Background and Objectives. Expression of the multidrug resistance P-glycoprotein, a transmembrane drug transporter, is influenced by recently described polymorphisms of the human MDR1 gene. Hematopoietic cells, such as lymphocytes, and hematopoietic stem cells, express P-glycoprotein, but the effect of MDR1 gene polymorphisms on P-glycoprotein activity in stem cells is unknown. We investigated whether T-129C, G26677T and C3435T polymorphisms influence P-glycoprotein function in stem cells.

Design and Methods. P-glycoprotein function was evaluated in immunomagnetically purified bone marrow CD34+ cells from 33 healthy bone marrow donors by the flow cytometric rhodamine 123-efflux assay. For T-129C and C3435T, bone marrow donors were genotyped by polymerase chain reaction amplification followed by MspAI and DpnII digestion analyses, respectively. For the analysis of C2677T, exon 21 was sequenced.

Results. P-glycoprotein function was not different among the C3435T genotypes (CC, 38.2±3.5%; n=17; CT, 42.2±3.3%; n=11; and TT, 45.0±5.3%; n=5) nor was it among the C2677T genotypes (CC, 39.4±2.4%; n=27; CT, 43.7±6.4%; n=5; and TT, 54.3%; n=1). Among the 33 subjects, three were heterozygotes for the –129C allele (CT) and no mutant homozygote was identified. P-glycoprotein was similar in heterozygotes (TC, 50.6±2.9%) and wild-type subjects (TT, 39.5±2.4%).

Interpretation and Conclusions. These findings suggest that the known functional MDR1 gene polymorphisms are not major determinants of P-glycoprotein function in hematopoietic stem cells. Other genetic variants might influence P-glycoprotein activity in this cell type.

© 2002, Ferrata Storti Foundation

Key words: P-glycoprotein, MDR1, polymorphism, hematopoietic stem cell.

---

The MDR1 gene-encoded P-glycoprotein (P-gp) is an energy-dependent transmembrane drug efflux pump responsible for the multidrug resistance (MDR) phenotype of leukemia.1 P-glycoprotein is able to eliminate a variety of xenobiotics from the intracellular cytoplasm by actively extruding structurally diverse, hydrophobic amphipathic substances from the cell.2 These xenobiotics include a wide range of drugs, such as antineoplastic agents (e.g. anthracyclines, Vinca alkaloids, epipodophyllotoxins, actinomycin D and taxanes) and antiretroviral drugs.3 The expression of P-gp by leukemic cells correlates with resistance to chemotherapy and is an independent risk factor for treatment failure in de novo and relapsed acute myeloid leukemia.1,2

In addition to neoplastic cells, a variety of normal human tissues (kidney, colon, jejunum, blood-brain barrier, liver, and hematopoietic tissue) are known to express P-gp at different levels.3 In the hematopoietic compartment, P-gp is expressed by peripheral blood lymphocytes, especially CD56+ natural killer and CD8+ cytotoxic T-cells, as well as bone marrow (BM) CD34+ hematopoietic stem cells (HSC).4 The role of P-gp in these cell subsets is still controversial, but it might be involved in the cytolytic activity of lymphocytes and in the protection of HSC against toxic compounds.2,4

Recently, strong evidence emerged showing that P-gp expression is genetically determined. A recent study asked the question whether individual differences in P-gp expression are under genetic control.5 Sequencing of the MDR1 gene yielded 15 novel single nucleotide polymorphisms (SNPs). Among these novel variations, a synonymous C to T transition at nucleotide position 3435 in exon 26 significantly influenced duodenal P-gp, downregulating its expression.5 In addition, it was demonstrated that the mutant MDR1 3435T allele is associated with decreased P-gp activity in CD56+ nat-
concentration of 5% fetal calf serum to a final RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 5% fetal calf serum to a final concentration of 5 × 10^6 cells/mL. Moreover, recent data demonstrated that the MDR1 3435T allele has important clinical significance in predicting a better response to antiretroviral treatment in HIV-1-infected patients.7

This SNP was found to be in linkage disequilibrium with a non-synonymous SNP at exon 21, a G to T transition at nucleotide 2677, leading to an amino acid substitution from alanine to serine at position 893, which was, in contrast, associated with higher P-gp expression in vitro and low fexofenadine plasma concentration.8 Moreover, a T to C transition at nucleotide –129 was also described, and heterozygotes (TC) presented lower P-gp expression in human placenta than did wild-type individuals (TT).9

Based on these results, it is conceivable that functional MDR1 genotypes might influence P-gp function in other hematopoietic cells, such as HSC. Data on this subject are, however, still lacking. In the present study we investigated whether P-gp function in CD34+ HSC is influenced by functional MDR1 gene SNPs.

**Design and Methods**

**Subjects**

Thirty-three consecutive healthy BM donors for BM transplantation (17 males and 16 females; mean age, 26.2 ± 2.4 years) with peripheral blood counts in the normal range and who were not taking any medication were included. Based on phenotypic characteristics and ethnic background, each individual was classified as Caucasian (26 subjects), Mulatto (4 subjects), Black (2 subjects) or Asian (1 subject). Bone marrow samples were obtained from posterior iliac crest aspiration at the time of transplantation, after informed consent. This study was approved by the local Ethics Committee.

**CD34+ cell separation**

Bone marrow mononuclear cells were isolated by density gradient centrifugation and CD34+ cells purified by indirect magnetic labeling (MACS, Miltenyi Biotec, Auburn, CA, USA), as described elsewhere.10 Briefly, BM mononuclear cells were incubated with γ-globulin and hapten-conjugated anti-CD34 monoclonal antibody (clone QBEND/10) and then incubated with colloidal super-paramagnetic MACS microbeads conjugated to an anti-hapten antibody. Cells were washed and resuspended in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 5% fetal calf serum to a final concentration of 5 × 10^6 cells/mL.

**Rhodamine 123-efflux assay**

P-glycoprotein activity was determined by means of rhodamine 123 (Sigma, St. Louis, MO, USA) efflux, a fluorescent dye that is a substrate for P-gp, as previously described.3,11 Briefly, 200 µL of purified cell suspension (5 × 10^6 cells/mL) were incubated with rhodamine 123 (final concentration of 200 ng/mL) for 20 min. After washing, cells were incubated in a rhodamine 123-free medium in the presence or absence of verapamil (Sigma), a P-gp inhibitor (final concentration of 10 µM) for 1.5 hour. Finally, cells were washed in a verapamil-containing medium and stained with phycoerythrin-conjugated anti-CD34 (anti-HPCA-2, clone 8G12, Becton Dickinson, San José, CA, USA) monoclonal antibody and peridinin chlorophyll protein-conjugated anti-CD45 (anti-HLe-1, clone 2D1, Becton Dickinson). Acquisition and analysis were performed in a FACScan flow cytometer (Becton Dickinson) equipped with an argon-ion laser with a wavelength setting of 488 nm. To investigate dye efflux in CD34+CD45+ cells, this cell subset was further identified using multiple gating methods, according to the ISHAGE protocol for enumeration of CD34+ hematopoietic stem cells.12 Rhodamine 123 efflux was calculated based on the proportion of dye-effluxing cells in the verapamil-free experiment, in comparison to the control cells (treated with verapamil). Rhodamine 123 efflux was measured in CD34+CD45+ cells only (Figure 1).

**MDR1 polymorphisms genotyping**

Subjects were genotyped for the MDR1 gene C3435T and T-129C polymorphisms by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) assay using DNA extracted from peripheral blood leukocytes by standard methods. For C3435T analysis, a 244-bp fragment of the MDR1 gene (GenBank Accession No. AC005068) was generated by PCR amplification with the primers: (forward) 5'-GAT CTG TGA ACT CTT ACA TTA GGC-3' and (reverse) 5'-GAA GAG AGA AGA TAC TCC-3'. For T-129C analysis, a 178-bp fragment was generated by PCR amplification with the primers: (forward) 5'-GAT CTG TGA ACT CTT GTT TTC A-3' and (reverse) 5'-GAA GAG AGA AGA TAC CTT ACA TTA GGC-3'.

Restriction enzyme digestion produces two fragments with 172-bp and 72-bp when the –129T allele is present (cutting MspI (New England Biolabs, Herts, UK) restriction enzyme digestion produces two fragments with 172-bp and 72-bp when the –129T allele is present, whereas the 3435C allele is present, whereas the 3435T mutant abolishes the restriction site (Figure 2).

For T-129C analysis, a 178-bp fragment was generated by PCR amplification with the primers: (forward) 5'-TGA TTT GCT GGG CAG GAA CAG-3' and (reverse) 5'-AAT CTT GGA AGA AGA TAC TCC-3'. DpnII (New England Biolabs) restriction enzyme digestion produces two fragments with 54-bp and 124-bp when the –129T allele is present (cutting
position 124), whereas the –129C mutant creates a new restriction site at position 92, producing three fragments of 32-bp, 54-bp and 92-bp (Figure 2).

In addition, the MDR1 gene was screened for the presence of the C2677T polymorphism by automatic sequencing of exon 21 in both directions. A 280-bp fragment was generated by PCR amplification with the primers: (forward) 5'-TATGGTTGGCAACTACA-3' and (reverse) 5'-CATGAAAGATGTCTTT-3' and the PCR product was sequenced with the Big Dye terminator kit and analyzed on the ABI 377-96 automated sequencer (Applied Biosystems). Flow cytometric analysis and genotyping were performed by researchers blinded to each others' results.

Statistical analysis
The Kruskal-Wallis non-parametric test followed by Dunn's multiple comparison test was used to compare P-gp function between genotypes. A p value < 0.05 was considered to be statistically significant.

Results
Seventeen subjects were homozygous for the 3435C allele (age, 24.8±2.5 years; male:female, 8:9), 11 were heterozygous (29.8±5.2 years; m:f, 6:5), and 5 were homozygous for the 3435T allele (23.4±4.0 years; m:f, 2:3). Rhodamine 123-efflux assay did not demonstrate significant differences for P-gp function in bone marrow CD34+ cells from donors with different MDR1 C3435T genotypes (CC, 38.2±3.5%; CT, 42.6±3.6%; and TT, 45.0±5.3%; Figure 3).

Thirty subjects were homozygous for the –129T allele (25.9±2.4 years; m:f, 15:15), and three were heterozygous (30±3 years; m:f, 2:1). No mutant homozygous subject was identified. Although P-gp function in CD34+ cells tended to be higher in TC subjects, the difference did not reach statistical significance probably due to the low number of heterozygotes (TT, 40.8±2.2%; and TC, 50.6±2.3%; Figure 3).

In addition, 27 subjects were homozygous for the 2677C allele (26.5±2.6 years; m:f, 14,13), five were heterozygous (CT; 25.2±5.1 years; m:f, 3:2), and only one subject was homozygous for the 2677T allele (24 years; female); this subject was also homozygous for the 3435T allele. P-glycoprotein activity in CD34+ HSC did not differ among genotypes (CC, 39.4±2.4%; CT, 43.7±6.4%; and TT, 54.3%; Figure 3).

Discussion
As myelosuppression is a common undesirable consequence of chemotherapy, the variability of P-gp function in HSC is an important issue to address since the majority of anti-cancer drugs are P-gp substrates and drug-adjusted doses could be con-
MDR1 polymorphisms and P-glycoprotein activity

sidered to avoid treatment-related toxicity. Moreover, as leukemic cells arise from their normal counterparts, genetic variation of P-gp expression may influence its expression in leukemic cells and the response to treatment. Multidrug resistance, mediated by P-gp, contributes greatly to chemotherapy failure in de novo and relapsed acute myeloid leukemia, mainly in CD34+ leukemic cells. In addition, the rhodamine 123-efflux assay seems to be the most effective method for determining the impact of P-gp expression on the outcome of acute myeloid leukemia. However, as the functional MDR1 T-129C, G2677T and C3435T polymorphisms do not seem to alter P-gp function in CD34+ HSC, the analysis of these gene variations would not have any implication either in avoiding chemotherapy-related marrow toxicity or in improving acute myeloid leukemia response to treatment, in which other genetic determinants might play a role.

The present data suggesting that functional MDR1 SNPs are not major determinants of P-gp function in hematopoietic CD34+ stem cells contrast with those from previous studies showing a correlation between 3435T carriernship and P-gp function in duodenal and CD56+ natural killer cells and between -129C and 2677T carriernship and P-gp activity in human placenta. In agreement with our findings though, there are the recently published data demonstrating that cyclosporin A plasma concentration and rejection incidence in stable renal transplant recipients are independent of MDR1 C3435T genotype. As cyclosporin A is a P-gp substrate, the variability in its absorption and clearance has been attributed to intestinal P-gp expression. However, similar doses of cyclosporin A were needed to maintain the same plasma concentration in patients with different MDR1 C3435T genotypes. Furthermore, Kim et al. found conflicting results demonstrating that 3435T and 2677T alleles are associated with higher P-gp expression in vitro. Taken together, these data revealing a heterogeneous impact of functional MDR1 polymorphisms on P-gp activity in different cell types are an interesting finding that is worth investigating further in other tissues in which P-gp function is known to play a role.

In conclusion, this work shows for the first time...
that P-gp function in bone marrow CD34+ HSC is not influenced by known functional MDR1 polymorphisms, suggesting that other genetic mechanisms might be involved in the regulation of P-gp activity in this cell type.

Contributions and Acknowledgments

RTC, RFF and RPF were the main investigators involved in the design of the study, data analysis and interpretation. They wrote the paper with MAZ. RFF, MAZ and RPF were responsible for acquisition of funding for this work. ABG and SMG performed the flow cytometry and genotype analyses, respectively, and both participated in data analysis and interpretation. All the authors contributed to the intellectual content of the manuscript. Authorship order is based on time, work and intellectual and scientific contribution of each participant.

The authors thank Dr. Wilson A. Silva Jr. for sequencing analysis and Denise P. Gallo for her technical assistance.

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) grant 98/14247-6. R. T. Calado was a recipient of FAPESP grant 00/13885-0.

References