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Research paper

Visualization and quantification of cytotoxicity mediated by antibodies using imaging flow cytometry

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ABSTRACT

Conventional approaches for the detection of antibody dependent cell-mediated cytotoxicity (ADCC) activity rely on quantification of the release of traceable compounds from target cells or flow cytometry analysis of population-wide phenomena. We report a new method for the direct imaging and quantification of ADCC of cancer cells. The proposed method using imaging flow cytometry combines the statistical power of flow cytometry with the analytical advantages of cell imaging, providing a novel and more comprehensive perspective of effector/target cell interactions during ADCC events. With this method we can quantify and show in detail the morphological changes in target and effector cells, their apoptotic index, the physical interaction between effector and target cells, and a directional transfer of cytosolic contents from effector to target cells. As a model system we used the therapeutic anti-CD20 antibody rituximab to target CFSE labeled Ramos human Burkitt's lymphoma cells, to CMTPX-labeled human monocytic U-937 effector cells. We expect that similar studies using different effector and target cell populations may contribute to the pre-clinical evaluation of therapeutic antibodies and help to identify mechanisms that could be beneficial in the immunotherapy of cancer.

1. Introduction

In recent years the generation of therapeutic antibodies has experienced significant growth, a trend that is expected to continue, with many new developments in antibody engineering focused on the improvement of antibody effector functions (Wang and Weiner, 2008). In particular, the mechanism of antibody dependent cell-mediated cytotoxicity (ADCC) has been shown to play a major role in the therapeutic activity of rituximab (Rituxan™, Roche), trastuzumab (Herceptin™, Roche) and other monoclonal antibodies in development. Studies suggest that enhanced antibody

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell mediated phagocytosis; E:T, effector to target ratio; CFSE, carboxy-fluorescein diacetate, succinimidyl ester; DRAQ5, 1,5-bis{[2-(di-methylamino) ethyl]amino}-4,8-dihydroxyanthracene-9,10-dione; LDH, lactate dehydrogenase; EDF, extended depth of field; FBS, fetal bovine serum; NHL, non-Hodgkin's lymphoma.

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mediated effector/target cell interactions and their cytotoxic effects on the target cells correlate with better overall response to treatment (Dalle et al., 2008). ADCC mediates the elimination of cancer cells through a mechanism that requires the presence of the antibody, target cells (expressing the antigen), and effector cells (bearing Fcγ-receptors) such as macrophages, NK cells, monocytes, or neutrophils. In the case of rituximab, it has been reported that leukemic target cells opsonized with antibody can be phagocytosed by macrophage effector cells (Glennie et al., 2007; Leidi et al., 2009). In vitro studies that aid in the prediction of clinical efficacy and in understanding the mode of action of therapeutic antibodies targeting cancer cells are of growing interest.

Current methods for ADCC determination rely on the evaluation of the loss of cell membrane integrity by quantification of the release of traceable compounds from target cells or by evaluation of the target cell viability by flow cytometry. The ^{51}Chromium (^{51}Cr) release assay relies on the quantification of the radioactive material liberated from ^{51}Cr-loaded target cells. It is a sensitive method and has been considered the gold standard for cell-mediated cytotoxicity (Brunner et al., 1968). However, its dependence on radioactivity, the variability in labeling efficacy, and substantial spontaneous leakage of the ^{51}Cr label in certain cell types has limited the usefulness of this method (Jakubek et al., 1983; Wisecarver et al., 1985). One non-radioactive alternative to the ^{51}Cr assay is the quantification of release of the fluorescent dye calcein-AM from target cells (Metelitsa et al., 2002). Another alternative is the colorimetric lactate dehydrogenase (LDH) assay which measures enzyme release after disruption of the cell membrane. It has the advantage that it is colorimetric and non-radioactive, but in ADCC assays this method does not differentiate target from effector cell-derived LDH release. Flow cytometry has also been used to measure cytotoxicity based on the uptake of DNA fluorescent probes after the disruption of the plasma membrane of target cells. This method has also been used for the simultaneous determination of ADCC and phagocytosis (ADCP) in three-color flow cytometry assays (Karagiannis et al., 2007). Although this method can effectively discriminate the viability of the subpopulations of effector cells, target cells, and interacting effector–target cells, it cannot distinguish effector–target conjugates from events in which the effector cell has phagocytosed the target. Fluorescence microscopy is used to discriminate these two cases, but this technique can miss rare events and has limited statistical power due to the limited number of events that can be analyzed.

To comprehensively evaluate ADCC activity and effector/target cell interactions in the presence of therapeutic antibodies we used the ImageStream imaging flow cytometry technology (Amnis Corp., Seattle, WA). This technology collects multiple high-resolution images (darkfield, brightfield, and various fluorescent) per cell in flow at high rates of image capture, enabling statistically robust microscopy applications. This technology has been used previously to measure simultaneously NK cytotoxicity and the phenotype of effector cells (Kim et al., 2007), to determine chemically induced apoptosis in cancer cell lines (George et al., 2004) and to study the membrane exchange between effector and target cells in the process of trogocytosis (Megjugorac et al., 2007; Beum et al., 2008). Here we used this technology to monitor simultaneously the viability of target cells, effector cells, and to analyze a subset of interacting effector/target cells, providing a novel and more comprehensive perspective of ADCC events mediated by a therapeutic antibody.

2. Materials and methods

2.1. Cell lines, fluorescent dyes, and antibody

The human monocytic cell line U-937 and the human Burkitt's B-cell lymphoma cell line Ramos were purchased from American Type Culture Collection (ATCC, Manassas, VA). Both cell lines were grown in RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 100 U/ml penicillin, 10 μg/ml streptomycin, and 10% (v/v) heat inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) at 37 °C in 5% CO_2. CellTracker™ Red (CMTPX, excitation 577 nm/emission of 602 nm) fluorescent dye (Molecular Probes, Life Technologies) was used to label the effector cells U-937 by incubating 30 min in the presence of RPMI 1640 with 10% FBS with 2 μM CMTPX at 37 °C in 5% CO_2. The cells were washed twice and further incubated for 30 min in RPMI 1640 with 10% FBS to remove the excess dye. Carboxyfluorescein succinimidyl ester (CFSE green, excitation 492 nm/emission of 517 nm) fluorescent dye (Molecular Probes, Life Technologies) was used to stain the target Ramos lymphoma cells. Briefly, cells were washed, resuspended 0.1% BSA in PBS with 0.2 μM CFSE and incubated 5 min at 37 °C. The reaction was quenched by adding 5 volumes of ice-cold 5% FBS in PBS and incubated for 5 min on ice. Cells were then washed twice with 5% FBS in PBS and resuspended in the growth media for the experiment. The mouse/human chimeric anti-CD20 IgG1 rituximab was purchased from Genentech (Genentech, San Francisco, CA).

2.2. Incubation of effector and target cells with the antibody

A total of 10^6 U-937 human monocyte effector cells labeled with the CMTPX red fluorescent dye were incubated with 2×10^5 Ramos human Burkitt's B-cell NHL target cells stained with the CFSE green fluorescent dye (5:1—E:T ratio) in the presence or absence of 5 μg/ml of rituximab for 1 h or 2 h at 37 °C in 5% CO_2. We used 5 μg/ml concentration of rituximab because it has been reported to be in the range to achieve ADCC activity (Manches et al., 2003). After the incubation, the cells were washed and fixed with 2% paraformaldehyde in PBS and the nuclei were stained with the DRAQ5 dye (1,5-bis{[2-(dimethylamino) ethyl]amino}-4, 8-dihydroxyanthracene-9,10-dione, Biostatus, Ltd., Leicestershire, UK) diluted 1/200 (12.5 μM, final concentration) for 30 min. We used DRAQ5 nuclear dye because it can stain nuclei of live or dead fixed cells.

2.3. Data acquisition with ImageStream

Samples were run in the ImageStream multispectral imaging flow cytometer (Amnis Corporation, Seattle, WA) and images were acquired for 10,000 events/sample. Cells were excited using a 488 nm laser with intensity ranging from 75 to 200 mW, depending on the staining. Brightfield, side scatter, fluorescent cell images were acquired at 40×