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Selection of resistant acute myeloid leukemia SKM-1 and MOLM-13 cells by vincristine-, mitoxantrone- and lenalidomide-induced upregulation of P-glycoprotein activity and downregulation of CD33 cell surface exposure

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ABSTRACT

Bone marrow cells and peripheral blood mononuclear cells obtained from both acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) patients contain upregulated levels of cell surface antigen CD33 compared with healthy controls. This difference enables the use of humanized anti-CD33 antibody conjugated to cytotoxic agents for CD33 targeted immunotherapy. However, the expression of the membrane-bound drug transporter P-glycoprotein (P-gp) has been shown to be critical for resistance against the cytotoxicity of a humanized anti-CD33 antibody conjugated to maytansine-derivative DM4. The aim of the present study was to examine whether the expression of P-gp in AML cell lines is associated with changes in CD33 expression. For this purpose, we established drug resistant variants of SKM-1 and MOLM-13 AML cell lines via the selection of parental cells for resistance to vincristine, mitoxantrone and lenalidomide. All three substances induced a multidrug resistance (MDR) phenotype in SKM-1 cells associated with strong upregulation of P-gp and downregulation of CD33. However, in MOLM-13 cells, the upregulation of P-gp and downregulation of CD33 were present only in cells selected for resistance to vincristine and mitoxantrone but not lenalidomide. Inverse expression of P-gp and CD33 were observed in all resistant variants of SKM-1 and MOLM-13 cells. The MDR phenotype of resistant variants of SKM-1 and MOLM-13 cells was associated with alterations in apoptotic regulatory proteins and downregulation of the multidrug resistance associated protein 1 and breast cancer resistance protein.

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1. Introduction

Leukemia cells from more than 90% of patients with acute myeloid leukemia (AML) express CD33 antigen, which is not expressed in normal hematopoietic stem cells or in non-myeloid tissues (Cianfriglia, 2013; Naito et al., 2000). Upregulated levels of CD33 are observed also in bone marrow cells and peripheral blood cells obtained from myelodysplastic syndrome (MDS, which could developed to AML) patients compared with healthy controls (Jilani et al., 2002). CD33 is a 67 kDa glycoprotein present on the surface of myeloid cells and is a member of the sialic acid binding immunoglobulin-like lectin family of proteins (Jurcic, 2012). After binding to an appropriate antibody, CD33 is rapidly internalized in leukemia cells (Cianfriglia, 2013; Naito et al., 2000). This action enables the use of humanized CD33 antibody conjugated to cytotoxic agents for targeted immunotherapy (Cianfriglia, 2013; Jurcic, 2012; Linenberger, 2005; Stasi, 2008; Voutsadakis, 2002; Walter, 2014; Walter et al., 2012). However, P-glycoprotein (P-gp, product of the *MDR1* gene) efflux activity has been described as critical for resistance against the cytotoxicity of humanized anti-CD33 antibody conjugated to maytansine derivative DM4, a

Abbreviations: AML, acute myeloid leukemia; BCRP, breast cancer resistance protein; CSA, cyclosporine A; DOX, doxorubicin; GAPDH, glyceraldehyde phosphate dehydrogenase; HSP90, heat shock protein 90; KCA, ketoconazole; Len, lenalidomide; LC_{50}, median of lethal concentration; MDR, multidrug resistance; MDS, myelodysplastic syndrome; MRP, multidrug resistance associated protein; MTX, mitoxantrone; P-gp, P-glycoprotein; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; VER, verapamil.

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potent tubulin inhibitor (Tang et al., 2009). P-glycoprotein, an ABCB1 member of the ABC transporter family, is a drug efflux pump with specificity for the transport of anthracyclines (e.g., doxorubicin – DOX), vinca alkaloids (e.g., vincristine – VCR), actinomycines (e.g., actinomycin D, dactinomycines), taxols (e.g., paclitaxel), alkylating agents (mitomycin C), peptide antibiotics (gramicidin, valinomycin), HIV-1 protease inhibitors and many others (reviewed in (Breier et al., 2005, 2013)). When expressed in neoplastic cells, P-gp induces multidrug resistance (MDR) to P-gp-substrates (Breier et al., 2005). Function of P-glycoprotein seems to be regulated by p38 and PI3 K/Akt kinases pathways (Barancik et al., 2001, 2006). Both these protein kinases play a role in resistance toward CD33 targeted AML cell damage (Haag et al., 2009; Rosen et al., 2013). There are several other ABC transporters that may be involved in the development of drug resistance such as multidrug resistance associated proteins (members of the ABCC gene subfamily) and breast cancer resistance protein (BCRP, a product of the *BCRP* gene and an ABCG2 member of the ABC transporter family). The substrate specificity of P-gp MRPs and BCRP partially overlaps (Litman et al., 2000).

Several lines of evidence indicate that P-gp may exert a secondary antiapoptotic activity that is independent from its drug efflux activity (Breier et al., 2013; Pallis and Russell, 2000; Ruefli and Johnstone, 2003; Tainton et al., 2004). This secondary activity may lead to a reduction in the sensitivity of P-gp positive cells to substances that are not P-gp substrates such as cisplatin (Gibalova et al., 2012).

In addition, massive structural remodeling of cell surface saccharides was detectable in the P-gp positive mouse leukemia L1210 cell line compared with their P-gp negative counterparts (Bubencikova et al., 2012; Sulova et al., 2010, 2009). Similar differences in the composition of cell surface sugars were observed in two P-gp positive L1210 cell variants obtained by either their adaptation to VCR or transfection with a gene encoding human P-gp compared to their parental P-gp negative cells (Bubencikova et al., 2012; Sulova et al., 2010). Thus, altered cell surface sugar composition seems to be a response to the presence of P-gp in the cell and is independent of how P-gp expression is achieved. It is reasonable to speculate that the alteration in cell surface saccharides is part of several complex changes that occur on the plasma membrane when P-gp is overexpressed. These complex changes may also involve alterations in the expression and plasma membrane localization of different glycoprotein antigens including CD33. This latter idea is consistent with the observation of an inverse correlation between CD33 expression and P-gp efflux activity in AML blasts obtained from AML patients (Walter et al., 2007). P-glycoprotein is believed to be induced via ligand activation of pregnane X receptors (PXR) and constitutive androstane receptor (CAR) (Cerveny et al., 2007).

Therefore, the aim of the present paper is to examine whether the overexpression of P-gp induced in AML cells by repeated culturing in the presence of increasing concentrations of anticancer (vincristine, mitoxantrone – MTX) and immunomodulatory agents (lenalidomide – LEN) is associated with changes in expression and membrane localization of CD33. For cell models, we used human AML cell lines (SKM-1 and MOLM-13) derived from AML patients whose diseases developed from MDS.

2. Methods and material

2.1. Cell culture conditions

Two cell lines derived from patients with AML developed from MDS (supplied by Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) were used in this study: (i) SKM-1 (Nakagawa et al., 1993) (ACC 547) – derived from the peripheral blood of a 76-year-old patient (AML M5; for additional characteristics including CD33 positivity, see information supplied by supplier[1]) and (ii) MOLM-13 (Matsuo et al., 1997) (ACC 554) – derived from the peripheral blood of a 20-year-old patient (AML FAB M5a; for additional characteristics including CD33 positivity, see information given by supplier[2]). Cells (inoculums 5×10^5) were grown in RPMI 1640 medium (GE Healthcare) containing 12% fetal bovine serum (Biotech, SR), 100,000 units/L of penicillin and 50 mg/L of streptomycin (both Sigma Aldrich, USA) for two days in a humidified atmosphere containing 5% CO_2 at 37 °C. To produce resistant variants, SKM-1 and MOLM-13 cells were treated over a 6-month period with repeated passages in media containing stepwise increases in their concentrations of anticancer agents. The following agents were used: VCR (Sigma Aldrich USA) in a concentration range of 0.1–50 nmol/L, MTX (Sigma Aldrich USA) in a concentration range of 0.1–10 nmol/L and LEN (Celgene, USA) at a concentration range of 0.2–100 nmol/L. During this selection for resistance, cells were monitored for P-gp expression and activity, CD33 transcript levels and CD33 protein localized on cell surface.

2.2. Cell death effect of VCR and MTX and LEN on sensitive and resistant variants of SKM-1 and MOLM-13 cells

Cells (5×10^4 cells/well) were cultured with or without VCR (0–10 nmol/L), MTX (0–10 nmol/L) and LEN (0–1 mmol/L) in 96-well cell culture plates. LEN, VCR and MTX were added directly to 200 μL of culture media. After 24 h, cell viability was assessed using the MTT assay (Gerlier and Thomasset, 1986), which was performed by adding MTT ([3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyltetrazolium bromide]) to a final concentration of 0.25 mg/mL per well. The cells were then incubated with MTT for 2 h. Next, the plates were centrifuged for 15 min (2500 rpm), and the cell sediment was extracted with dimethyl sulfoxide. The absorbance at 540 nm was measured using a Universal Microplate Spectrophotometer mQuant (BioTek Instruments, Inc. USA). Dose–response curves were fitted according to an exponential decay equation (Eq. (1)) by non-linear regression as previously described (Kupsakova et al., 2004):

$$N = 100\% \times \exp[\ln(0.5) \times (c/LC_{50})] \quad (1)$$

where N represents the percentage (from a control in the absence of drugs) of cell viability after culturing in the presence of drugs at a concentration c. The LC_{50} is the concentration of a substance when $N = 50\%$.

The experimental data were fit by nonlinear regression using the SigmaPlot Graphing Software (version 8.00). Statistical significance was analyzed using an unpaired Student's t-test.

2.3. Determination of MDR1, CD33, PXR, CAR and BCRP gene expression by RT-PCR in sensitive and resistant variants of SKM-1 and MOLM-13 cells

Total RNA from susceptible and drug resistant SKM-1 and MOLM-13 cell lines was isolated using Trizol Reagent (Life Technology, Slovakia) according to the manufacturer's instructions. Reverse transcription was performed with 2 μg of DNAse I (Thermo Scientific, Germany) treated RNA and the RevertAidTM H Minus First-Strand cDNA Synthesis Kit (Thermo Scientific, Germany) according to the manufacturer's protocol. PCR was performed in

[1] http://www.dsmz.de/catalogues/details/culture/ACC-547.html?tx_dsmzresources_pi5[returnPid]=192.

[2] http://www.dsmz.de/catalogues/details/culture/ACC-591.html?tx_dsmzresources_pi5[returnPid]=192.

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