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ANALYSIS OF EXPANSION MESENCHYMAL STROMAL CELLS IN PATIENTS WITH LOW RISK MYELODYSPLASTIC SYNDROME

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ABSTRACT

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal hematopoietic disorders characterized by ineffective hematopoiesis, cytopenias and dysplasia and one or more lineages. The stratification of MDS is made based on the percentage of bone marrow blasts, number of cytopenias and karyotype at diagnosis. Somatic mutations in the p53 tumor suppressor gene are found in approximately 50% of all human tumors, making it the most commonly mutated gene. The expression of p53 protein and the study of mutations is especially needed in the prognosis of MDS. In this context, the study aims to evaluate the expansion of mesenchymal stromal cells (MSCs) and the expression of p53 protein in patients with SMD, low risk, according to the International Prognostic System (IPSS), in order to demonstrate the importance of these evaluations also diagnostics. This is a cross-sectional analytical study with review 3 adult patients of both sexes, the diagnosis of low-risk MDS receiving outpatient treatment at the University Hospital Walter Cantídio (HUWC). MSCs were characterized by immunophenotyping and screening of mutation of the p53 gene by Real Time PCR System (Applied Biosystems). For data analysis, the statistical software was used GraphPadPrism 5.0. Statistical differences between groups were checked by Student t or Mann-Whitney's test significance level was $p < 0.05$ for all analyzes. The results showed a smaller expansion of MSCs in the bone marrow of patients with MDS compared with a control group. A survey of mutation of the p53 gene was negative in all patients. The results demonstrate an impairment in the growth of MSCs in patients with MDS, collaborating with the hypothesis that medullary microenvironment in MDS may be compromised contributing greater understanding of disease mechanisms. However studies with larger sample should be conducted in order to establish the best results.

Key words: MDS; hematopoietic cells; mesenchymal cells; TP53 mutation.

INTRODUCTION

Mesenchymal stromal cells (MSCs) are a group of clonogenic, cells present in the bone marrow stroma, with potential to differentiate into various cell lineages. They propitiate the production and differentiation of hematopoietic stem cells in the bone microenvironment. In the bone marrow match 0.01% to 0.0001% [1,2]. MSCs are multipotent expressing positivity for CD73, CD90 and CD105 markers, and lack of expression of CD14, CD34, CD45, CD19, HLA-DR, CD3, CD11b, CD8, CD4, CD16 and CD56 in 95% of the cells in cultures. MSCs can be isolated from bone marrow by various methods, expandable pontencial maintaining their pluripotency and growth, with a doubling time which varies with the donor [3,4].

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of clonal hematopoietic disorders characterized by ineffective hematopoiesis, cytopenias and dysplasia and one or more lineages. The stratification of MDS is made based on the percentage of bone marrow blasts, number of cytopenias and karyotype at diagnosis. Somatic mutations in the p53 tumor suppressor gene are found in approximately 50% of all human tumors, making it the

most commonly mutated gene. The expression of p53 protein and the study of mutations is especially needed in the prognosis of MDS [4].

Several in vitro studies show that the bone marrow of patients with MDS has a high rate of cell proliferation and cell death (apoptosis). The paradox in a hypercellular marrow peripheral cytopenias in MDS can be attributed to several mechanisms, such as changes in its own hematopoietic cells, changes in the expression of molecules involved in apoptosis (Fas, Bcl-2, caspase), abnormalities in the cell cycle as well as presence of changes in the stroma [4,5] component.

The SMD has a high rate of ineffective hematopoiesis, manifested by anemia, neutropenia and / or thrombocytopenia. Besides the fact that the impairment also appears to occur in the bone marrow microenvironment, and MSCs. The ineffective hematopoiesis, is characterized by increased apoptosis, present in approximately 75% of patients with MDS [6,7,8].

In this context, this study aims to evaluate the expansion of MSCs in cultures of patients with low-risk MDS and compare with those of healthy donors. Moreover, determining the expression of p53 gene in patients with MDS MSCs.

CASUÍTICA AND METHODS

Casuistry

This is a cross section of 3 adult patients, two females and one male, the diagnosis of low-risk MDS in a clinical service specializing in Fortaleza - Ceará. Risk stratification was performed by the International Prognostic Scoring System Revised (IPSS-R). Patient samples were obtained from bone marrow, during the period January to December 2013. Clinical data related to age, sex, blood count, bone marrow biopsy and bone were collected for analysis of medical records. The inclusion criteria in this study were samples at diagnosis, free of any type of treatment and availability of suitable cells for analysis.

All samples were obtained only after patients or guardians agree to participate and sign the "Statement of Consent", approved by the Federal University of Ceará Research Ethics Committee of the University Hospital Walter Cantídio (HUWC).

The control group (n=4) of MSCs was obtained from the Cell Culture Laboratory and Molecular Analysis of Hematopoietic Cells, Center for Experimental Research / Hospital de Clinicas de Porto Alegre.

Isolation, cultivation and expansion of MSCs

The procedure for isolation, cultivation and expansion of MSCs was performed at the Laboratory of the Bank Umbilical Cord Blood Center of Ceará-Hemoce. The criteria adopted for the characterization of MSCs were those of the International Society for Cellular Therapy (ISCT) [9].

MSCs were isolated from bone marrow samples from patients with MDS (3 samples) and control subjects (6 samples) in culture medium poor in high concentrations of glucose and amino acids and proteins (fetal bovine serum). After counting the cells of the bone marrow aspirate about 1x 〖〗 10 ^ 6 cells / ml were subjected to culture in bottles of 25 cm ^ 2 in α-MEM medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with antibiotics and with 15% fetal bovine serum (fetal bovine Serum Standard - ^ TM HyClone, Logan, UT, USA). Cells were cultured in a humidified 37 ° C incubator with 5% CO 〖〗 _2. After 3 to 5 days, it was able to remove nonadherent cells and new culture medium added. Every 2 or 3 days, the medium was changed and the cell culture was maintained until reaching a confluence of 70-90%.

When they reach this confluence, MSCs were subjected to treatment with 1 ml of trypsin-EDTA 1x (0.05% Tripsin 0.53 mM EDTA, Gibco 〖〗 ^ TM Carlsbad, CA, USA) for 2-4 minutes at 37 ° C. After inactivation of trypsin, cell suspension was washed, resuspended in culture medium and plated at a density of 5x 10 〖〗 ^ 4 cells / cm ^ 2. Upon reaching the 3rd passage, the cells were subjected to the analyzes provided.

immunophenotyping

In flow cytometry, the cell suspension passes through a channel system which generates a laminar flow cell. A light beam hits these cells suffering deviation according to the physical characteristics of the same: cell size, granularity, internal complexity of the cell.

The monoclonal antibodies used is conjugated with three different fluorochromes: phycoerythrin (PE phycoeritrin the English), fluorescein isothiocyanate (FITC, fluorescein isothiocyanate English), PerCP (peridinin chlorophyll English). Positive and negative controls were included for proper calibration of the device, analyze the results and define the positivity of the sample.

The labeling of cells occurred after culturing MSCs reach the third pass, they were trypsinized, centrifuged, and the supernatant was discarded, leaving

approximately 1.5 mL of media then held for cell counting. To perform labeling cells with monoclonal antibodies it takes a minimum of 5x 〖〗 10 ^ 5 cells per tube, so after counting was performed in adjusting the final volume of cell suspension to that amount of cells were in a volume of 100 ul in which were added 5µl of a fluorochrome-labeled antibody (FITC, PE or PerCP). After addition of the antibody sample was incubated in the dark for 15 minutes, then washed with 1x PBS, centrifuged and supernatant discarded, the cell pellet was added 100 ul of 1x PBS. Once the cell suspension has been marked by the technique described, proceeded to the acquisition of fluorescence intensity in the cytometer.

Immunophenotyping of cells was performed using monoclonal antibodies which recognize antigens on the cell surface membrane. For the identification of these cells was assembled a panel containing the following markers CD105 PE (Serothec, Oxford, England), CD73 PE, CD45 FITC, CD14 PE, CD34 FITC, CD90 PE, CD13 PE (Becton Dickinson, San Jose, CA, USA), CD140B PE, CD146 PE and CD31 FITC.

The sequencing of the TP53 gene

Mutational analysis of the TP53 gene was performed in the Laboratory of Molecular Biology of the Transplant Center Bone Marrow (CEMO) Cancer Institute (INCA) in Rio de Janeiro, by direct sequencing. Exons 3 - 9 gene were amplified by PCR from DNA extracted from MSCs. The PCR primers and conditions for amplification of genomic DNA followed established by the International Agency for Research on Cancer (p53.iarc.fr/ProtocolsAndTools.aspx). All PCR products were confirmed by 1.5% agarose gel, purified using the Wizard SV Gel kits and PCR Clean-Up (both Promega) and sequenced by an automatic sequencer 16 capillaries (ABI PRISM ® 3100 Genetic Analyzer, Applied Biosystems). The sequence data files were analyzed using Mutation Surveyor (SoftGenetics) software. All variants were found compared with databases: Cosmic, dbSNP, and 1000 genomes UniProtKB

Statistical Analysis

Results were expressed as mean ± standard error of the mean. For data analysis, the statistical software was used GraphPadPrism 5.0. Statistical differences between groups were checked by Student t or Mann-Whitney tests. The level of significance was set at $p< 0.05$ for all analyzes.

RESULTS

A total of three patients with low-risk MDS were analyzed for the expansion of mesenchymal cells and compared with a control group consisting of individuals considered healthy. Of the three patients studied one being female 74 years old, diagnosed with SMD hypocellular variant hypocellular marrow and 0.8% blasts; bone marrow biopsy with 20% diseritropoese and dismegacariocitopoese and Normal reticulin; Karyotype 46, XX; immunohistochemistry for p53 and negative for CD34 positive megakaryocytes; IPSS intermediate 1 with good clinical outcome. Patient with 58 year old female with pancytopenia; hypocellular marrow with 4% blasts; with hypercellular bone marrow biopsy, 50% of diseritropoese and dismegacariopoese; karyotype 46XX. The male patient of 78 years; CRDM; IPSS intermediate 1; karyotype 46, XY, normocellular marrow with moderate and mild diseritropoese and disgranolopoese dismegacariopose and presence of 0.9% blasts; hypercellular bone marrow biopsy with diseritropoese, disgranulopoese and dismegacariopoese and reticulin grade 1; immunohistochemistry for p53 positive focal nuclear pattern.

TABLE 1 - Clinical characteristics of patients with myelodysplastic syndrome diagnosis (n = 3)

VARIABLES	PATIENT 1	PATIENT 2	PATIENT 3
Age (years)	78	54	78
Gender	Female	Male	Female
Cytogenetics, n (%)	Karyotype Normal	Karyotype Normal	karyotype Normal
IPSS	Intermediate 1	Intermediate 1	Intermediate 1
IPSS- R	Low	Low	Low
hematological Prâmetros			
RBC /1012/L	3.72	3.22	3.81
Hemoglobin, g/dL	10.8	10.6	12.1
Hematocrit, %.	32.7	31.0	34.7
leukocytes /L	3.700	2.924	3273
Platelet/L	116.000	42090	49530

In Figure 1 we can see confirmation of the origin of MSCs through the characteristic profile by immunophenotyping.

In relation to research the expansion of mesenchymal cells in patients compared to the control group we observed a significant decrease in the group of MDS patients compared to the control group. The analysis of mutations in the p53 gene was negative in patients with MDS MSCs.

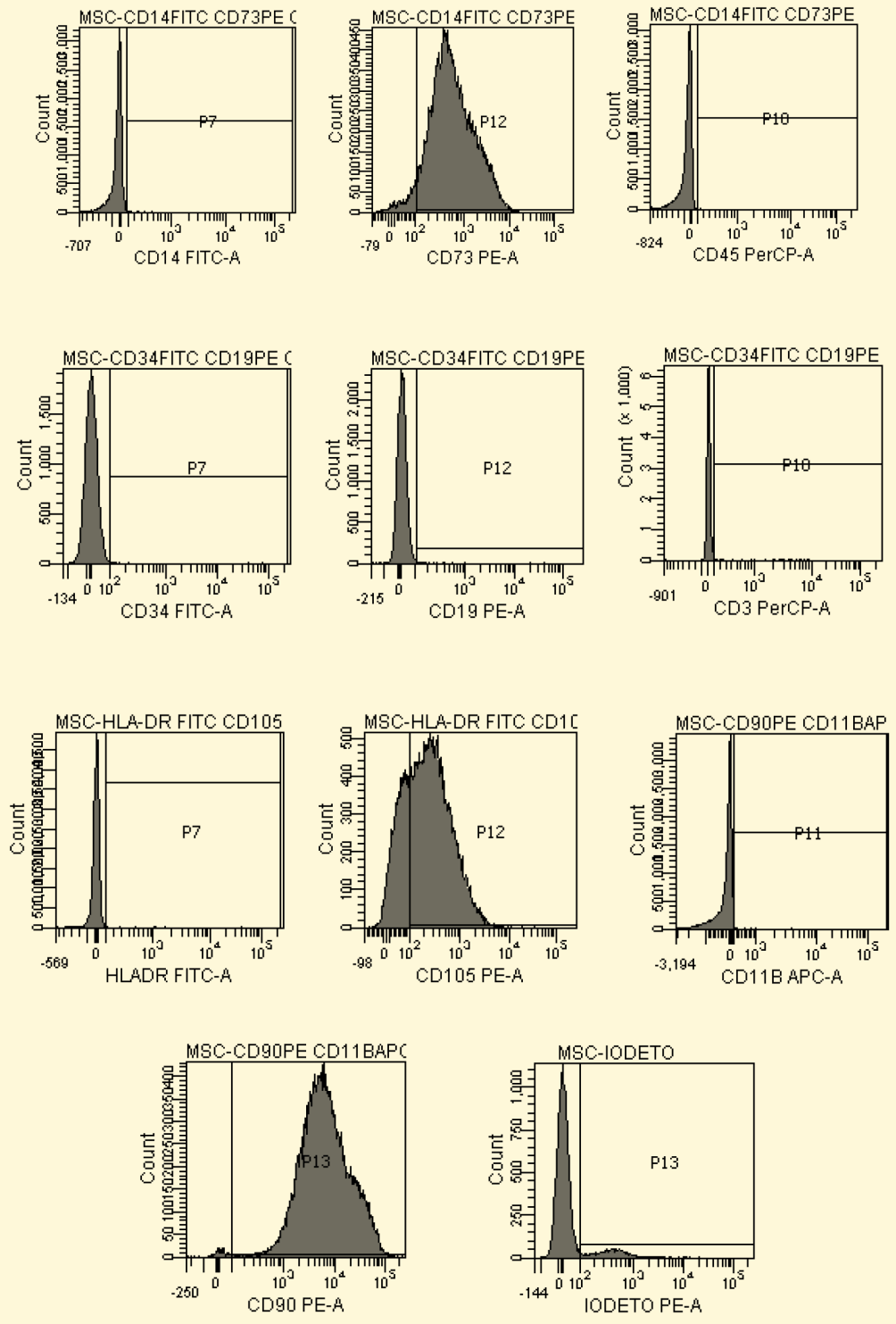

FIGURE 1 - Phenotypic analysis of MSCs in patients with low-risk MDS (n = 3). Feasibility: 89.7% (10.3% dead cells)

TABLE 2 - Analysis of expansion of MSCs in patients with low-risk myelodysplastic syndrome and apparently healthy individuals

MONONUCLEAR CELLS RECOVERED FROM THE BAG AND FILTER		p0	p1	p2	p3
CONTROL	15.700.000	1.099.000	48.081.250	9.676.351.563	84.635.821.667
CONTROL	150.000.000	12.400.000	640.666.667	125.730.833.333	541.480.788.889
CONTROL	28.000.000	6.981.333	1.087.924.444	381.226.857.407	2.328.025.342.568
CONTROL	194.000.000	19.788.000	1.261.485.000	147.698.868.750	746.371.616.750
CONTROL	7.400.000	740.000	46.250.000	12.738.020.833	
CONTROL	304.500.000	23.548.000	1.138.153.333	314.414.858.333	
PATIENT	1.075.000	2.200.000	6.306.667	40.867.200	250.652.160
PATIENT	4.060.000	4.300.000	32.480.000	329.130.667	3.774.031.644

TABLE 3 - Characterization of mutation of TP53 in MSCs in patients with low-risk MDS (n = 3)

PATIENT	MUTATION IN TP53
1	Absent
2	Absent
3	absent

DISCUSSION

In culture, MSCs are a population of cells with the morphological appearance of fibroblasts, adherent to plastic. The half-life is limited, with an average doubling time of 33 hours and a maximum overlap of about 40. Expands As the number lost their multipotential capacity and undergo apoptosis. The cell cycle studies in cultured human MSC show that while a small fraction of these cells proliferating (approximately 10% of cells are in S + G2 + M phase) are most cells in the G0/G1 phase, comprising a minority of resting cells [10,11].

Some aspects regarding the interactions between the neoplastic clone and the bone microenvironment has been rumored as one of the mechanisms of the pathophysiology of MDS. However, studies on the subject are scarce and therefore requiring research characterizing the bone marrow stromal cells in healthy individuals and in patients with malignant hematological diseases [12].

The development of MDS is a complex process, for which we propose a model with successive steps. In this model, an abnormal clone could interact with hematopoietic marrow microenvironment providing the altered neoplastic growth with normal shifting [13] hematopoiesis.

Studies evaluating the functionality and molecular phenotyping aspect of MSCs in patients with MDS have been documented. However the results are conflicting. In this study the degree of purity of MSCs was 89.7% of the cells present in the sample, we can affirm that the data obtained are in effect for these cells. We found that the pattern of growth of MSCs in patients with low-risk MDS was different from healthy subjects. There was a significant reduction in the MSCs expanssão of MDS patients compared to healthy bone marrow. The growth pattern of MSCs is controversial because some studies have described altered expansion [11,14], while others have observed a similar growth of normal bone marrow [15] standard. The discrepancies may result attributed to the large variation in the growth of MSC in MDS subtypes or methodological used, among others.

Regarding the immunohistochemical study of MSCs found that there was no difference in the pattern of patients with low-risk MDS, relative to healthy individuals. These results corroborate with the literature, which state that most studies agree that MSCs from MDS patients are identical to normal [2,15] markers. Studies, but has shown that the expression of CD90, CD104 and lower CD105é MSCs in MDS patients [4,10,11]. Finding attributed to alteration of the marrow stroma and hematopoietic cells.

Regarding the analysis of mutation of p53 gene mutation was not observed in MCSs in patients with MDS. Additional studies are needed to elucidate the mechanisms involved in the regulation of MCSs in MDS, so that we can establish the prognostic value of MCSs, the pathophysiology in this disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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