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Original Article

Anti-myeloma effects of ruxolitinib combined with bortezomib and lenalidomide: A rationale for JAK/STAT pathway inhibition in myeloma patients

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ABSTRACT

JAK proteins have been linked with survival and proliferation of multiple myeloma (MM) cells; therefore, JAK inhibition could be a therapeutic strategy for MM. We evaluated *JAK1* and *JAK2* expression in MM patients and the effects of JAK/STAT pathway inhibition on apoptosis, cell cycle, gene and protein expression in RPMI-8226 and U266 MM cell lines. 57% of patients presented overexpression of *JAK2* and 27%, of *JAK1*. After treatment with ruxolitinib and bortezomib, RPMI-8226 and U266 presented 50% of cells in late apoptosis, reduction of anti-apoptotic genes expression and higher number of cells in SubG0 phase. Co-culture with stromal cells protected RPMI-8226 cells from apoptosis, which was reversed by lenalidomide addition. Combination of ruxolitinib, bortezomib and lenalidomide induced 72% of cell death, equivalent to bortezomib, lenalidomide and dexamethasone, combination used in clinical practice. Many JAK/STAT pathway genes, after treatment, had their expression reduced, mainly in RPMI-8226, with insignificant changes in U266. In this scenario, JAK/STAT pathway could pose as a new therapeutic target to be exploited, since it is constitutively active and contributes to survival of MM tumor cells.

Introduction

Multiple myeloma (MM) is a plasma cell neoplasm and, despite recent advances in its treatment, it stills remains an incurable disease [1,2].

The activation of JAK proteins has been demonstrated in MM and other types of hematologic cancers, such as Chronic Myeloproliferative Neoplasms, promoting survival and proliferation of tumor cells [3–6]. Moreover, JAK/STAT pathway interacts with other signaling pathways and these paths intersect at several levels, each amplifying the activation of the other [7]. For instance, the JAK/STAT pathway promotes signaling through Ras protein via transcriptional activation of SOS. SOS binds to RasGDP, a negative Ras signaling regulator, and reduces its activity, promoting the activation of this pathway. Conversely, stimulation of the RTK/Ras pathway causes the activation of MAPK. MAPK specifically phosphorylates a serine near the C-terminal portion of most STATs [8,9].

Ruxolitinib is a JAK1/2 inhibitor [10] and has been approved for polycythemia vera and myelofibrosis treatment [11]. It is a competitive ATP inhibitor, binding to the catalytic site of the cytokine receptor kinase domain [10]. No clinical trial testing the combination of bortezomib and ruxolitinib was carried out to date in MM.

Ruxolitinib is able to affect the JAK/STAT pathway directly and the PI3K/Akt and Ras/Raf/MAPK pathways indirectly, while bortezomib inhibits the NFκB pathway; that is, four major signaling pathways could be affected by this drug combination. Therefore, inhibition of the JAK/STAT pathway through the use of ruxolitinib associated with bortezomib might, in theory, not only affect cellular signaling cascade through STAT proteins, but also routes the Ras/Raf/MAPK and PI3K/Akt/mTOR pathways since they are interconnected. The effect of the combination of bortezomib and ruxolitinib can be an alternative for those patients who do not respond

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well to currently available therapies and exhibit increased expression of *JAK1* and *JAK2*.

The aim of this study was to evaluate *JAK1* and *JAK2* expression in MM patients and the effects of JAK/STAT pathway inhibition on apoptosis, cell cycle, gene and protein expression in RPMI-8226 and U266 MM cell lines, with and without co-culture with normal stromal cells.

Materials and methods

Ethical aspects

The present study (0011/13) was approved by the Research Ethics Committee of the Federal University of São Paulo. Written consent was obtained from all patients.

Patients

Samples consisted of bone marrow aspirates from 30 newly diagnosed MM patients without prior chemotherapy, corticosteroids or bisphosphonates treatment. Three bone marrow donors were used as controls.

Cell lines

MM cell lines (RPMI-8226, U266, SKO-007 and SK-MM2) were cultivated in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 1% NEAA and 1% penicillin/streptomycin. The HS5 normal bone marrow cell line was cultivated in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 1% sodium pyruvate and 1% penicillin/streptomycin. All cell lines were maintained at 37 °C, in the presence of 5% CO_2.

RNA extraction

Extraction of RNA was carried out with the RNeasy Mini kit (Qiagen, Valencia, California, USA). Following the extraction, RNA was quantified by DS-11 spectrophotometer (Denovix, Wilmington, Delaware, USA).

cDNA synthesis

Two micrograms of total RNA obtained from patients bone marrow aspirates and MM cell lines were reverse transcribed with Superscript III reverse transcriptase (Invitrogen, Carlsbad, California, USA), following the manufacturer's instructions.

Real time PCR

The quantification of *JAK1* (Hs01026996_m1) and *JAK2* (Hs01078136_m1) expression was evaluated by real time PCR in the 7500 Real Time PCR System (Applied Biosystems, Foster City, California, USA). The constitutive gene *GAPDH* was chosen as the endogenous control. The $2^{-\Delta\Delta Ct}$ formula was used to calculate the relative expression of the target gene [12]. Expression of *JAK1* and *JAK2* in patient samples was considered as over and under expressed when there was an increase or decrease of relative gene expression, respectively, of at least two fold, compared to control samples.

Drug treatment

1×10^5 cells were treated with ruxolitinib (R) (Selleckchem, Houston, Texas, USA), bortezomib (B) (Jansen-Cilag, Beerse, Belgium), lenalidomide (L) (Nacto Pharma, Telegana, India) and dexamethasone (D) (Aché Laboratórios Farmacêuticos, São Paulo, Brazil), alone and in combination, for 24, 48 and 72 h, and analyzed by flow cytometry. Drug concentration chosen for the subsequent assays was the one able to induce approximately 50% of cell death, after single "in vitro" treatment. For sequential treatment, 1×10^5 RPMI-8226 and U266 cells were plated and treated with ruxolitinib or bortezomib alone. After 72 h, the second drug was added (for example, bortezomib was added to cells that had received ruxolitinib) and, after another 72 h, cells were analyzed by flow cytometry.

Flow cytometry

Cell death was assessed by flow cytometer BD FACSCaliburTM (Becton Dickinson, Franklin Lakes, NJ, USA). Cells were labeled with annexin V and/or propidium iodide (PI) (Becton Dickinson, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. Only the upper right quadrant was analyzed, which corresponds to the doubly labeled cells (annexin V and PI positive).

Real time PCR for intrinsic apoptosis pathway genes

Following synthesis of cDNA, a polyA tail was added to the cDNA (cDNA polyA), which was amplified using the PrimeSTAR® HS DNA Polymerase (2.5 U/μL - Takara Bio Inc., Kusatsu, Japan). Then, qPCR was performed as described before for the following genes: *TP53* (Hs010334249_m1), *CASP9* (Hs00154261_m1), *BAX* (Hs00180269_m1), *BAD* (Hs00188930_m1), *PUMA* (Hs00248075_m1), *NOXA* (Hs00560402_m1), *BCL2* (Hs00608023_m1), *MCL1* (Hs01050896_m1) and *BCL-XL* (Hs00236329_m1) in the 7500 Real Time PCR System (Applied Biosystems, Foster City, California, USA).

Cell cycle

After treatment of RPMI-8226 and U266 cells with ruxolitinib and bortezomib, cells were centrifuged at 2000 rpm for 2 min, resuspended in hypotonic solution of PI (20 mg of PI at 10 mg/mL, RNAse 20 mg/mL, PBS and Triton X) for 30 min at 37 °C and protected from light. The percentages of cells in SubG0, G0/G1, S and G2/M phases were identified by flow cytometry.

Co-culture of RPMI-8226 MM cells and HS5 stromal cells

The following drugs were used: ruxolitinib (R), bortezomib (B), lenalidomide (L) and dexamethasone (D). 1×10^5 HS5 cells were plated and, after 48 h, 1×10^5 RPMI-8226 cells were added to the wells. After 48 h, drug treatment was performed. After 72 h of treatment, plasma cells were sorted in a MACS device - Magnetic Cell Sorting of Human Cells (Miltenyi Biotec, Bergisch Gladbach, Germany). Tumor plasma cells (CD138 +) were labeled with anti-CD138 monoclonal antibody and, once isolated, were evaluated by flow cytometry.

PCR array

For each situation, analysis was performed in duplicates and one of the control plates (cells without treatment) was used as normalizer. The amount of cDNA used was 50 ng for each TaqMan® Array Human JAK/STAT Pathway, 96-Well Plate (Applied Biosystems, Foster City, California, USA), following the manufacturer's instructions.

Western blotting

1×10^6 RPMI-8226 and U266 cells were treated with a combination of ruxolitinib and bortezomib (R + B), and, after 24 h, protein extraction was carried out with the Total Protein Extraction Kit (Merck Millipore, Billerica, Massachusetts, USA), following the manufacturer's instructions. 25 μg of protein were separated by polyacrylamide gel electrophoresis using PROTEAN® Precast Mini Gel (Bio-Rad, Hercules, California, USA). Protein was transferred to a polyvinylidene membrane and the primary antibodies used were: Beclin-1 (3495), XBP-1s (12782) and α/β tubulin (2148) (for all primary antibodies: Cell Signaling, Danvers, Massachusetts, USA; dilution 1: 1000). The membrane was visualized in UVITEC Cambridge equipment (UVItec Limited, Cambridge, UK).

Statistical analysis

The data are presented as means + S.E.M (standard error of the mean) of at least three experiments performed independently, considering $p < 0.05$ as statistically significant. The correlation between clinical variables and *JAK1* and *JAK2* gene expression was performed using the Chi-square test of Pearson (X^2) or Mann-Whitney test. The overall survival curve was drawn using the Kaplan-Meier method, followed by Log-rank test. To compare *JAK1* and *JAK2* expression, and data from cell cycle and cell death, we used One Way ANOVA test with Bonferroni post-test. For the apoptosis intrinsic pathway genes expression, the statistical test used was Fisher's *t* test. For treatment analysis, we also used One Way ANOVA test with Bonferroni post-test.

Results

JAK1 and JAK2 gene expression

All four MM cell lines expressed *JAK1* and *JAK2* mRNA. RPMI-8226 had lower expression of *JAK1* when compared to U266 ($p < 0.05$), without statistically significant difference for *JAK2* expression (Fig. 1). SKO-007 had lower expression of *JAK1* when compared to RPMI-8226 ($p < 0.05$) and U266 ($p < 0.001$), while SK-MM2 had statistically significant lower expression for both genes when compared to RPMI-8226 and U266. Because of gene expression values, ease of cultivation and molecular background (see discussion), we chose RPMI-8226 and U266 cell lines to be used in the subsequent experiments. In order to confirm our cell line findings, we examined a panel of patient's samples. When *JAK1* and *JAK2* expression were analyzed in patients' samples, 8 (27%) showed overexpression of *JAK1* and 17 (57%), of *JAK2* (Fig. 2A), keeping with the cell lines findings and confirming a biological role for *JAK1* and *JAK2* in MM. To determine whether the mRNA level of *JAK1* and *JAK2* had prognostic significance, the expression data was correlated with clinical factors. For correlation between clinical