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Stromal cells attenuate the cytotoxicity of imatinib on Philadelphia chromosome-positive leukemia cells by up-regulating the VE-cadherin/β-catenin signal

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ABSTRACT

β-Catenin is a key regulator of leukemia stem cell maintenance and drug resistance. Herein, we investigated the protective effects of the stromal cell-mediated VE-cadherin-β-catenin signal on Ph+ leukemia cells during imatinib treatment. We found stromal cells could desensitize imatinib and up-regulate VE-cadherin expression on Ph+ leukemia cells (K562 and SUP-B15 cells), which further stabilized and activated β-catenin. Knockdown of VE-cadherin with shRNA diminished the β-catenin protein and partly resensitized Ph+ leukemia cells to imatinib despite the presence of stromal cells, suggesting VE-cadherin is a potential target in the treatment of Ph+ leukemia.

1. Introduction

Philadelphia chromosome-positive (Ph+) acute leukemia, including chronic myeloid leukemia (CML) blast crisis and Ph+ acute lymphoblastic leukemia, are common hematopoietic malignancies with poor prognoses [1]. The CML blast crisis is highly refractory to treatment. The rate of response to chemotherapy in patients with myeloid blast crisis is approximately 20%, and the rate of complete remission is less than 10%. In patients with lymphoid blast crisis, the rate of response is approximately 50%, but remissions are short-lived. Patients with Ph+ acute lymphoblastic leukemia have slightly lower complete remission rates compared with patients with Ph-negative ALL but have markedly reduced remission durations (less than one year in average), and few if any such patients have been cured with conventional chemotherapy [2]. The causative molecular event in Ph+ leukemia is the transposition of ABL and BCR sequences to form a BCR-ABL fusion gene, leading to the expression of a constitutively active ABL protein-tyrosine kinase [3]. An ABL protein-tyrosine kinase inhibitor (TKI), imatinib, the first molecule-targeting drug, was introduced into clinical practice approximately 10 years ago and has proven to be very effective at killing Ph+ acute leukemia cell lines and inhibiting BCR-ABL transformed cells in vitro [4–6]. However, the response rate for patients with Ph+ acute leukemia is lower than that of patients with chronic phase CML when imatinib is used as a single agent, and Ph+ acute leukemia patients had a higher relapse rate [7].

The low response to imatinib and high relapse rate of patients with Ph+ acute leukemia indicates that Ph+ acute leukemia cells are relatively resistant to imatinib in vivo, although these cells are sensitive to imatinib in vitro. Several mechanisms of resistance against imatinib have been reported in patients with CML, including mutations in the tyrosine kinase domain and up-regulation of BCR-ABL transcription [8,9]. However, neither mutation nor amplification of BCR-ABL is detected in a significant number of patients. Second-generation TKIs, such as dasatinib [10], nilotinib [11] and bafetinib, potentially overcome most imatinib resistance mechanisms. However, like imatinib, they are less effective against quiescent leukemia stem cells (LSC) [12].

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Given its key roles in mediating the establishment of drug resistance [13] and regulating LSC maintenance, quiescence and proliferation [14], β-catenin is a transcription co-factor with T cell factor/lymphoid enhancer factor (TCF/LEF) [15] in the Wnt pathway and an adaptor protein that binds to the cadherins. The serine kinase GSK-3 phosphorylates β-catenin at Ser33, Ser37 and Thr41, leading to the ubiquntilization and degradation of β-catenin. The cytoplasmic domain of cadherins regulates the structural integrity and function of the cadherin complex by binding to β-catenin through α-catenin and the actin cytoskeleton or by binding to p210-catinin [16]. The phosphorylation of β-catenin at Tyr142 or the tyrosine phosphorylation of the intracellular domain of cadherin by Src family kinases, alters the binding affinity of β-catenin to the cytoplasmic domain of cadherins. VE-cadherin is one of the classic cadherins; it is primarily expressed in endothelial cell adherens junctions and controls the hematopoietic stem cell (HSC) niche and hematopoiesis [17]. Wang et al. [18] reported that VE-cadherin is a stem cell marker of Ph+ leukemia cells. VE-cadherin can directly bind and stabilize β-catenin. The interplay between VE-cadherin and β-catenin may modulate the stem cell properties of LSCs and the establishment of drug resistance.

Stromal cells can protect leukemia cells during chemotherapy via VCAM-1 mediated cell-cell contact [19], AKT pathway activation [20] and the secretion of chemokines and cytokines. Previous data have shown that stromal cells arrest leukemia cells in the G0/G1 phase of the cell cycle [21]. However, the mechanisms of this issue remain to be elucidated. Our data showed that Ph+ leukemia cells present a low level of VE-cadherin expression and that stromal cells regulate the survival of Ph+ leukemia cells during imatinib treatment by up-regulating the expression of VE-cadherin, further increasing the β-catenin level and activating the β-catenin signal. These observations suggest a novel mechanism for bone marrow stromal cells in maintaining leukemia cells during exposure to imatinib.

2. Materials and methods

2.1. Co-culture of stromal and leukemia cells

The Ph+ leukemia cell line SUP-B15 was obtained from ATCC (Manassas, VA, USA). The K562 leukemia cell line and the mouse bone marrow stromal cell line OP-9 was provided by the Cell Bank of Chinese Academy of Science (Shanghai, China). The lentivirus packing cell line 293FT was purchased from Invitrogen (Carlbad, CA, USA). Human bone marrow stromal cells (HBMSCs) were isolated and expanded as described in the supplemental data. The leukemia cells were maintained in IMDM medium with 10% FBS. The OP-9 bone marrow stromal cells and HBMSCs were cultured in α-MEM medium with 10% FBS. To establish a co-culture of stromal and leukemia cells, SUP-B15 or K562 cells were seeded onto 70% confluent stromal cells and maintained by subculturing a portion of SUP-B15 or K562 cells onto new stromal cells weekly for more than 2 months.

2.2. Flow cytometry

Anti-CD34 (8G12), CD38 (HB7), CD19 (HIB19), VE-cadherin (55-7H1) and Ki-67 (B56) antibodies were purchased from BD Pharmingen (San Diego, CA, USA). All the antigens were labeled according to the manufacturer's instructions. K562 cells were gated with anti-human CD33 antibody, SUP-B15 cells were gated with anti-human CD19 antibody, and nucleic acid dye 7-AAD (eBioscience, San Diego, CA, USA) was used to determine cell viability. The samples were analyzed on a FACS Calibur flow cytometer (BD Bioscience, San Jose, CA, USA) using CellQuest Pro software (BD Bioscience). For each sample, at least 2×10^5 events were acquired for downstream analysis.

2.3. Production of lentiviral stocks and knockdown of VE-cadherin by shRNA

Sequences targeting the mouse VE-cadherin shRNAs were cloned into the GFP carrying pLB lentiviral vector, and the vectors containing sequences targeting VE-cadherin shRNAs were co-transfected with pSPXA2 (*gag/pol and rev*) and pMD2.G (VSV-G, vesicular stomatitis virus-G pseudotype) plasmids into 293FT packaging cells (Invitrogen) using lipofectamine 2000. The supernatant containing lentiviral particles was collected after an additional 48-hr incubation, filtered through 0.45-μm filters to remove cell debris and further concentrated via ultracentrifugation using a SW32 rotor (Beckman, Palo Alto, CA, USA) at 70,000 × *g* and 4 °C for 2 h. K562 or SUP-B15 leukemia cells were seeded into 6-well plates; concentrated virus was added, and the cells were spun down at 2000 rpm for 2 h in the presence of 6 μg/ml polybrene (Sigma). After 7 days of virus infection, the GFP+ cells were sorted using FACSAriaIII flow cytometry (BD Bioscience).

2.4. Stable overexpression of VE-cadherin in Ph+ leukemia cells

The coding DNA sequences of human VE-cadherin were amplified from the cDNA of human umbilical vein endothelial cells (Millipore, Temecula, CA, USA) and inserted downstream of the CMV promoter of the pCDNA3.1+ vector. K562 and SUP-B15 leukemia cells were electrotransfected using a Neon transfection system (Invitogene). After transfection, stable-transfected cells were selected with 700 μg/ml G418 for 2 months.

2.5. Cell proliferation assay

Cell proliferation was evaluated using the conversion of the water-soluble tetrazolium salt WST-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan) to formazan. Briefly, 96-well plates were seeded with 20,000 cells/well. After culture, 10 μl of CCK-8 solution were added to each well. The samples were incubated at 37 °C for 4 h, and the absorbance was measured at 450 nm using a microplate reader (Thermo Multiskan MK3, Waltham, USA).

2.6. TOP/FOP Dual-luciferase reporter assay

The activity of β-catenin was determined with a TOP/FOP dual-luciferase reporter assay. The FOPflash and TOPflash TCF reporter plasmids were obtained from Millipore. K562 and SUP-B15 leukemic cells were electrotransfected with FOPflash or TOPflash TCF Firefly luciferase reporter plasmids, and transfection efficiencies were determined via the co-transfection of pRL-TK Renilla luciferase control plasmid (Promega, Madison, WI, USA). Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) on a GloMax96 luminometer (Promega). The activity of β-catenin was indicated by the ratio of TOPflash: FOPflash luciferase activities, while the Renilla luciferase activity was used to correct for differences in transfection efficiency.

2.7. Leukemic cell adhesion assay

K562 or SUP-B15 leukemic cells were stained with Calcein AM (Invitrogen) according to the manufacturer's protocol. Then, leukemic cells were added to 96-well plates coated with OP-9 cells or HBMSC stromal cells. The cells were incubated for 30 min at 37 °C. After incubation, the "before wash" fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Non-adherent cells were removed after 3 washings, and the "after wash" fluorescence was measured. The percentage of adhesion was calculated using the following formula: [(fluorescence after wash)/(fluorescence before wash)] × 100%.

2.8. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using Trizol Reagent (Invitrogen), and cDNA was synthesized using an iScript Reverse Transcription kit (Bio-Rad, Hercules, CA, USA). The mRNA expression was analyzed using qRT-PCR in triplicate with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) in an ABI 7500 Realtime PCR system, and the data were normalized to the β-actin expression level using the standard curve method.

2.9. Immunoblot and co-immunoprecipitation analysis

The cells were lysed in ProteoJET mammalian cell lysis solution (Fermentas, Vilnius, Lithuania) supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). For cytoplasmic and nuclear protein extraction, the cells were treated using a ProteoJET nuclear protein extraction kit (Fermentas) according to the manufacturer's instructions. The samples were clarified, denatured with SDS buffer and boiled for 5 min. The proteins were separated using SDS-PAGE and transferred onto nitrocellulose membranes. Proteins were detected using the following antibodies: VE-cadherin (D87F2, 1:1000, Cell Signaling Technology, Danvers, MA, USA), β-catenin (D10A8, 1:1000, Cell Signaling Technology), Histone H3.1 (1:500, Bioworld, Nanjing, China), CD133 (Abcam, 1:1000, Cambridge UK), β-tubulin (1:1000, Cell Signaling Technology) and β-actin (1:5000, Bioworld), followed by incubation with peroxidase-conjugated secondary antibodies (Sigma–Aldrich, 1:80,000) and chemiluminescence detection using X-ray film. For immunoprecipitation, whole-cell lysates were precleaned with protein G beads and then were incubated overnight at 4 °C with anti-VE-cadherin; then, protein A/G beads (Merck Calbiochem, Darmstadt, Germany) were added, followed by additional 1-h incubation. Immunocomplexes were extensively washed and then resuspended and boiled in SDS loading buffer.