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NK sensitivity of neuroblastoma cells determined by a highly sensitive coupled luminescent method

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Abstract

The measurement of natural killer (NK) cells toxicity against tumor or virus-infected cells especially in cases with small blood samples requires highly sensitive methods. Here, a coupled luminescent method (CLM) based on glyceraldehyde-3-phosphate dehydrogenase release from injured target cells was used to evaluate the cytotoxicity of interleukin-2 activated NK cells against neuroblastoma cell lines. In contrast to most other methods, CLM does not require the pretreatment of target cells with labeling substances which could be toxic or radioactive. The effective killing of tumor cells was achieved by low effector/target ratios ranging from 0.5:1 to 4:1. CLM provides highly sensitive, safe, and fast procedure for measurement of NK cell activity with small blood samples such as those obtained from pediatric patients.

Keywords: NK cells; Cytotoxicity; Polio virus receptor; Coupled luminescent method; Neuroblastoma cells

Natural killer (NK) cells display cytolytic activity against different types of tumor cells and virus infected cells [1]. The commonly used assays such as chromium-51 or europium release for measurements of NK activity are time consuming. Moreover, these methods require target cell labeling with substances that are either toxic to cells or hazardous in handling (radioactive material) [2–4]. In contrast, coupled luminescent methods (CLMs) may avoid such problems. In CLMs, the activity of the enzyme or enzymes of interest is coupled in some manner to the production or consumption of a high-energy molecule, such as adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide reduced form (NADH), which is a luminescent substrate for one or more of the biological luciferases. Luciferases are enzymes which produce light as they consume such high-energy molecules. Properly designed coupled luminescent assays are able to combine the advantages of specific assays for enzyme function with the very great sensitivity of luminescent detection methods. As such CMLs do not require labeling of target cells (since glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is a natural component and of abundance in all living cells) and may provide more sensitive procedure than conventionally used methods [4]. The latter feature may be of special importance in cases with small blood samples including pediatric patients with solid tumors.

To test the utility of CLM for NK measurements, we used a model of neuroblastoma (NB) that represents one of the most common solid pediatric tumors [5]. With CLM, we showed that NK cells at effector to target (E:T) ratios ranging from 0.5:1 to 4:1 lysed tumor cells effectively. The method was further evaluated by experiments dealing with neutralization of poliovirus receptor (PVR; CD155) on NB cells. PVR is a known determinant for the susceptibility of NB cells to NK-mediated killing [6].

Materials and methods

Cell lines and reagents. The human MYCN-amplified NB cell line UKF-NB-2 and UKF-NB-4 were established in our laboratory from bone marrow metastases of NB patients with stage IV disease [7–9]. The vincristine (VCR) resistant cell subline designated UKF-NB-2^{r}VCR^{10} was

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established by exposing parental cells to increasing concentrations of the drug [9]. The resistant subline was grown for more than 6 months in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS) and containing 10 ng/ml VCR (UKF-NB-2^{r}VCR^{10}). Cells were subcultured at 5-day intervals. All culture media and media supplements were purchased from Seromed (Berlin, Germany). The cells were propagated in IMDM supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin, and 100 mg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. The NK cell sensitive erythroleukaemic cell line K562 (ATCC No. CCL-243) was maintained in IMDM supplemented with 20% FCS and used as standard for NK cell cytotoxicity.

NK cell preparation. Human peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy volunteers by Ficoll–Hypaque centrifugation. Freshly isolated PBMC were incubated for 2 h at 37 °C to allow adherence of monocytes to the bottom of the culture flasks. The cell suspension was collected and NK cells were separated according to manufacturer's protocol using the MACS NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). The separated NK cells were treated with 100 U/ml recombinant human IL-2 (Cell Concepts, Umkirch, Germany) for 5 days. Flow cytometric analysis to determine purity of NK cells showed that more than 90% of the cells were $CD56^{+}CD3^{-}$ (data not shown).

Measurement of cell surface receptors. For quantitative analysis of the expression of PVR and major histocompatibility complex class I-related chain A/B (MICA/B), a one color cytofluorometric analysis (FACScan; Becton Dickinson, Heidelberg, Germany) was carried out. Cells were stained with mouse monoclonal to PVR, ab3142 (Abcam, Cambridge, UK), and anti-human MICA/MICB monoclonal antibody (mAb), BAMO1 (Immatics, Tuebingen, Germany), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG second reagent (Becton Dickinson, San Jose, CA).

Cytotoxicity assay. NK cells were tested for cytolytic activity against indicated target cells using the "aCella-Tox" kit (Cell Technology, Mountain View, CA) that employs the coupled luminescent technology for the detection of cytotoxicity (Fig. 1) [4]. Briefly, target cells were plated in triplicate (5000 cells/well) in a 96-well white plate (Greiner Bio-One, Frickenhausen, Germany). Effector cells (NK cells) at indicated E:T ratios were added. Spontaneous effector and target cell death was accomplished by including control wells of effector cells at numbers corresponding to those of their various effector/target cell ratios and target cells according to the concentrations used for the assay. Twenty microliters of lytic reagent (0.5% NP-40/100 μl sample) was added to the target cells positive control (total G3PDH release) 15 min to end of assay incubation. At the end of incubation, 100 μl of 2× enzyme assay reagent was added to each well. Fifty microliters of 1× detection reagent was immediately added to each well. The plate was read at once in a luminometer (Glomax; Promega, Mannheim, Germany). For the mAb mediated neutralization experiments, 10 μg/ml of anti-PVR mAb was used. Isotype control IgG (Becton Dickinson, San Jose, CA) was used as negative control. IMDM supplemented with 1% heat-inactivated FCS was used as assay medium. The percent cytotoxicity was calculated as follows: [(experimental G3PDH release-spontaneous G3PDH release from effector cells alone-spontaneous G3PDH release from target cells alone)/(maximum G3PDH release from target cells-spontaneous G3PDH release from target cells)] × 100. The spontaneous target cell release was always <20% of maximum release.

Results

Establishing the method

We first determined the linear response of "aCella-Tox" within K562 and NB cell lines. This was accomplished by titrating the cells in the assay medium from 30,000 to 1000 cells/well for K562 and from 20,000 to 250 cells/well for NB. Twenty microliters of the lytic agent, NP-40, was added to each well. Lysed cells were further incubated for the length of the assay (4 h) before adding the enzyme assay reagent and detection reagent as described in Materials and method. The luminescence was then measured in a luminometer. The cell concentrations that fell in the linear range of the assay were up to 20,000 cells/well for both

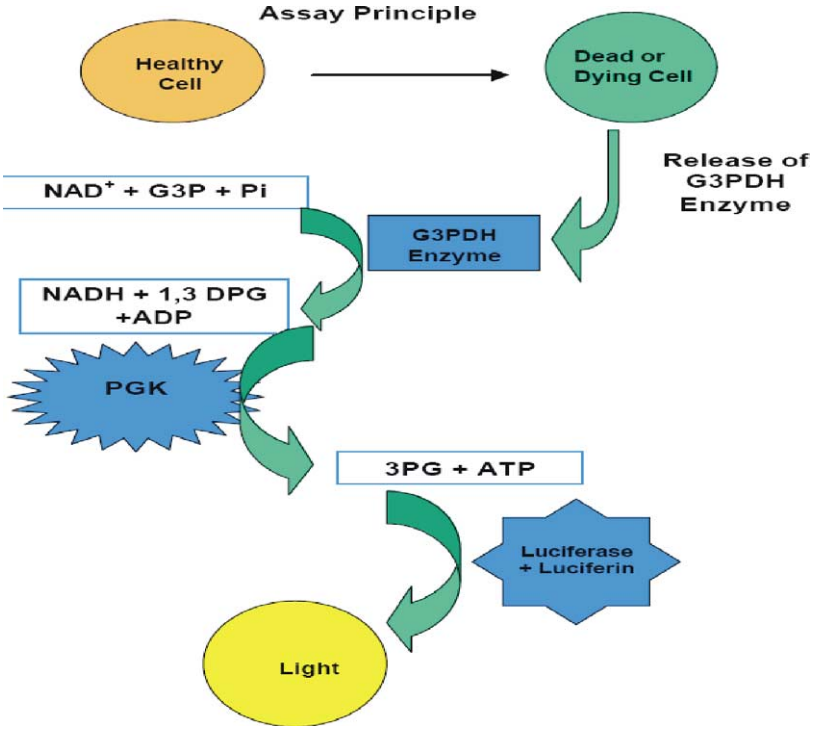

Fig. 1. Assay principle of the coupled luminescent method of cytotoxicity based on G3PDH release.

K562 and NB cell lines (data not shown). Here, we noticed that the sensitivity of the "aCella-Tox" kit is dependent on the concentration of cells used. The fewer the number of cells used, the more sensitive is the kit. The assay was then standardized using NK cells against K562 at different E:T ratios and different incubation times. As shown in Fig. 2, target cells were lysed at E:T ratio as low as 0.5:1. Optimal lysis of target cells was achieved after 4 h of coincubation with NK cells at an E:T ratio of 4:1.

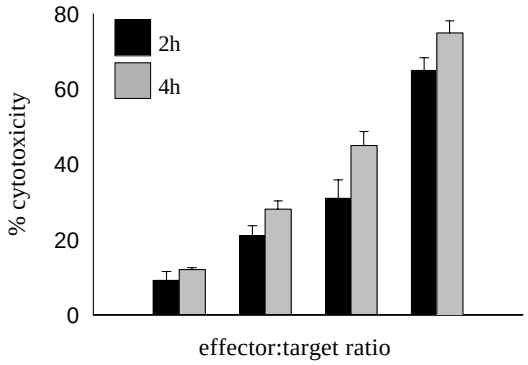

Fig. 2. Cytotoxicity of NK against K562 assessed by G3PDH release. Five thousand K562 target cells were coincubated with IL-2 activated NK cells at the indicated E:T ratios for 2 and 4 h at 37 °C. The results are means ± SD of three independent experiments.

Expression of PVR on neuroblastoma and its role in NK cell-mediated cytotoxicity

We previously showed, using europium release assay, that UKF-NB-2 and UKF-NB-3 cell lines are susceptible to NK lysis [10]. The mechanism involved in this lysis was however not clarified. Recently, it was shown that the susceptibility of a subset of NB cells to NK-mediated lysis is dependent on the expression level of PVR specifically recognized by DNAM-1 [6,11–13]. We analyzed the surface expression of PVR on UKF-NB-2, UKF-NB-4, and UKF-NB-2^{r}VCR^{10}. As shown in Fig. 3, PVR was markedly expressed in all cell lines tested (mean fluorescent units were 17 ± 3 for UKF-NB-2; 23 ± 2.5 for UKF-NB-4; and 28 ± 2 for UKF-NB-2^{r}VCR^{10}). UKF-NB-2^{r}VCR^{10} showed a higher expression of PVR than the parental UKF-NB-2 and UKF-NB-4.

Contrasting these results, the expression of other NK-activating receptor (NKG2D) ligands like MICA/B was not found on the surface of NB cells (Fig. 3). To further validate CLM, we tested whether the differential expression of PVR on NB cells correlates with NK sensitivity. The cells expressing higher PVR were more lysed than those with lower PVR expression (Fig. 4A). UKF-NB-2^{r}VCR^{10} cells were 2–4-fold more effectively killed than the parental UKF-NB-2. NK cell lysis was however inhibited by mono-

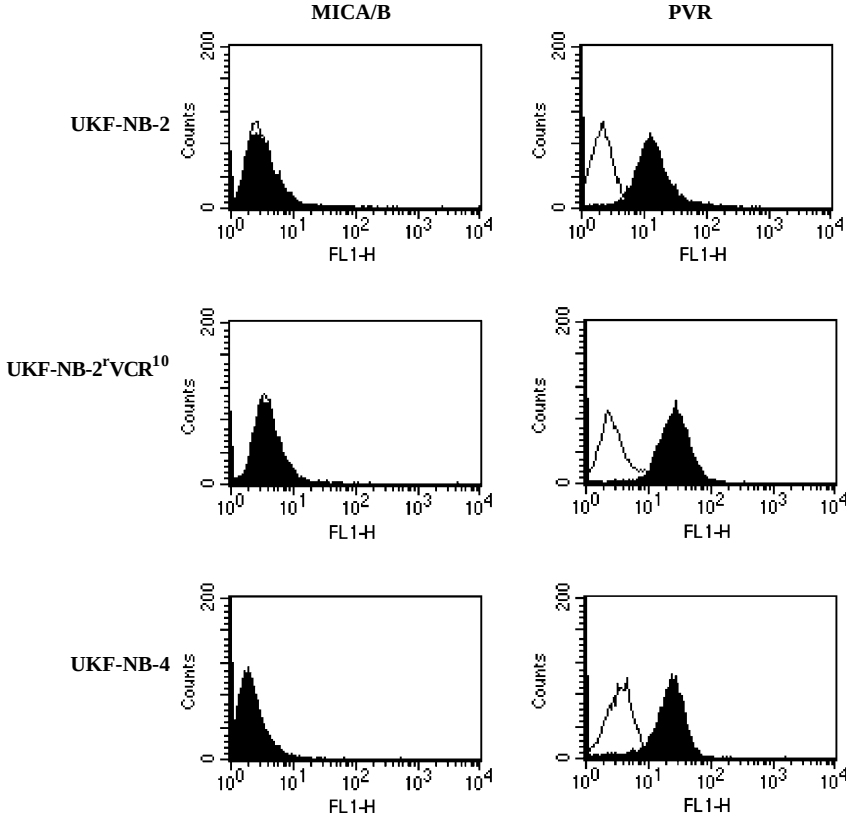

Fig. 3. Analysis of PVR and MICA/B surface expression on NB cell lines. NB cell lines were analyzed by one-color fluorescence and cytofluorometric analysis for expression of PVR and MICA/B. Empty histogram shows isotype control staining, filled histogram shows PVR and MICA/B expression. The results presented are representative of at least five separate experiments.

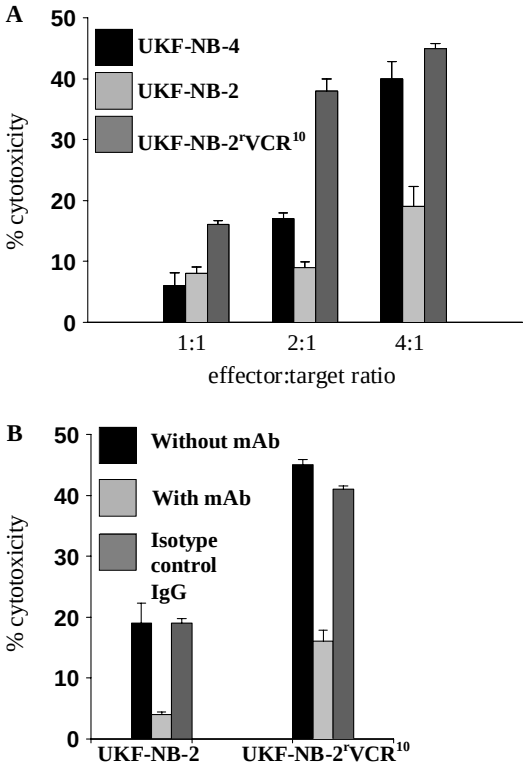

Fig. 4. Role of PVR in NK cell-mediated cytotoxicity against NB cells assessed by G3PDH release. (A) Five thousand NB cells were coincubated with IL-2 activated NK cells at the indicated E:T ratios for 4 h at 37 °C. (B) NK cell cytotoxicity against indicated NB cell lines at an E:T ratio of 4:1 in either the absence or presence of 10 μg/ml anti-PVR mAb. IgG isotype control was used as negative control. The results are means ± SD of three independent experiments.

clonal Ab mediated neutralization of PVR expressed on NB cells (Fig. 4B). Taken together, these results indicate that NK cell cytotoxicity of NB cells is dependent on the level of PVR expressed on tumor cells (Fig. 4).

Discussion

Properly designed coupled luminescent assays are able to combine the advantages of specific assays for enzyme function with the very great sensitivity of luminescent detection methods. In CLM, the inherent sensitivity of luciferase detection is enhanced by the amplification effect of enzyme turnover, which produces thousands, millions, or billions of high-energy molecules for each molecule of enzyme. The luminance signal is produced by firefly luciferase acting on ATP, which in turn is produced by the coupled reactions of G3PDH and phosphoglycerokinase (PGK), two consecutive enzymes of the glycolytic pathway. G3PDH, a very abundant enzyme in all known cells, is measured to quantify release (and therefore cell death and/or membrane damage), while PGK, which is generally not so abundant in cells, is supplied in a reaction cocktail, along with glyceraldehyde-3-phosphate (G3P), nicotinamide adenine dinucleotide oxidized form (NAD+), inorganic phosphate (Pi), dithiothreitol (DTT), adenosine diphosphate (ADP), the components of the luciferase reaction, and appropriate buffers and salts. The fact that G3PDH is a natural component of cells, and does not need to be introduced into the cells in any manner, distinguishes this assay from all methods which require prelabeling of the cells, transfection, transformation, or other methods of introducing proteins or other molecules into the target cells in order to generate a signal in a later step [4].

To validate CLM, we used a NB model. Most NB cells are generally resistant to NK cells lysis [10] when compared to K562 that are readily lysed even with NK cells that have not been activated with lymphokine such as IL-2. All NB cell lines tested were resistant to non-activated NK lysis (data not shown), but were readily lysed by IL-2 activated NK cells. This may be due at least in part to the failure of NB cells to express NKG2D ligands, as were observed for MICA/B in the present study. This is consistent with previous results which showed that NKG2D-activating receptor on the surface of primary neuroblasts and NB cell lines are downregulated [14]. On the other hand, PVR was readily expressed by NB cells. We therefore selected PVR to further validate CLM. PVR is a ligand recognized by DNAM-1 receptor [6,12]. DNAM-1 (CD226) which is expressed in virtually all human NK cells, T cells, monocytes, platelets, and a subset of B-lymphocytes is another surface molecule that has been shown to participate in the induction phase of NK cell activation. DNAM-1 is known to be involved not only in NK cell activation but also in cell–cell adhesion [15–17]. This suggests that the adhesion of NK cells to NB cells could be mediated by DNAM-1–PVR interaction. The present results support previous observations [6] demonstrating that the assessment of PVR expression could be used as an immunological marker for the susceptibility of NB cells to NK cell-mediated attack.

Interestingly, we show with CLM the higher susceptibility of drug resistant NB cells to NK lysis than non-resistant cells. UKF-NB-4, which possesses the intrinsic multidrug resistance (MDR) phenotype [18], and the drug-induced resistant UKF-NB-2^{r}VCR^{10} [9] cell line were more susceptible to NK-mediated lysis than the parental UKF-NB-2 cell line. This is of interest since induction of drug resistance in tumor cells as shown seems to be associated with changes in their sensitivity to NK cell-mediated lysis. Several studies demonstrated that multidrug resistant leukemic cells develop decreased sensitivity to NK cell-mediated lysis mainly at the level of killer/target recognition [19,20]. On the other hand, multidrug resistant cells derived from some solid tumors may exert increased sensitivity to NK cell-mediated lysis by different mechanisms [21,22]. The increased sensitivity of multidrug resistant NB cells was due to their increased PVR expression. This might suggest a role of drug resistance of NB cells in NK cell-mediated cytotoxicity.

In conclusion, this study demonstrated the use of a highly sensitive, safe, and fast method to determine NK cell cytotoxicity. The features of CLM may be of particular

importance in cases with small blood samples including pediatric patients with solid tumors or viral infections.

Acknowledgments

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