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ScienceDirect

Leukemia Research 31 (2007) 579–589

Leukemia Research

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Gene stage-specific expression in the microenvironment of pediatric myelodysplastic syndromes

Rosimeire A. Roela [a], Dirce M. Carraro [b], Helena P. Brentani [b], Jane H.L. Kaiano [b], Daniel F. Simão [b], Roberto Guarnieiro [c], Luiz Fernando Lopes [b], Radovan Borojevic [d], M. Mitzi Brentani [a,∗]

[a] *Faculdade de Medicina da Universidade de São Paulo, Departamento de Radiologia (LIM24), Av Dr Arnaldo 455 sala 4112, São Paulo 01246903, Brazil*

[b] *Centro de Tratamento e Pesquisa, Hospital do Câncer, São Paulo, Brazil*

[c] *Instituto de Ortopedia e Traumatologia, Hospital das Clínicas da FMUSP, São Paulo, Brazil*

[d] *Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Received 31 July 2006; received in revised form 11 September 2006; accepted 10 October 2006
Available online 7 November 2006

Abstract

Using cDNA microarray assays we have observed a clear difference in the gene expression pattern between bone marrow stromal cells obtained from healthy children (CT) and from pediatric patients with either myelodysplastic syndromes (MDS) or acute myeloid leukemia (AML) associated with MDS (MDS–AML). The global gene function profiling analysis indicated that in the pediatric MDS microenvironment the disease stages may be characterized mainly by underexpression of genes associated with biological processes such as transport. Furthermore, a subset of downregulated genes related to endocytosis and protein secretion was able to discriminate MDS from MDS–AML.
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Keywords: Pediatric myelodysplastic syndromes; Hematopoetic microenvironment; Gene profile; Bone marrow stromal cells; Acute myeloid leukemia associated with myelodysplastic syndromes; Gene stage-specific expression

1. Introduction

The myelodysplastic syndromes (MDS) are a group of clonal stem cell disorders characterized by cytopenia, ineffective hematopoiesis and hypercellular bone marrow [1,2]. Overall, MDS is the most common hematological malignancy in the elderly [3]. In children the incidence is lower but effective treatment of both adults and children in the advanced stages of the disease is problematic [3,4]. The clinical course of MDS can be divided into several distinct phases related to the percentage of leukemic blasts in bone marrow. In the early, indolent stage, affected individuals manifest only cytopenia. This phase is referred to as refractory anemia (RA), and the 5–20% increase of leukemic blasts characterizes patients with RA with excess of blasts (RAEB). Patients with a percentage of blasts higher than 20% are diagnosed as having MDS-associated leukemia [5].

The development of an effective treatment for MDS will require the characterization of the molecular mechanism that underlies stage progression [4]. Many chromosomal abnormalities (e.g. 5q- and monosomy 7) have been detected in adult MDS patients and several studies of gene profile by cDNA microarray have been conducted on hematopoietic cells from adult MDS patients and the specific changes identified are likely to be biologically important markers for the various stages of this disorder [2,3].

The healthy hematopoietic microenvironment is composed of bone marrow stromal cells which are mainly fibroblasts, adipocytes, endothelial cells and macrophages and their secreted extracellular matrices, growth factors and cytokines [6]. This environment organized in differentiation

∗ Corresponding author. Tel.: +55 11 3082 6580; fax: +55 11 3082 6580.
E-mail address: mbrentani@lim24.fm.usp.br (M.M. Brentani).

0145-2126/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2006.10.007

niches contributes to regulate the self-renewal, commitment, differentiation, and proliferation of stem cells and hematopoietic progenitors [7]. However, the contribution by the non-hematopoietic medullar compartment or the hematopoietic microenvironment to the pathogenesis of MDS remains to be clarified.

In vitro studies of the functional integrity of the hematopoietic microenvironment in MDS have been controversial. Although, some of them suggested that this microenvironment is functionally normal [8,9], there is increasing evidence indicating that there are alterations in the function of microenvironment cell layers from MDS marrow of adult patients [10]. Moreover, bone marrow mesenchymal stem cells from MDS patients seem to be cytogenetically abnormal [11,12]. In agreement with this evidence, we have previously reported that a fibroblast-enriched cell layer derived from the bone marrow of MDS children displayed alterations in supporting the growth of hematopoietic stem cells (CD34+) as compared to bone marrow stromal cells from healthy controls. In addition, we have detected differences in gene profiling determined by cDNA array analysis between normal and MDS marrow fibroblasts. Deregulated genes were mainly associated with the extracellular matrix [13]. The aim of the present study was to perform a comparative large-scale gene expression analysis of bone marrow stromal cells from healthy children and patients with pediatric MDS or acute myeloid leukemia (AML) associated with MDS (MDS–AML) in order to understand the molecular complexity of the hematopoietic microenvironment that might contribute to the development and/or progression of MDS.

2. Patients, materials and methods

2.1. Patients

Local ethics committee approval (informed consent in accordance with the Helsinki protocol) was obtained for the use of patients' bone marrow aspirates from the cancer treatment and Research Center, Hospital do Câncer, and of normal donor samples from Instituto de Ortopedia e Traumatologia, Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo. Bone marrow aspirates were obtained from the posterior iliac crest of one healthy adult (reference of cDNA microarray), of 6 patients (5 males and 1 female ranging in age from 1 to 12 years) at the time of diagnosis and of three healthy age-matched children (all males) submitted to corrective orthopedic surgeries defined from now on as controls (CT). Four patients were typed as MDS (all RAEB) and two as MDS–AML according to FAB classification criteria elaborated by the Pathology Committee of the Brazilian Cooperative Group on Pediatric Patients with Myelodysplastic Syndrome (BCG-PED-MDS). Two MDS and all MDS–AML patients presented Down syndrome.

2.2. Primary cell culture

Mononuclear cells were isolated from bone marrow aspirates and plated onto Iscove's medium (Sigma, St. Louis, MO, USA) supplemented with 20% fetal bovine serum (FBS, Invitrogen Life Technologies, Carlsbad, CA, USA) and antibiotics. The cells from the adherent fraction were classified as myofibroblasts by the expression of muscle α-actin, collagen IV, laminin and fibronectin after the third passage. These cells were considered to be free of malignant hematopoietic clones such as macrophages which are trypsin-resistant, as previously reported by our group [13].

2.3. cDNA microarray assembly, hybridization and analysis

Open reading frame-expressed sequence tags (ORESTES) representing 4608 human genes were selected from the Human Cancer Genome Project (HCGP) bank (Fundação de Amparo à Pesquisa do Estado de São Paulo FAPESP/Instituto Ludwig de Pesquisa sobre o Câncer) and immobilized in a customized cDNA platform [14]. Inserts were amplified by PCR using M13 reverse and forward primers from the cDNA clones. Amplicons purified by gel filtration and clones were printed as three or six replicates onto Corning slides using a Flexys Robot (Genomic Solutions, Ann Arbor, MI, USA) at the Ludwig Institute for Cancer Research, São Paulo, Brazil. A total of 192 reference sequences were also spotted on the slides. This cDNA microarray platform, complying with MIAME format, was submitted to the Gene Expression Omnibus (GEO) data repository under the accession number GPL1930 (www.ncbi.nlm.nih.gov/projects/geo).

Total RNA from patient and normal donor bone marrow stromal cell primary cultures were isolated using Trizol reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. RNA quality was verified by agarose gel electrophoresis (Sigma) l upon visualization with ethidium bromide (Sigma). Only RNA samples with a ratio > 1 for 28S/18S ribosomal RNA were further processed. A two-round RNA amplification procedure was carried out by combining antisense RNA amplification with a template-switching effect according to a previously described protocol [15,16]. At the start, 3 μg total RNA was used to yield around 60 μg of amplified RNA (aRNA). Three to 5 μg aRNA was then used in a reverse transcriptase reaction in the presence of Cy3- or Cy5-labeled dCTP (GE Heathcare, Piscataway, NJ, USA) and SuperScript II (Invitrogen Life Technologies). Normal bone marrow stromal cells from an adult donor were used as reference for hybridizations.

Equal amounts of MDS, MDS–AML or CT samples and reference cDNA labeled probes were concurrently hybridized against cDNA microarray slides. Dye swap was performed for each sample analyzed to control for dye bias. Pre-hybridization was carried out in a humidified chamber at

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