A DELFIA® time-resolved fluorescence cell-mediated cytotoxicity assay performed on the PHERAstar® *FS*

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- **A europium time-resolved fluorescence cytotoxicity assay in a 96 well format is analyzed by an HTS reader**
- **The data herein shows that estrogen dramatically reduces cell death induced by NK92 natural killer cells**
- **This time-resolved fluorescence assay is shown to be an alternative to the radioactive chromium release assay**

Introduction

Estrogens are steroid hormones that act via estrogen receptors to exert both intranuclear and extranuclear effects in human cells. By this way, estrogens promote different cellular processes like proliferation and metastases in human cancers; in addition, estrogen has been shown to play a role in tumors, developing the ability to block immunosurveillance. Immunosurveillance results primarily from apoptosis of neoplastic cells induced by natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). These cytolytic lymphocytes (CLs) use perforin and granzyme-containing granules. Granzyme B (GrB), is thought to play a leading role in granzyme-mediated cytotoxicity to induce lysis of target cells.

It was shown that in breast cancer cells, increasing concentration of estrogens induce increasing levels of granzyme B inhibitor and SerpinB9/Proteinase Inhibitor 9 (PI-9), while progressively blocking cell death induced by NK92 natural killer cells. Several assays are available to monitor apoptosis and other forms of cell death. These assays focus on changes that occur during apoptosis – the annexin V and propidium iodide assays are based on changes in membrane properties and permeability, and the TUNEL assay is based on apoptosis-related DNA fragmentation.

These and other assays are extremely good in evaluating apoptosis in a homogenous cell population, but are less useful when evaluating apoptosis in only one type of cell when a mixture of cells is present. One common example of cell death in a mixed cell population occurs in the process termed immunosurveillance in which cells of the immune system, such as cytotoxic T lymphocytes (CTLs) and Natural Killer (NK) cells, induce apoptosis of target cells. Assaying apoptosis in mixed cell populations usually requires the cells of interest to be pre-loaded with a reagent, and the loss of membrane integrity that occurs during cell death results in the reagent being released. The most widely used assay is to pre-load cells with radio-labeled chromium. However, 51Cr is an unstable photon emitter and requires costly and cumbersome lead shielding for safe use.

Here the PHERAstar® *FS* HTS microplate reader is used in a time-resolved fluorescence assay to study the loss of membrane integrity. In this assay, target cells are loaded with fluorescence enhancing ligand, BATDA, which penetrates the cell membrane quickly and is hydrolyzed to form a hydrophilic ligand (TDA) that is released from cells only after cytolysis (Figure 1).

In the presence of a solution containing Eu^{3+}, the released TDA forms a highly fluorescent and stable chelate (EuTDA), whose levels are measured. It is shown that this assay produces results that are similar to the classical chromium release assay.

Assay Principle

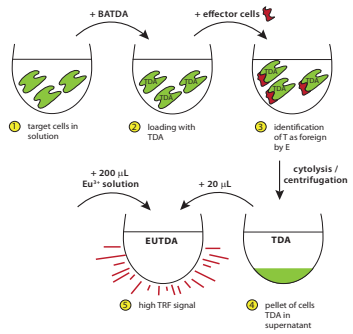

Fig. 1: Principle of the DELFIA® cytotoxicity assay.

BATDA will pass the cell membrane of the target cells. Intracellular esterases cleave ester bondages leading to a hydrophilic ligand that cannot pass the membrane (TDA). Effector cells recognize their target cells as foreign and the cytolysis process is initiated. After cytolysis the TDA is released and is found to be in the supernatant after centrifugation. A 20 µl aliquot of supernatant is added to 200 µl of Eu^{3+} solution resulting in a high time-resolved fluorescent signal that is measured on the PHERAstar *FS*.

Materials & Methods

- 96-well V-bottom black microplates
- DELFIA® EuTDA Cytotoxicity Reagents including BATDA Reagent, Lysis buffer and Europium Solution, PerkinElmer
- PHERAstar *FS*, BMG LABTECH

The cell mediated cytotoxicity assay was carried out following the manufacturer's protocol with a few modifications. Target cells (MCF-7, human breast cancer cells) were incubated at 10^6 cells/mL in phenolred free MEM plus 5% CD-calf serum with BATDA for 10 min at room temperature, followed by 4 washes.

The target cells were then mixed with effector cells at different effector cells/target cells (E/T) ratios. Cytotoxicity assays were carried out in 96-well V-bottom plates containing 10,000 target cells in a total volume of 200 µL/well. The plates were subjected to centrifugation for 30 seconds at 1,000 rpm to facilitate contact between target and effector cells.

After incubating for 2 hours, supernatant from each reaction (20 µl) were added to 200 µl of the Eu^{3+} solution.

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Following agitation of the plates for 5 min, time-resolved fluorescence was measured by using an application specific DELFIA® module with an excitation wavelength of 337 nm and an emission wavelength of 615 nm using a PHERAstar *FS* microplate reader.

Calculation

Percent specific lysis was calculated as:

$$100 \times \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}}$$

Spontaneous release

Target cells are incubated with medium instead of effector cells.

Maximum release

Target cells are incubated with lysis buffer instead of effector cells.

Results & Discussion

Using the described time resolved fluorescence assay that detects release of a fluorescent substrate from cells that lose membrane integrity, estrogen treatment for 24 hours dramatically reduced cell death induced by NK92 cells (Fig. 2).

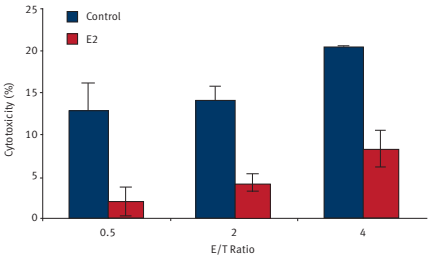

Fig. 2: Estrogen (E2) protects MCF-7 cells against cytolysis induced by NK92 cells. MCF-7 target (T) cells were treated with ethanol vehicle (filled bars) or 10 nM E2 (open bars) for 24 hours, followed by incubation with NK92 effector (E) cells at different E/T ratios.

Proteinase Inhibitor 9 (PI-9) inhibits granzyme B and NK cells use the granzyme pathway to kill target cells. However, the diverse effects of estrogens raise the possibility that other actions of estrogen might be responsible for blocking NK cell-induced cytotoxicity. To evaluate the role of PI-9 in estrogen-mediated protection against NK cell induced cytotoxicity, we used RNAi to knock down PI-9 expression. Knockdown of PI-9 with PI-9-specific siRNA abolished the ability of estrogen to block NK92 cell-induced killing (Fig. 3). These data demonstrate that estrogen's ability to block NK cell mediated cytolysis of MCF-7 cells derives from its ability to induce PI-9.

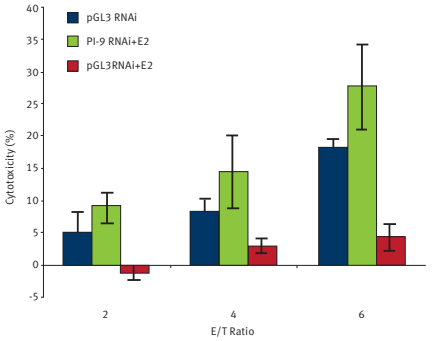

Fig. 3: RNAi knockdown of PI-9 blocks estrogen protection against NK cell mediated cytotoxicity. MCF-7 cells were transfected with the control pGL3 luciferase siRNA, or with the PI-9 siRNA. After 24 hours, ethanol vehicle or estrogen (E2) was added and the cells were maintained for an additional 24 hours and incubated with the indicated ratios of effector NK92 cells to MCF-7 target cells and assayed for cytoxicity using the time resolved fluorescence assay.

Conclusion

With the DELFIA® cytotoxicity assay measured on the PHERAstar *FS* it is possible to investigate NK cell mediated cytolysis. The data indicate a clear relationship between increasing estrogen and reduced cell lysis. Furthermore it was shown that estrogen protects the MCF-7 cells by utilizing Proteinase inhibitor-9.