable cells was up to >95% and diluted to 10^9/L in CM. Cell viability was examined by the propidium iodide (PI, St. Louis, MO, USA) nuclear staining method. Cells were stained with 50 μL of a 50 μg/ml PI solution and analyzed with FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cells stained with PI were considered as dead cells.

CFDA-SE labeling of target cells and PBMCs

Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE Cat# C-1157, Molecular Probes, USA) stock solution (5 mM) was dissolved in DMSO (Fisher Scientific, Fair Lawn, NJ, USA). 2×10^7 PBMCs in 0.5 ml PBS was mixed with equal volume of 5 μM CFDA-SE in a total volume of 1 ml PBS and the final concentration of CFDA-SE was 2.5 μM, and the cell/CFDA-SE mixture was incubated at 37℃ for 8 min under gentle agitation. The labeling reaction was stopped by incubation with an equal volume of pre-cooled fetal bovine serum (4℃) (FBS, Hyclone, Logan, UT, USA) for 1 min. The CFSE labeled cells were washed twice with PBS containing 10% FBS and recounted, then used as proliferative experiment. The procedure of labeling target cells was done same as PBMCs, However, for target cells of various cancer cells that load CFSE concentration differently, they were labeled at the different optimal CFDA-SE concentration and incubated for different durations, respectively, as previously described (Wang et al., 2005). The conditions of labeling target cells with CFDA-SE are shown in Table 1. CFSE labeled target cells were immediately used in the cytotoxicity assay. The target cells and PBMCs concentration was adjusted to 6×10^7 and 1×10^9 cells/L, respectively in CM.

Induction of CIK cells

CIK cells were generated as previously described (Du et al., 2000). CFSE labeled or unlabeled PBMCs were suspended in CM at a concentration of 10^6 cells/ml. Cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2, 1000 kU/L IFN_γ was added on the 1st day. After 24 h incubation, 50 mg/L monoclonal antibodies (mAb) against CD3 (BD Pharmingen, NJ, USA), 300 kU/L IL-2 (Gibco), and 100 kU/L rIL-1α (Gibco) were added. Fresh IL-2 (150 kU/L) and CM were added every 3 days to maintain a cell density of 1.5 to 2.0×10^9 cells/L for 21 days. CFSE-unlabeled cells were used as the control for proliferation assay and effectors for cytotoxicity assay. The viability of cells was determined by PI nuclear staining, with the viability of expanded cell population on day 21 being 85 to 90%.

Proliferation assay and phenotype analysis of CIK cells

CIK cells loading CFSE cultured for 21 days were harvested and washed twice with PBS, then divided into aliquots, some of which for proliferation assay and the rest (10^6 cells) were stained with anti-CD3 conjugate PerCP (Clone No.SK7), anti-CD56 conjugate PE (Clone No.MY31). The samples were incubated for 30 min at 4℃ in dark, and washed once in PBS at 300 *g* for 5 min. The supernatants were discarded and the cell pellets were resuspended in 0.5 ml PBS prior to flow cytometry. The phenotype and percentage of divided CIK cells population was analyzed on a FACSCalibur (BD Bioscience, San Jose, CA, USA), usually 20,000 events, excluding cellular debris gating on the forward scatter versus side scatter cell

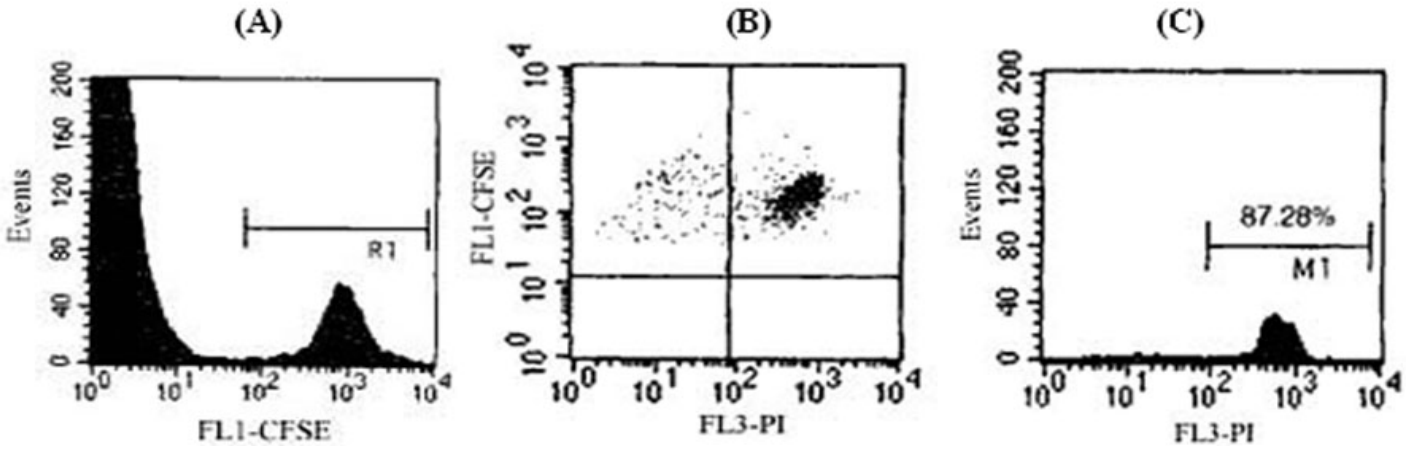

Figure 1. Flow-cytometric analysis of CFSE (FL1) and PI (FL3) labeled target cells (K562, HL-60, HeLa, A375 and MCF-7). (A) CFSE positive target cells were exposed and gated (R1) in an FL1 histogram. (B) Target cells (R1) were further analyzed in an FL1/FL3 dot plot, dead target cells (CFSE+PI+) were visualized in upper right quadrant. (C) An FL3 histogram was then used to determine the proportion of dead target cells as 87.28%. PI-treated target cells, not incubated with effectors, were used to set marker for dead cells (M1).

population.

All data were acquired by CellQuest™ software ver.5.2.1 (BD Bioscience, USA), with FL1 as the CFSE emission channel (530/30 nm band pass filter), FL2 as the PE emission channel (585/42nm band pass filter), and FL3 as the PerCP emission channel (670nm Long Pass), using logarithmic amplifiers, respectively. The sequential loss of CFSE fluorescence was used to measure cell division. The fluorescence intensity distribution of the CFSE-stained sample was run under the same instrument conditions as the unstained cells. The peak position of CFSE labeled PBMCs, but not induced was used to set up a cut-off level for CIK cells. The percentage of total proliferated PBMCs, the maximum number of cells division, and the numerical values for proportions of proliferated cells at each cell division were obtained by using Proliferation Wizard Basic Mode analysis of ModFit LT software ver.3.0 (Verity Software House, USA). The parent generation was set as the median fluorescence intensity using an unstimulated CFSE labeled control sample. All fluorochrome labeled mAbs were purchased from BD Biosciences (CA, USA).

Flow cytometric assay of cytotoxicity

Unlabeled CIK cells were used as the effector (E), and K562, HL-60, HeLa, A375 and MCF-7 cells served as targets (T). Effector and target cells were mixed in CM at different E:T ratio (100:1, 50:1, 25:1, 12.5:1, 6.25:1) in triplicates, with 6000 target cells added per well. In parallel, target cells were incubated alone to measure spontaneous cell death. The mixed cells were spun transiently at 120 *g* for 1 min, and incubated at 37°C for 4 h in 96-well U-bottomed plates (Becton Dickinson, Franklin Lakes, New Jersy, USA) in a final volume of 200 µL per well in a humidified 5% CO_2.

To investigate the possible loss of fluorescence intensity after a period of culture, freshly labeled K562 cells were analyzed in the flow cytometer and compared with K562 cells incubated for 18 h. Possible cross-contamination of effector cells was tested by measuring fluorescence intensity in the effector cell population after 18 h co-cultivation with freshly stained target cells. At the end of the incubation time, the total contents of the U bottom plate were transferred to 12 × 75 mm Falcon tubes (Becton Dickinson Labware, Lincolin Park, NJ). The tubes were then put in an ice water bath and incubated with 50 µL of 50 µg/ml PI for 5 min, followed by flow cytometric analysis within 1 h. CFSE /PI double stained target cells were excited by an argon ion laser emitting at 488 nm. CFSE were detected in the FL1 channel (530/30 nm band pass filter), while PI were detected in the FL3 channel (670nm Long Pass).

All samples were analyzed on a FACSCalibur (BD Bioscience, USA), using software CellQuest ver.5.2.1(BD Bioscience) for the acquisition and data analysis. The total number of events (cells) was determined by analyzing the data using a dot plot and rectangular regions to define the cell populations. During data acquisition, "a live gate" was set on the CFSE-stained target cell population using an FL1-histogram (Figure 1A), and 1000 target events were collected. For data analysis, target cells (R1) were further analyzed in an F1/F3 dot plot, dead target cells (CFSE+PI+)were visualized on upper right quadrant (Figure 1B). An FL3 histogram (Figure 1C) was used to determine the percentage of lysed target cell. PI-treated target cells, not incubated with effectors, were used to set marker for dead cells (M1). The percentage of target cell death (cytotoxicity) was calculated as:

$$\text{Cytotoxicity (\%)} = \frac{\text{Dead target cells in the sample (\%)} - \text{Spontaneously dead target cells (\%)}}{100 - \text{Spontaneously dead target cells (\%)}} \times 100$$

Statistical analyses

Time-kinetics of spontaneous target cells death was calculated by linear regression analysis using statistical SAS software, while statistical comparison of cytotoxicity was performed with 't' test. $P<0.05$ was considered statistically significant.

RESULTS

CFSE labeling target cells

To observe the variation of the fluorescence intensity of CFSE-labeled target cells with time, the loss of the intensity was studied by comparing freshly labeled target cells with labeled target cells incubated for 18 h. A

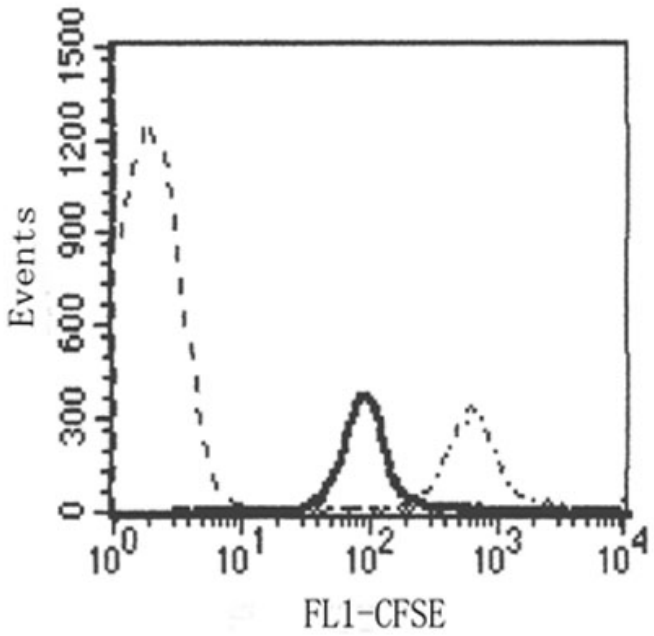

Figure 2. Histogram overlay showing K562 target cells immediately after CFSE labeling (dotted line), target cells incubated for 18 h after CFSE labeling(black line) and unlabeled effector cells (break line).

decrease in fluorescence intensity after 18 h incubation could be observed with the mean fluorescence intensity dropping from 3439 to 1774. Moreover, a clear separation between labeled target cells and unlabeled effector cells was observed (Figure 2).

Assay of spontaneous targets cell death

In the absence of effectors, the percentage of spontaneous target cells death was determined by PI staining. PI is an intercalating DNA dye that detects the loss of plasma membrane integrity, with a late event associated with cell death. Its level of incorporation depends on the extent of membrane alteration. The double stained $CFSE^+/PI^+$ populations represented spontaneous dead target cells, while the $CFSE^+/PI^-$ cells were live cells. From triplicate experiment, the spontaneous death of K562 cells were found to be less than 5% within 12 h of incubation and less than 10% within 24 h (Figure 3).

Proliferation assay and phenotype analysis of CIK cells

PBMCs were labeled with CFDA-SE and then cultured in the presence of various types of cytokines for 21 days. The distribution of cells according to the number of divisions they completed was shown by the different sized peaks corresponding to serial 2-fold decreases of fluorescence intensity. CFSE fluorescence profile of the entire cell population showed that these cells underwent up to 6 divisions after 21 days of expansion. The percentage of total divided cell population amounted to 97.12%, of which 17.28% cells went through 6 rounds and 6.84% cells entered the seventh generation (Figure 4). We estimated the proliferation of CIK cells using the Proliferation Index (PI), which can be calculated by a formula:

PI = The total numbers posterior to proliferation / The total numbers prior to proliferation.

PI was 4.56 as calculated by proliferation wizard basic mode analysis of the ModFit software. The fluorescence intensity distribution of the CFSE-stained and unstained cells was shown as overlap on the histogram (Figure 5). The profile of unstained sample, including CFSE-unstained PBMCs but non-induced control and CFSE-unstained CIK cells induced for 21 days, was both located in 10^0 - 10^1 of x-axis on the histogram. The profile position of CFSE stained without induced control reduced in parent generation of CIK cells. These results indicate that the division of CIK cells was monitored by CFSE labeling combined with flow cytometry.

CD3 and CD56 are surface markers of T cells which exert highly cytotoxic effects on tumor cells. The immunophenotype assays revealed that the percentage of CD3 + CD56+T cells in the population of CFSE labeled PBMC increased from 5.39 to 83.91% after induction of CIK for

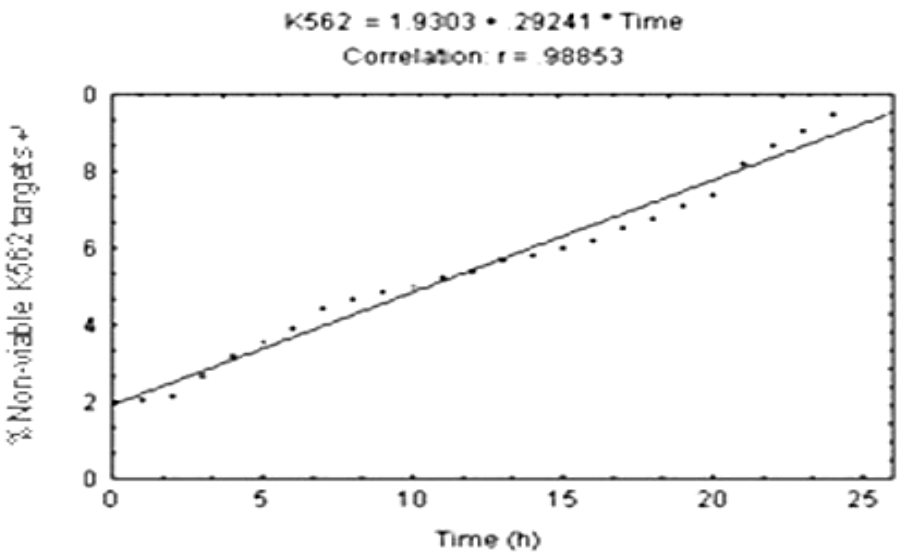

Figure 3. Correlation between background target cell death and incubation period for K562 cell line from triplicate experiments.

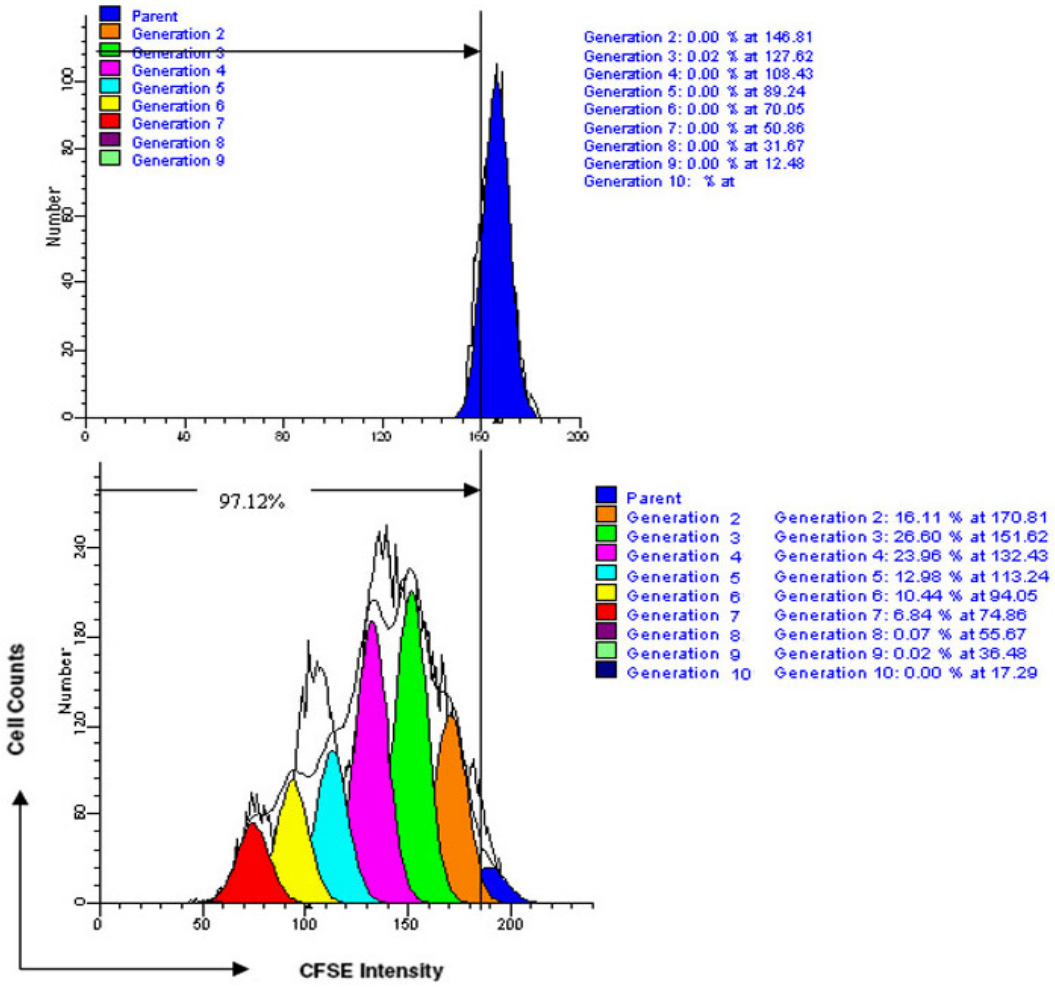

Figure 4. Fluorescence intensity and proliferation kinetics model of CIK cells induced for 21 days. The results shown were representative of six independent experiments.

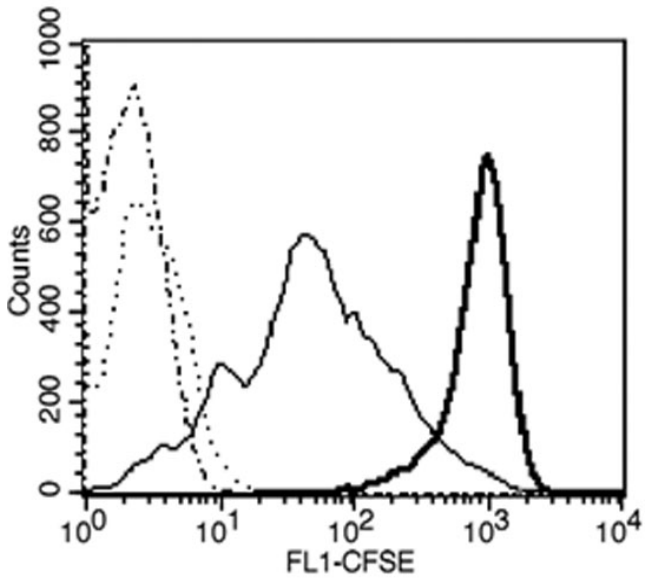

Figure 5. An overlay on histogram showing the fluorescence intensity distribution of CFSE-labeled and unlabeled cells acquired under the same instrument conditions, CFSE-labeled PBMCs but non-induced (black line), CFSE-labeled CIK cells induced for 21 days (solid line), CFSE-unlabeled PBMCs but non-induced (break line), CFSE-unlabeled CIK cells induced for 21 days (dotted line).

21 days, The heterogeneous PBMC population consisted of 94.03 CD3(+), 0.12 CD3(-) CD56(+), 83.91 CD3(+) CD56(+) and 35.12% CD3(+)CD56 (-). Moreover, in parallel CFSE-unlabeled control, the percentage of CD3+CD56+ on T cells increased from 1.98 to 81.15% after induction of CIK for 21 days (Figure 6). Thus, the labeling of CIK cells with CFSE did not interfere with their immunophenotypes.

Cytotoxicity assay

In order to optimize the E:T ratio, the cytotoxicity of the E:T ratios 100:1, 50:1, 25:1, 12.5:1 and 6.25:1 were compared after incubation of effectors and targets for 4 h. The maximal lytic activity was reached at the ratio 100:1 and 50:1 (Figure 7). The mortality of the ratio 100:1 was approximately same as that of the ratio of 50:1 for each tumor (P >0.05). The percentage of target cells death decreased gradually at ratio ranging from 50:1 to 6.25:1. The cytotoxicity of CIK cells against tumor cells of K562, HL-60 and HeLa were significantly different at ratio ranging from 50:1 to 6.25:1. The cytotoxicity for different target cells however varied at the same E:T ratios. CIK cells after 21 days of induction exerted an efficient killing effect on cancer cells. 83.52, 81.39, 70.23, 57.06 and 41.85% of K562 target cells were killed at the five different E:T ratios, respectively. The maximum cytotoxicity of CIK cells against HL-60, HeLa, MCF7 and A375 were 60.15, 57.32, 23.03 and 19.53%, respectively.

Furthermore, the cytotoxicity of CIK cells against cancer cells was significantly different depending on the E:T ratio. The cytotoxicity of CIK cells showed a significant and positive correlation with effector: target (E:T) ratio ranging from 50:1 to 6.25:1, while the optimal E:T ratio was 50:1 to 25:1. These data suggest that heterogeneous CIK cell population, which demonstrates characteristic immunophenotypes, has strong cytotoxic effects on cancer cell *in vitro*. Meanwhile, it proves that flow cytometric assay combined with CFDA-SE labeling is a suitable means for the determination of cytotoxicity.

DISCUSSION

CIK cells are highly efficient cytotoxic effector cells against diverse carcinoma cells (Kim et al., 2009b, 2007a, b; Kornacker et al., 2006; Sun et al., 2005; Takayama et al., 2000; Wang et al., 2008) and might serve as an alternative adoptive immunotherapeutic strategy for the eradication of residual cancer cells and prevention or delay of tumor relapse (Andreesen et al., 1998; Sangiolo et al., 2009). The high anti-tumor activity of CIK cells is mainly due to the high proliferation of CD3+/CD56+ double positive cells (Marten et al., 2001), which possess both strong cytotoxic activity of T cells and MHC-unrestricted antitumor advantages of NK cells. In this study, we explored the potential utility of an alternative nonradioisotopic marker of cell, the cytoplasmic dye CFDA-SE combined with flow cytometry, to develop a novel method for tracking in real-time the number of divisions each CIK cell has undergone, and assessing accurately the phenotype and cytotoxicity of CIK cells.

CFDA-SE is a non-polar compound that diffuses freely into the cell, where esterase cleaves the acetyl groups and the remnant becomes markedly fluorescent, leaving carboxyfluorescein succinimidyl ester (CFSE) that binds covalently to intracellular macromolecules and well retained within the cell (Lyons, 2000). Since its introduction to the flow cytometric analysis of lymphocyte proliferation by serial halving of the fluorescence intensity, the vital dye CFDA-SE has become widely used in cell labeling, tracking, and migrating *in vitro* and *in vivo* (Lyons, 1999; Lyons and Doherty 2004; Ko et al., 2007; Lee et al., 2008; Gil et al., 2009; Parish et al., 2009). Our results suggest that the CFSE labeling can be used to document division and differentiation events occurring in CIK effector cells upon stimulation with various cytokines *in vitro*. After induction for 21 days, the majority of CIK cells co-express CD3+CD56+ surface markers. The percentage of CD3+CD56+T cells in the population of PBMC increased from 5.39 to 83.91%. The immunophenotypes for CFSE-stained CIK cells were normal compared to unstained control CIK cells. More also, 97.13% of these cells had differentiated and underwent 6 rounds of successive divisions, leading to the significant

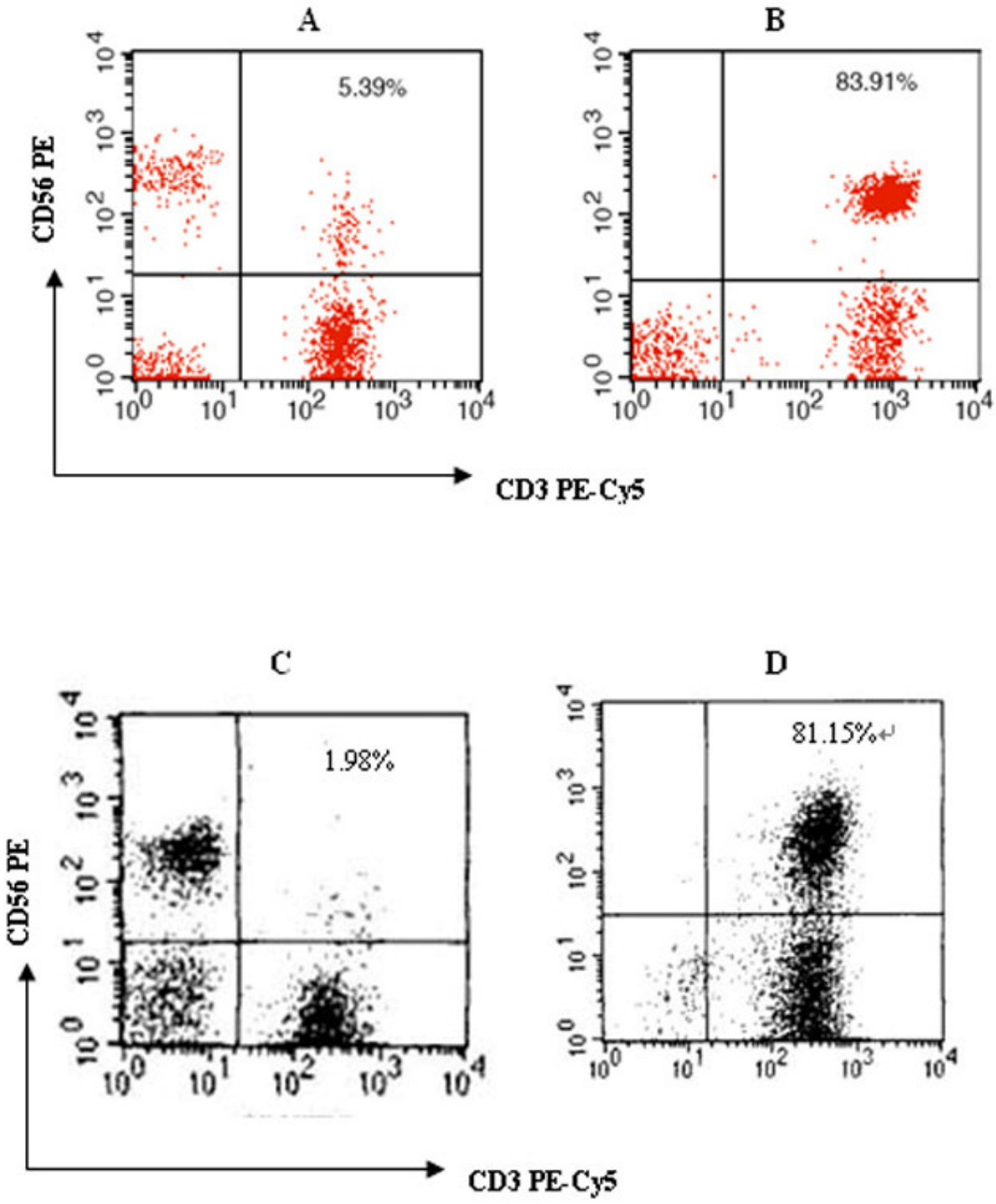

Figure 6. Immunophenotypes of CIK cells induced for 21 days, (A) Pre-induction for CFSE labeled CIK cells; (B) Post-induction for CFSE labeled CIK cells. (C) Pre-induction for CFSE unlabeled CIK cells. (D) Post-induction for CFSE unlabeled CIK cells.

increase of expanded CIK cells. We found that only a small fraction of PBMCs were undivided. These data support therefore the fact that CIK cells possess a higher proliferation capacity *in vitro*. The combination of CFSE labeling and flow cytometry can analyze the proliferation of CIK cells on a single cell level and indicate kinetically the progress of cell division. Thus, this approach provides a convenient means to analyze cell division and differentiation.

The conventional test for measuring cell-mediated

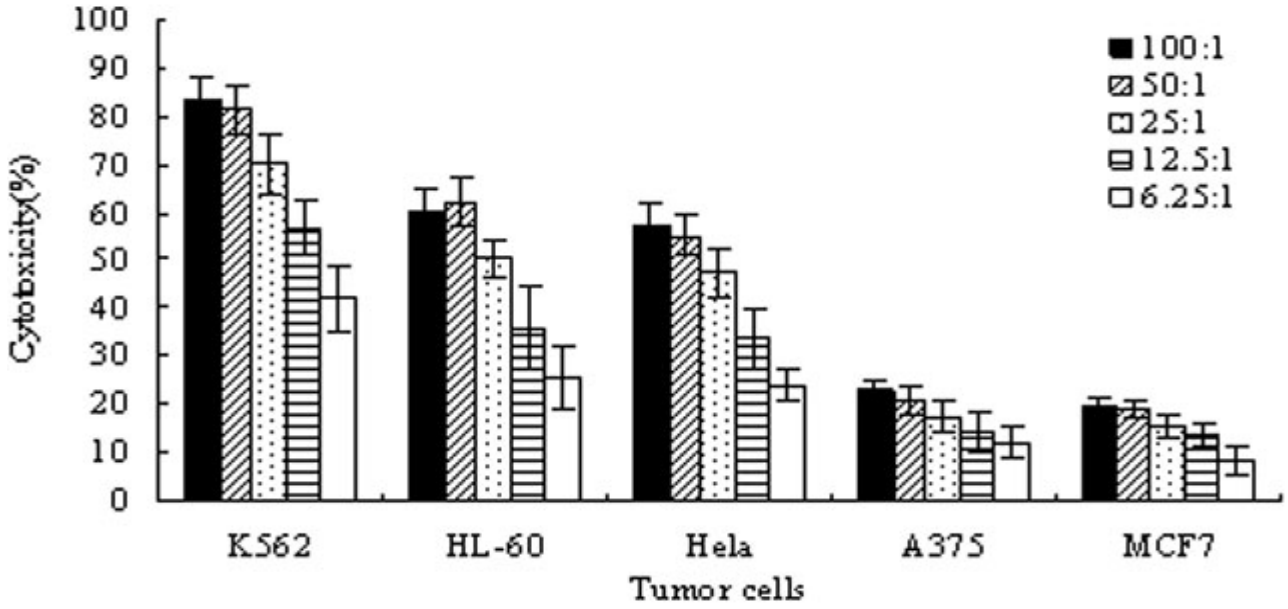

Figure 7. Percentage cytotoxic activity of CIK cells against tumor cells by flow cytometric assay. Effectors and targets were incubated at 37℃ with 5% CO_2 at E:T ratios of 100:1, 50:1, 25:1, 12.5:1 and 6.25:1. Mean (± S.D) values from six independent experiments. The mortality of the ratio 100:1 is approximately same as that of the ratio of 50:1 for each tumor (P>0.05), while the cytotoxicity of CIK cells against tumor cells of K562, HL-60 and HeLa is significantly different at ratio ranging from 50:1 to 6.25:1.

cytotoxic activity *in vitro* is based on chromium-51 (^{51}Cr) release assay (Morales and Ottenhof, 1983), which is often regarded as the gold standard assay (Cederbrant et al., 2003). However, it has several drawbacks including:

(1) Difficulty in labeling cells with low cytoplasm/nucleus ratio
(2) A high spontaneous release of ^{51}Cr from target cells over time
(3) A delay between actual cell damage and release of ^{51}Cr-bound intracellular proteins into the supernatant
(4) Measurement of lysis at the population level but not at the individual cell level
(5) Health risk; the care and handling associated with radioactive isotope usage
(6) Requirement for quite large amount of effector cells.

On the other hand, some alternative assays based on the activity of the intracellular enzymes, example, MTT (3-(4, 5dimethlthiazol-2-y) 2, 5-diphenyl tetrazolium bromide) assay, acid phosphatase assay and LDH (lactate dehydrogenase) assay indirectly reflect cytotoxic activity so that the measurement errors may be increased. Consequently, these problems inherent in the use of ^{51}Cr or others assay have promoted the development of nonradioactivity flow cytometric assays (Marcusson-Ståhl and Cederbrant, 2003; Blom et al., 2009; Wang and Zheng, 2002; Kim et al., 2007). ;Zaritskaya et al., 2010
In this study, the cytotoxic activity of CIK cells gene-rated from healthy human PBMCs was determined using multiparametric flow cytometry. In this method, the target cells were distinguished from the effector cells by pre-labeling them with a fluorescent dye CFSE. When membrane damage occurs, the dye is almost instantaneously lost and the cells are no longer able to uptake or retain the charged dye (Hoefel et al., 2003; Lyons, 2000; Parish, 1999). Therefore, the effector cells are not stained after target cells and effector cells are mixed for co-culture, while CFSE is released from target cells, thus eliminating cross-contamination problem. Target cell death is detected with PI which labels the DNA of damaged cells. CFSE and PI emission fluorescence are detected in FL1 and FL3 channel, respectively. As CFSE and PI have significant fluorescence crosstalk, particularly between the FL1 (530/30 nm) and the FL2 (585/42 nm) filters, it is advantage to use FL3 (670 nm LP) over the FL2 to detect PI because of the reduced crosstalk with CFSE. The experimental results showed that CIK cytotoxicity was detected at low E: T ratio in the period of 4 h incubation and increased gradually with the E: T ratio rising. The flow cytometric method also demonstrated that CIK cells exhibit highly efficient cytotoxic effects on both NK-sensitive and NK-resistant tumor cells. The differential cytotoxicity of CIK cells against the different targets used in this study may be due to the different sensitivity of these targets cells to CIK. The percentage of spontaneous target cells death is one of standards evaluating a method for cytotoxicity assay. The mortality of K562 cells was less than 5% within 12 h of incubation. Thus, CFSE can be applied to a long-term cytolysis

assay since it has little effect on cell vitality or apoptosis, consistent with previous reports (Hodgkin et al., 1996; Hasbold et al., 1998; Sheehy et al., 2001).

The CFSE/PI flow cytometry is a therefore more sensitive means to detect cytotoxicity than ^{51}Cr release assay, particularly at low E:T ratios and short-term incubation (Lecoeur et al., 2001; Jedema et al., 2004; Godoy-Ramirez et al., 2005). PI is able to enter the cell as soon as damage of the cell membrane occurs and stain the nuclear DNA, hence, all compromised target cells are stained immediately (Papa et al., 1988). These early apoptotic cells stained by PI however, exhibit weak membrane alteration and do not release ^{51}Cr. Moreover, we noticed that the percentage of lysis of compromised target cells was approximately the same at the 50:1 and 100:1 ratio. An explanation is that as cell targets fragment are killed and lose their nuclei, they are no longer be detected in the assay, thus leading to under-estimated killing unlike the cumulative ^{51}Cr release assay (Lecoeur et al., 2001). Hence, the CFSE/PI assay is suitable for determining early apoptotic cell death at lower E:T ratios.

In summary, we exploited an approach based on flow cytometric assay for the measurement of cytotoxic activity. This assay combines the identification of effector cells by CFSE staining and the detection of target cell lysis by PI incorporation. It is rapid, simple, and reliable, and can be easily performed on a single laser flow cytometer. It also has practical and scientific advantages compared with the classical ^{51}Cr release assay for the following reasons: First, it allows for analysis of target cells at the single cell level. In contrast, ^{51}Cr-release method measures an entire target cell population and may reflect partial ^{51}Cr release from the entire target population or total ^{51}Cr release from a fraction of the targets cells (Jodi et al., 1999). Also, CFSE binding is stable and makes this test usable for long-term cytotoxicity assay. In addition, the assay eliminates the need for a radioactive label, thus reducing potential radioactive hazards and the cost of purchasing and discarding radioactive materials. Finally, the flow cytometric assay is reliable and reproducible, producing consistent and comparable results. Our strategy is a flow cytometric assay to simultaneously measure the proliferation and cytotolytic activity of CIK cells, thus the method developed in this study demonstrates some novelty.

Abbreviations:

CIK, Cytokine-induced killer; **PBMCs,** peripheral blood mononuclear cells; **IL-2,** interleukin-2; **IL-1α,** interleukin-1; **IFNγ,** interferon-gamma; **anti-CD3mAb,** monoclonal antibody against CD3; **CTL**, cytotoxic T lymphocytes; **CFDA-SE**, carboxyfluorescein diacetate succinimidyl ester; IMDM, Iscove's modified Dulbecco's medium; **FCS**, fetal calf serum; **CM**, complete medium; FBS, fetal bovine serum; PI, proliferation index; **CFSE**, carboxyfluorescein succinimidyl ester; **LDH**, lactate dehydrogenase.

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