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Research paper

The expression of P-glycoprotein in leukemia cells is associated with the upregulated expression of nestin, a class 6 filament protein

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ABSTRACT

Multidrug resistance (MDR) is a serious obstacle to the effective chemotherapeutic treatment of leukemia. Expression of plasma membrane P-glycoprotein (P-gp), a transporter involved in drug efflux, is the most frequently observed molecular causality of MDR. We observed the coexpression of P-gp and the filament protein nestin in the acute myeloid leukemia (AML) cell lines SKM-1 and MOLM-13 following the induction of P-gp expression using vincristine. Nestin is considered a marker of neural stem cells and neural progenitor cells. The aim of this study was to determine whether there is causal relationship between the expression of P-glycoprotein and the expression of nestin in both of these AML cell lines. The expression of P-gp was induced in SKM-1 cells by selective pressure using vincristine (VCR), mitoxantrone (MTX), azacytidine (AzaC) and lenalidomide (LEN). Whereas the selective pressure of VCR, MTX and AzaC also induced P-gp expression in MOLM-13 cells, LEN was found to be ineffective in this regard. In all cases in which P-gp expression was induced in SKM-1 and MOLM-13 cells, its expression was associated with the induction of nestin mRNA expression and the presence of a 200–220 kDa nestin-immunoreactive protein band in western blots. Silencing P-gp expression using s10418 siRNA (known as the P-gp silencer) was associated with the downregulation of the nestin transcript level, demonstrated using RT-PCR. Nestin mRNA was also observed in two P-gp-positive variants of L1210 cells that were obtained either by selection with VCR or by transfection with a retrovirus encoding human P-gp. Detectable levels of nestin transcripts were not observed in P-gp-negative parental L1210 cells. Taken together, these results indicated that the induction of P-gp expression is causally associated with the expression of nestin in leukemia cells.

1. Introduction

The multidrug resistance (MDR) of neoplastic cells represents a complex phenotype characterized by specific differences in the expression levels of diverse proteins, which contribute to their reduced sensitivity to anticancer drugs [1]. The expression of P-glycoprotein (P-gp) is most often observed molecular cause of MDR [2]. P-glycoprotein (P-gp), known also as CD243 [3] in the cluster of differentiation surface antigens, is an ABCB1 member of the ABC transporter family that pumps a large number of endogenous or foreign substances with diverse chemical structures out of neoplastic cells [2,4]. The expression of P-gp generally induces strongly reduced sensitivity to P-gp substrates. Moreover, P-gp expression has been found to be associated with changes in several cellular regulatory pathways that reflect the rebuilding of cellular architecture, particularly that of the plasma membrane [5]. Major remodeling of cell-surface sugars linked to membrane proteins that bind lectins has been associated with the expression of P-gp induced either by vincristine (VCR) selective pressure or by transfection with a retrovirus encoding human P-gp [6–8]. Large-scale changes in cellular homeostasis that are associated with P-gp expression contribute to the MDR phenotype and may be responsible for the resistance to other substances that are not P-gp substrates [5]. The expression of P-gp may also alter the differentiation status of cells [9]. We consistently observed the downregulation of the

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level of the immature AML myoblast marker CD33 in P-gp-positive acute myeloid leukemia (AML) cell variants compared with that of their P-gp-negative counterparts [10]. P-gp is expressed in nearly all hematopoietic progenitor cells, including long-term culture-initiating cells. The highest levels of P-gp in these progenitor cells occur in those displaying the characteristics of pluripotent stem cells [11].

Nestin (a class 6 filament protein), a product of the *Nes* gene [12–14] that is known as a neural stem-cell and progenitor-cell marker [15] has also been detected in other cell types and tissues during mammalian embryogenesis, such as skeletal muscle cells, the umbilical cord, blood, cardiac muscle cells, sertoli and interstitial testicular cells, odontoblasts, hair follicle sheath cells, hepatic cells and renal progenitor cells (reviewed in [16]). Nestin expression may also occur in solid tumors, in which it is associated with the initiation, promotion, and progression of disease and poor chemo-responsiveness [17,18]. Interestingly, nestin has been shown to be expressed in several leukemia cell lines [19] and in mature $CD138^+/CD38^+$ plasma cells in multiple myeloma [14].

The positive correlation of nestin expression and P-gp expression has been reported in human neural stem/progenitor cells [20]. The induction of P-gp expression in acute myeloid leukemia SKM-1 and MOLM-13 cells by selection with VCR has been associated with the coexpression of nestin transcripts [21]. The latter finding indicated that the coexpression of P-gp and nestin can also occur in leukemia cells.

P-gp-positive SKM-1 and MOLM-13 cells were established by periodical cultivation in medium with stepwise increasing concentrations of VCR, mitoxantrone (MTX) [10] and azacytidine (AzaC) [22]. SKM-1 cells but not MOLM-13 cells expressed P-gp when lenalidomide (LEN) was used to induce P-gp expression [10]. The aim of the present study was to determine whether nestin is coexpressed with P-gp in P-gp-positive variants of SKM-1 and MOLM-13 cells. To investigate the connection between P-gp and nestin expression, we determined whether transfecting L1210 mouse leukemia cells with a retrovirus encoding human P-gp [7] also induced nestin expression, and whether silencing P-gp expression using a P-gp silencer induced changes in nestin expression.

2. Materials and methods

2.1. Cell culture conditions

The following AML cell lines were used in this study: P-gp-negative SKM-1 (ACC 547 obtained from *Deutsche Sammlung von Mikroorganismen und Zellkulturen* GmbH, DSMZ Germany); MOLM-13 (ACC 554 obtained from DSMZ Germany); and their P-gp-positive variants SKM-1/VCR, SKM-1/MTX, SKM-1/LEN, SKM-1/AzaC, MOLM-13/VCR, MOLM-13/MTX and MOLM-13/AzaC with resistance that was established by culturing them in media with stepwise increasing concentrations of VCR, MTX, AzaC (all obtained from Sigma Aldrich, USA) and LEN (Celgene, USA) [10,22]. The cells 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 purchased from Sigma Aldrich) for two days in a humidified atmosphere containing 5% CO_2 at 37 °C. Additionally, we used mouse lymphoid leukemia P-gp-negative L1210 cells (obtained from DSMZ Germany) and their P-gp-positive variants L1210/VCR and L1210/T that were obtained either by cultivation in stepwise increasing concentrations of VCR [23] or by transfection with a retrovirus encoding human P-gp [7], the pHaMDRwt retrovirus (Addgene product 10957 USA), which contains the full-length P-gp cDNA [24]. L1210 cell variants were cultured in RPMI 1640 medium containing 4% fetal bovine serum and 1 μg/mL of gentamycin (Gibco, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were passaged every two days.

2.2. Effect of VCR, MTX, doxorubicin (DOX) and cisplatin (cisPt) on the viability of drug-sensitive and -resistant variants of SKM-1 and MOLM-13 cells

SKM-1 and MOLM-13 cells (5×10^4 cells/well) were cultured with or without VCR (0–250 nmol/L), MTX (0–250 nmol/L), DOX (0–250 nmol/L) and cisPt (0–15 μmol/L) in 96-well cell culture plates. DOX, VCR, cisPt and MTX were added directly to 200 μL of the culture medium. After 48 h, cell viability was assessed using the MTT assay [25], which was performed by adding MTT ([3-(4,5-dimethylthiazol-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 using dimethyl sulfoxide. The absorbance at 540 nm was measured using a Universal Microplate Spectrophotometer mQuant (BioTek Instruments, Inc. USA). The IC_{50} values were obtained by respective dose-response curve fitting according to the following exponential decay equation (Eq. (1)) using non-linear regression, as previously described [26]:

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

where N is the percentage of viable cells present after cultivation in the presence of drugs at concentration c relative to that of control cells grown in the absence of a drug. The IC_{50} is the concentration of a substance when N = 50%.

IC_{50} values were accounted from three independent measurements. The significance of differences was analyzed using an unpaired Student's t-test.

2.3. Determination of ABCB1 and NES gene expression levels in P-gp-negative and P-gp-positive variants of SKM-1, MOLM-13 and L1210 cells using RT-PCR

The total RNA was isolated from P-gp-negative and P-gp-positive variants of SKM-1, MOLM-13 and L1210 cell lines using TRIzol Reagent (Life Technology, Slovakia) according to the manufacturer's instructions. Reverse transcription was performed using 2 μg of DNase I- (Thermo Scientific, Germany) treated RNA and a RevertAid H Minus First-Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's protocol. PCR was performed in a total volume of 25 μL using a PCR kit according to the manufacturer's protocol (Thermo Scientific, Germany). The level of β-actin or GAPDH expression was used as an internal standard for human (SKM-1, MOLM-13) or mouse (L1210) cells, respectively. After treating the samples at 94 °C for 3 min to inactivate the reverse transcriptase, the samples were subjected to 30 cycles of 95 °C for 30 s followed by 57 °C for 30 s for *ABCB1* and *GAPDH*, 57.2 °C for 30 s for NES or 54.4 °C for 30 s for β-*actin*, ending with 72 °C for 90 s and a final extension of 72 °C for 10 min. The PCR products were separated in a 1.5% agarose gel (Invitrogen, Thermo Fisher Scientific, Slovakia) and were visualized using GelRedTM nucleic-acid stain (Thermo Scientific, Germany). Images of the gels were captured and the band densities were quantified using a Typhoon 9210 imaging system (GE Healthcare, USA). The data were expressed as the relative level of each mRNA normalized to that of the housekeeping gene β-actin. Data were obtained from three independent experiments and the significance of differences was analyzed using an unpaired Student's t-test.

The sequences of the primers used in this study were as follows: for the β-*actin* gene, 5′-CTG GGA CGA CAT GGA GAA AA-3′ and 5′-AAG GAA GGC TGG AGA GTG C-3′, which produced a 564-bp product; for the *GAPDH* gene, 5′ AAC TTT GTC AAG CTC ATT TCC 3′