Leukemia Research 55 (2017) 41–48

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Unexpected cross-reactivity of anti-cathepsin B antibodies leads to uncertainties regarding the mechanism of action of anti-CD20 monoclonal antibody GA101

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ARTICLE INFO

Article history:
Received 3 November 2016
Received in revised form
17 December 2016
Accepted 4 January 2017
Available online 18 January 2017

Keywords:
Antibody-based immunotherapy
GA101
CD20
Cathepsin B
Lysosomal permeabilization
Lymphomas
Cell death

ABSTRACT

GA101, also known as obinutuzumab or Gazyva (Gazyvaro), is a glycoengineered type II humanized antibody that targets the CD20 antigen expressed at the surface of B-cells. This novel anti-CD20 antibody is currently assessed in clinical trials with promising results as a single agent or as part of therapeutic combinations for the treatment of B-cell malignancies. Detailed understanding of the mechanisms of GA101-induced cell death is needed to get insight into possible resistance mechanisms occurring in patients. Although multiple in vitro and in vivo mechanisms have been suggested to describe the effects of GA101 on B-cells, currently available data are ambiguous. The aim of our study was to clarify the cellular mechanisms involved in GA101-induced cell death in vitro, and more particularly the respective roles played by lysosomal and mitochondrial membrane permeabilization. Our results confirm previous reports suggesting that GA101 triggers homotypic adhesion and caspase-independent cell death, two processes that are dependent on actin remodeling and involve the production of reactive oxygen species. With respect to lysosomal membrane permeabilization (LMP), our data suggest that lack of specificity of available antibodies directed against cathepsin B may have confounded previously published results, possibly challenging current LMP-driven model of GA101 action mode.

1. Introduction

The CD20 protein, which is selectively expressed on precursor and mature B cells, has been validated as a target antigen for immunotherapy in B-cell malignancies. Rituximab, a Type I chimeric (human/mouse) IgG1 anti-CD20 antibody, was approved for the treatment of patients with B-cell non-Hodgkin lymphoma (NHL) as far back as the late nineties. It has been shown that rituximab binding to CD20 may cause cell death through three distinct mechanisms: (1) activation of complement-dependent cytotoxicity (CDC) after CD20 relocalization into lipid rafts; (2) initiation of antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis (ADCP) via Fc region binding to Fcγ receptors (FcγR) on innate immune effectors such as natural killer cells and macrophages; (3) direct induction of programmed cell death (PCD) [1–5]. Addition of rituximab to standard chemotherapy improves the clinical outcome of patients with NHL and chronic lymphocytic leukemia (CLL). However, a significant percentage (30–50%) of rituximab-treated patients fail to achieve complete or partial remission, and many experience disease relapse [3,4,6–14]. This has prompted research efforts to develop novel anti-CD20 antibodies with improved functional properties and greater clinical efficacy [5].

Obinutuzumab (GA101) is a Type II humanized mouse IgG1κ anti-CD20 antibody with a glycoengineered Fc fragment [15] to

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http://dx.doi.org/10.1016/j.leukres.2017.01.010
0145-2126/

enhance Fc receptor binding affinity (in particular for FcγRIIIa). GA101 is currently under phase III clinical trials for the treatment of patients with NHL [16,17] and has been approved for patients with previously untreated CLL [18–21]. In vivo studies have indicated superior anti-lymphoma activity for this third-generation anti-CD20 antibody over rituximab [15,22,23]. Positional mapping showed that the epitope of GA101 partially overlaps the epitope of rituximab and extends towards the C-terminus of CD20 which contributes to the high binding affinity of GA101 for CD20 [5,15,24]. Protein tomography analysis indicated that, contrary to rituximab bivalent binding to CD20, GA101 preferentially binds to CD20 monovalently, which favors intra- rather than inter-CD20 tetramer binding [5,24,25]. Both structural features and CD20-binding characteristics of GA101 make it different from rituximab in terms of mode of action: GA101 does not provoke CD20 relocalization in lipid rafts and subsequent CDC, but induces stronger homotypic adhesion (HA) and direct PCD [5,15]. In addition, the glycoengineered fragment of GA101 leads to enhanced ADCC and ADCP activity [1,2,15,26,27].

Although rituximab has been demonstrated to induce apoptosis of non-Hodgkin lymphoma cell lines in vitro [28,29], evidence from numerous investigations does not appear to support this observation especially in the in vivo context [30–37]. For its part, GA101 is thought to trigger non-apoptotic (necrotic-like) cell death without the need of CD20 cross-linking. The current proposal on the sequence of events leading to GA101-induced "programmed necrosis" is as follows: (i) binding of GA101 to the extracellular domain of CD20 followed by HA, (ii) peripheral reorganization of the actin cytoskeleton, (iii) lysosomal membrane permeabilization (LMP), (iv) cathepsin B release to the cytosol, (v) NADPH oxidase (NOX)-mediated (i.e., non-mitochondrial) reactive oxygen species (ROS) production, and (iv) loss of plasma membrane integrity [38,39].

However, not all cells die by caspase-independent cell death upon exposure to GA101 as both procaspase-3 overexpression and caspase-3 activation were reported in the human non-Hodgkin's lymphoma B cell line RL cells [40]. Moreover, cell surface exposure of phosphatidylserine (PS) was consistently observed upon GA101 treatment in a variety of cell lines, raising the possibility of a transition from apoptosis to secondary necrosis for a fraction of the B-cell population [15,40]. In fact, there is evidence that GA101 can induce mitochondrial membrane permeabilization (MMP) [39,41], but this event was considered as secondary to LMP and cathepsin B release [39]. The ability of GA101 to trigger necrosis, apoptosis or both may reflect features of the same cell death pathway that converge on mitochondria and/or lysosomes in a cell type- and/or context-specific manner. Alternatively, the choice of GA101-induced cell death by necrosis or apoptosis may depend on which of the two events occurs first: LMP or MMP.

Motivated by these concerns, we sought to undertake an accurate kinetic analysis of LMP and MMP in GA101-treated B cells. The present study was also carried out with the premise that detailed understanding of the role of mitochondria and lysosomes in GA101-induced cell death can provide insights for GA101 use in therapy and help overcome possible resistance mechanisms.

2. Material and methods

2.1. Cell culture and treatments

Raji or SUDHL-4 cells were cultured in RPMI 1640 medium (PAA) containing 10% fetal calf serum, 2 mM L-glutamine, 100 units penicillin, and 100 μg streptomycin. Cells were seeded at 0.5×10^6 cells/mL and treated with 10 μg/ml of GA101 (provided by Roche Pharma) or rituximab (MabThera Lot: H0623, Roche) for different periods of time.

2.2. HA assay

Cells were seeded in 8-well imaging chambers (Zell-Kontakt, Germany) and recorded every 5 min for 24 h using phase-contrast time-lapse videomicroscopy (inverted Axiovert 100 M, Zeiss) in an environment at 37 °C with 5% CO2. In other experiments, microphotographs of untreated cells and cells receiving pre-treatment with different inhibitors or treatment by GA101 were taken using an inverted Leica microscope. Images were processed using ImageJ software.

2.3. Cell death analysis by flow cytometry

In a first set of experiments, cells were treated with GA101 or Rituximab for 6, 12, 24 and 48 h or left untreated. Treated cells were harvested by centrifugation at 1800 rpm for 5 min and resuspended in 100 μL of Annexin V/propidium iodide labeling solution (Annexin-V-FLUOS Staining kit, Roche). Cells were then analyzed by flow cytometry (Becton Dickinson, LSR II) after an incubation period of 15 min in the dark at room temperature.

In a second set of experiments, cells were first pre-treated with different inhibitors for 1 or 2 h: Latrunculin B (Enzo Life Science), Tiron (Sigma-Aldrich), Z-VAD-FMK, Z-DEVD-FMK, Z-IETD-FMK, Z-LEHD-FMK (BD biosciences), CA-074-Me (Calbiochem), GW4869 (Calbiochem), Nec-1s (Calbiochem), diphenyleneiodonium (Calbiochem), ML141 (Santa Cruz). The cells were then left untreated or were treated with GA101 for 6 h and analyzed by flow cytometry after AV/PI labeling.

2.4. ROS detection

10^6 cells were first pre-treated for 2 h with different inhibitors: Latrunculin B, Tiron, DPI, or ML141, and then treated with GA101 and the ROS marker carboxy-H2DCFDA (25 mM, Molecular Probes) for 30 min, 1 h, 3 h, 6 h or left untreated. Carboxy-H2DCFDA is oxidized in the presence of ROS to carboxy-DCF, which emits green fluorescence. ROS detection was analyzed by flow cytometry and data were processed using the FlowJo software.

2.5. Subcellular fractionation

Cells (4×10^7) were harvested 4 h after treatment with GA101 and rinsed in cold SET buffer (250 mM sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4) by centrifugation at 1200 rpm for 5 min. Cells were resuspended in 0.5 mL of cold SET buffer containing ETDA-free antiprotease cocktail (APC, Roche) and then subjected to 30 strokes on ice using a Dounce homogenizer. A centrifugation at 1000*g* at 4 °C for 10 min was performed to separate supernatant from cell/nuclei pellet. The supernatant was collected and the pellet was resuspended in 0.5 mL of cold SET buffer containing APC. The suspension was then subjected to another 30 strokes and to centrifugation as described above. The supernatants were pooled and centrifuged at 20,000*g*, 4 °C for 20 min. The pellet was washed with 1 mL SET buffer containing APC and lysed with 50 μL of lysis buffer (Thermo Scientific) to obtain a mitochondria/lysosome fraction. The supernatant was ultracentrifuged at 100000*g* at 4 °C for 60 min. The supernatant (corresponding to the cytosol fraction) was collected and the pellet was resuspended in 50 μL of lysis buffer to obtain a vesicle-rich fraction. To obtain only the mitochondria/lysosome and cytosolic fractions, treated cells were subjected to the same protocol with SE buffer (250 mM sucrose, 1 mM EDTA, pH 7.4) instead of SET solution, skipping out the ultracentrifugation step.