Real-Time, Label-Free Measurement of Natural Killer Cell Activity and Antibody-Dependent Cell-Mediated Cytotoxicity

Abstract

The response of tumor cells (as target cells) to natural killer (NK) cell activity (as effector cells), in the presence or absence of immunoglobulin G isotype-specific antibody, was measured to determine if cell-mediated cytotoxicity (specifically antibody-dependent cell-mediated cytotoxicity (ADCC)) can be investigated using the Agilent xCELLigence system. It was shown that the addition of NK cells in suspension, over a monolayer of adherent tumor cells, does not produce impedance or Cell Index (CI) changes, because the NK cells do not come into contact with the biosensor. However, the secretion of perforins and granzymes by these nonadherent NK cells did activate caspases inducing tumor cell apoptosis. Dysfunctional and dying tumor cells detached from the biosensor, reducing the number of viable and adherent cells on the biosensor surface. Our findings provided compelling evidence for how the Agilent xCELLigence system can be used for the dynamic real-time monitoring of cell-mediated cytotoxicity and the impact of specific antibodies.
Introduction

Cell-mediated cytotoxicity is an important means by which the body protects itself from pathogenic attacks, providing an essential defense mechanism against viruses, bacteria, or parasites, as well as transformed and dysfunctional cells. It also plays an important role in maintaining homeostasis of the immune system. Cell-mediated cytotoxicity specifically involves leukocytes that recognize and destroy other cells or invaded microbes. Two types of leukocyte-effector cells carry out this activity:
- Lymphoid cells known as cytotoxic T-lymphocytes, and NK cells
- Myeloid cells, such as macrophages, eosinophils, and neutrophils

The most important role of cytotoxic T-lymphocytes is the elimination of cells infected with viruses. In addition to expressing their own cell-specific peptides on their cell surface, infected cells also exhibit processed pathogen-specific antigens in association with the major histocompatibility complex (MHC) class I molecules. Virus-infected cells present these pathogen-specific antigens to cytotoxic T-lymphocytes, which are capable of distinguishing between self and nonself peptides and can mobilize an antiviral response accordingly.

Cytotoxic T-lymphocytes and NK cells act in a complementary way to protect the body because NK cells specifically recognize and kill cells that exhibit reduced (or have lost) MHC class I expression.

Significantly, tumor cells also commonly express reduced levels of MHC class I molecules, which provides the basis for an effective antitumor effect of NK cells. Cell-mediated cytotoxicity is a result of the following complex cell-cell interactions:
- Direct cell-cell interactions producing internalization and phagocytosis of infected cells, or pathogens by macrophages and neutrophils
- Secretion of cytokines, such as Fas ligand and tumor necrosis factors, by cytotoxic T-lymphocytes
- Release of granule proteins, such as perforin and granzymes, by both cytotoxic T-lymphocytes and NK cells
- Release of toxic molecules, such as reactive oxygen intermediates and lysosomal enzymes, by myeloid cells

Cellular degranulation is also facilitated by a process known as opsonization of the target cells by cross-linking antibodies. In this complex event, antibodies, using their Fab fragments, first specifically attach to antigens on the surface of the target cells to be killed. Opsonization confers the required spatial proximity between target and effector cells by specifically binding the Fc fragments of the target cells directly to the Fc receptors of the corresponding effector cells (cytotoxic T-lymphocytes and NK cells). The subsequent cross-linking of Fc receptors is an essential part of this immune response, leading to target cell death known as antibody-dependent cell-mediated cytotoxicity (ADCC).

The rationale for a new method to measure NK cell activity and additive antibody-dependent cytotoxicity

Monitoring and quantifying the lytic activity of effector cells, such as cytotoxic T-lymphocytes and NK cells, is important for defining physiological and pathophysiological states, response to infectious viral agents, and autoimmune reactions. These types of investigations are also essential for characterizing the lysis process itself and for identifying lysis mediators. The phenomenon of cell-mediated cytotoxicity through ADCC can be studied in vitro, using either fresh lymphocytes isolated from blood or NK cell lines as effectors. Appropriate in vitro targets include pathogen-infected eukaryotic cells and tumor cell lines.

The most common method for measuring cell-mediated cytotoxicity is the release assay based on the loss of target cell membrane integrity. Up to four hours after effector cell addition resulting in target cell lysis, the radioactive release from target cells prelabeled with Chromium ($^{51}$Cr) or Indium ($^{111}$In) is measured, or the release of naturally occurring substances, such as lactate dehydrogenase (LDH), into the culture medium is assayed. Release of these substances serves as an indirect measure of the extent of cell damage due to effector cell-mediated target cell lysis.

Alternative methods also include flow cytometry, enzyme-linked immunosorbent assay-based granzyme measurement, and morphometric analyses by microscopy.
The RTCA SP, MP, and DP instruments are suitable for addressing how cells respond in a broad spectrum of research fields including drug development, toxicology, cancer biology, medical microbiology, and virology. The impedance-based technology of the xCELLigence system has been shown to work for various applications, such as cell proliferation and cytotoxicity, cell adhesion and spreading, cell culture quality control, receptor tyrosine kinase activation, mast cell activation, and G protein-coupled receptor (GPCR) activation. The most important application in the context of this application note is the detection of NK cell activity in combination with ADCC.

Materials and methods

Cell culture and antibodies

DU145 target cells and NK92 effector cells were obtained from ATCC. NK92 cells were genetically modified to stably overexpress the FcgR III. Both cell lines were grown in a standard cell culture incubator at 37 °C with 5% CO₂. DU145 cells were maintained in RPMI 1640 GlutaMAX media (Gibco) with 10% FCS (Perbio Science), 1% penicillin, and 1% streptomycin (Roche Applied Science). NK92 cells were cultured in MEM alpha medium with L-glutamine (Gibco), 10% FCS, 10% horse serum (Invitrogen), 0.1 mM 2-mercaptoethanol (Gibco), 0.2 mM myo-inositol (Calbiochem), 0.02 mM folic acid (Alfa Aesar), and 10 ng/mL interleukin-2 (Cell Systems). The monoclonal antibody for the ADCC assay was specific for human IGF-1R, binding the human FcgR III with its Fc fragment.
**Results and discussion**

**Dynamic monitoring of NK cell-mediated cytolysis of DU145 cells**

The DU145 prostate cancer line was seeded as the target at a density of 5,000 cells per well into an E-Plate 96 to assess cell-mediated cytolytic activity. Cells were allowed to attach and proliferate for 20.5 hours. By then, they had reached their logarithmic growth phase as evidenced by dynamic CI monitoring of the DU145 cell population using the RTCA MP instrument every 15 minutes (Figure 1A). At 20.5 hours, real-time measurements were paused, medium was removed, and DU145 cells were exposed to NK92 cells stably overexpressing FcgR III (CD16). These effector cells were added to the target cells in NK cell medium at varying effector-to-target (E:T) cell ratios, ranging from 0.47:1 to 30:1 (a seven-fold doubling dilution series). After the addition of NK92 cells, CI measurements were restarted and changes in proliferation kinetics of the DU145 cells were recorded every 15 minutes for 67.5 hours. There was an initial slight decline in CI values after adding the NK cells due to medium and temperature changes, as shown in Figure 1A. Within one hour after this decline in CI for all treated wells, there was a clear correlation between the number of added effector cells and the concomitant decrease in CI value (Figures 1A and 1B).

Comparing the data between NK92 cells (E) to DU145 (T) cells with E:T ratios from 0.47:1 to 30:1, the CI values showed that, over time, if fewer NK cells were added to the well, fewer target cells were lysed and there was a greater increase of CI. Treatment with NK92 cells at an...
cells (by its Fc fragment). Again, 5,000 DU145 cells were seeded into each well of an E-Plate 96, and logarithmic growth was monitored every 15 minutes for 20.5 hours. NK cells in NK cell medium were added at E:T ratios of 3.75:1 (Figure 2A) and 1.88:1 (Figure 3A). DU145 target cells were pre-incubated with different amounts of anti-IGF-1R for 30 minutes, resulting in final concentrations between 0.1 and 100 µg/mL. The effect of NK cells without antibody (untreated control), as well as the effect of the varying amounts of antibody were recorded every 15 minutes for 67.5 hours.

Target cells treated with lower numbers of NK cells (Figure 1A, dark green and other lines) reach distinctive plateau phases of growth at 88 hours, depending on the number of NK cells introduced to the DU145 cells. When comparing plots normalized to either the last time point before NK cell addition (Figure 1A), or to the control cell baseline (Figure 1B), all E:T cell ratios except the lowest E:T ratio of 0.47:1 produced a step-wise differential change in CI values between 33 and 88 hours of the experiment.

**Dynamic monitoring of anti-IGF-1R-dependent NK cell-mediated cytolysis of DU145 cells**

The insulin-like growth factor 1 receptor (IGF-1R) is a member of the tyrosine kinase receptor family, mediating the effects of IGF-1, a polypeptide similar to insulin. IGF-1 plays an important role during development and growth, and IGF-1 and IGF-1R are implicated in several types of cancer. The development of therapeutic antibodies against IGF-1R, based on their involvement in NK cell-mediated lysis of tumor cells, may be a strategy to treat or prevent cancers.

In the next experiments, cytolytic activity of NK92 cells on DU145 cells was examined in the presence of the anti-IGF-1R monoclonal antibody, which binds to both extracellular human IGF-1R (by its Fab fragments) and the Fcg receptor III (FcgR III) on NK92

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**Figure 2.** Effect of the anti-IGF-1R antibody on NK cell-mediated cytolysis of DU145 cells (E:T ratio = 3.75:1). Five thousand DU145 cells per well were seeded in triplicate into an Agilent E-Plate 96 and monitored every 15 minutes using the Agilent RTCA MP instrument. After 20.5 hours, NK92 cells were added to the wells at an E:T ratio of 3.75:1. Target cells were pre-incubated in advance with different concentrations of anti-IGF-1R antibody for 30 minutes. The effect of NK cell and anti-IGF-1R addition was monitored for 67 hours. A) Plot of normalized CI values of the entire course of the experiment (88 hours). Data are normalized to the last time point before NK cell addition and curves are plotted with control wells (DU145 cells only) set as baseline. B) Calculation of IC50 after 60 hours. C) Calculation of time-dependent IC50 from nine hours after NK cell addition to the end of the experiment at 88 hours. All plots were generated using the RTCA Software 1.1.
In comparison to nontreated control cells, NK cells induced a moderate decline in CI values on DU145 cells without antibody pretreatment (red line). This was due to NK-mediated cytolsis of DU145 cells (compare controls to antibody-treated wells in Figures 2A and 3A). DU145 pretreatment with antibody clearly produced a concentration-dependent decrease in impedance and CI values (summarized in Table 1). CI values of anti-IGF-1R-treated DU145 cells were lower than that of DU145 cells without antibody pretreatment, indicating that the presence of the antibody increased the cytotoxic effect of the NK cells (Figures 2A and 3A, Table 1).

Table 1. NK cell-mediated cytolsis of DU145 cells 48 hours after NK cell addition and DU145 cell pretreatment with increasing amounts of IGF-1R antibody. NK92 cells were added to DU145 cells at different effector cell:target cell (E:T) ratios. The percentage of cytolsis was calculated based on the normalized CI values 48 hours after DU145 antibody pretreatment and NK cell addition. The last time point before addition of the NK92 cells was chosen for normalization of data.

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<th>E:T Ratio</th>
<th>Anti-IGF-1R Antibody (µg/mL)</th>
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<tr>
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<td>30:1</td>
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Figure 3. Effect of the anti-IGF-1R antibody on NK cell-mediated cytolsis of DU145 cells. (E:T ratio = 1.88:1). Five thousand DU145 cells per well were seeded in triplicate into an Agilent E-Plate 96 and monitored every 15 minutes using the Agilent RTCA MP instrument. After 20.5 hours, NK92 cells were added to the wells at an E:T ratio of 1.88:1. Target cells were pre-incubated in advance with different concentrations of anti-IGF-1R for 30 minutes. The effects of NK cell and anti-IGF-1R pretreatment were monitored for 67 hours. A) Plot of normalized CI values of the entire course of the experiment (88 hours). Data are normalized to the last time point before NK cell addition and curves are plotted with control wells (DU145 cells only) set as baseline. B) Calculation of IC50 after 60 hours. C) Calculation of time-dependent IC50 from nine hours after NK cell addition to the end of the experiment at 88 hours. All plots were generated using the RTCA Software 1.1.
Anti-IGF-1R was hypothesized to increase the interaction between DU145 and NK cells by cross-linking overexpressed FcγR III on NK92 cells. The CI findings showed that a clear antibody dose-response dependency and the degree of NK92-mediated cell lysis is also a function of the E:T ratio: The more NK cells administered, the stronger the additive effect of the antibody (Table 1). In contrast, DU145 cell treatment with anti-IGF-1R in the absence of NK92 cells showed no reduction in CI values compared to control levels. In fact, antibody amounts over 6.25 µg/mL produced a slight increase in CI values, and corresponding DU145 proliferation curves clearly run above the curve representing nontreated control cells (Figure 4).

Calculation of half inhibitory (IC50) anti-IGF-1R concentrations

To further quantify the effects of the anti-IGF-1R antibody on the NK92 and DU145 cytolytic cell-cell interaction, normalized CI values obtained from the antibody dose-responses were used to calculate IC50 values using the RTCA Software 1.1. Data obtained from experiments using either 3.75:1 or 1.88:1 NK92:DU145 (E:T) cell ratios were used to calculate IC50 values of 2.2 and 7.5 µg/mL, respectively (Figures 2B and 3B). The IC50 value was significantly higher for the lower number of NK cells added. This indicated that approximately 3.5 times more antibody was needed to induce the same extent of target cell lysis when only half of the NK cell amount was administered.

Time-dependent IC50 values for more than 50 hours of the experiment were calculated using another tool in the RTCA Software 1.1, showing the excellent reproducibility of IC50 values over this long period (Figures 2C and 3C).

Conclusion

The present study shows the feasibility of using the xCELLigence system to monitor both NK cell-mediated tumor cell cytolysis and ADCC in a label-free, noninvasive manner, corroborating earlier findings by Glamann and Hansen. The experiments highlighted in this application note show both the quantitative effect of adding different amounts of NK92 cells, as well as the additive cytolytic effect of introducing an NK92-dependent anti-IGF-1R monoclonal antibody.


