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Imatinib restores VASP activity and its interaction with Zyxin in BCR–ABL leukemic cells

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ABSTRACT

Vasodilator-stimulated phosphoprotein (VASP) and Zyxin are interacting proteins involved in cellular adhesion and motility. PKA phosphorylates VASP at serine 157, regulating VASP cellular functions. VASP interacts with ABL and is a substrate of the BCR–ABL oncoprotein. The presence of BCR–ABL protein drives oncogenesis in patients with chronic myeloid leukemia (CML) due to a constitutive activation of tyrosine kinase activity. However, the function of VASP and Zyxin in BCR–ABL pathway and the role of VASP in CML cells remain unknown. *In vitro* experiments using K562 cells showed the involvement of VASP in BCR–ABL signaling. VASP and Zyxin inhibition decreased the expression of anti-apoptotic proteins, BCL2 and BCL-XL. Imatinib induced an increase in phosphorylation at Ser157 of VASP and decreased VASP and BCR–ABL interaction. VASP did not interact with Zyxin in K562 cells; however, after Imatinib treatment, this interaction was restored. Corroborating our data, we demonstrated the absence of phosphorylation at Ser157 in VASP in the bone marrow of CML patients, in contrast to healthy donors. Phosphorylation of VASP on Ser157 was restored in Imatinib responsive patients though not in the resistant patients. Therefore, we herein identified a possible role of VASP in CML pathogenesis, through the regulation of BCR–ABL effector proteins or the absence of phosphorylation at Ser157 in VASP.

1. Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative neoplasm of the hematopoietic stem cell, characterized by the presence of the fusion protein breakpoint cluster region-abelson (BCR–ABL), an oncoprotein with a constitutively tyrosine kinase activity. Significant efforts are being made to understand the molecular mechanisms of BCR–ABL signaling pathway [1]. Imatinib mesylate (Gleevec/Glivec, STI571) is a specific BCR–ABL tyrosine kinase inhibitor that can lead towards the elimination of the clone of BCR–ABL positive cells [2]; however there are patients that fail to respond to this treatment. Thus, the combination of tyrosine kinase inhibitors and other targets has been intensified in an attempt to induce apoptosis of resistant CML cells, including the strategies targeting BCL-2 protein family [3–6]. The cross-talk between the aberrant BCR–ABL signaling and BCL2 protein family has been described and has generated new therapeutic opportunities [7].

Several mechanisms influence disease evolution leading to uncontrolled cell proliferation, resistance to apoptotic and altered migration of CML cells. BCR–ABL oncoprotein induces multiple abnormalities of cytoskeletal function, resulting in an increased mobility and a reduced adhesion of leukemic cells to bone marrow stroma [8,9]. Dysregulation of actin polymerization and the depolymerization process are often associated with tumor development [10–12].

Cytoskeleton protein activity and the actin reorganization are essential to a variety of cellular processes including cell motility, cell division, cellular differentiation, and establishment and maintenance of cell adhesion [13,14]. VASP (vasodilator-stimulated phosphoprotein) and Zyxin are two important actin regulatory proteins; that act as a protein complex involved in driving actin dynamics in the context of cell division and the formation and maintenance of cell junctions [15,16]. Zyxin directs actin assembly by recruiting VASP to specific sites of adhesion [17,18].

VASP is a member of the Ena/VASP protein family. These family members share a conserved structure consisting of an N-terminal homology EVH1 domain, a central poly-proline region (PPR) and a C-terminal EVH2 domain [19]. The C-terminal of Zyxin has an N-terminal proline-rich domain and three LIM domains. The proline-rich domain may interact with SH3 domains of proteins involved in signal transduction pathways while the LIM domains are probably involved in protein-protein binding [19]. VASP interacts with Zyxin through the proline-rich region or through the LIM domain, though

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only the interaction with the LIM region is dependent on VASP's phosphorylation state [20]. VASP is a substrate of cAMP-dependent and cGMP-dependent protein kinase and phosphorylation at Ser157 in VASP regulates its interaction with actin [21]. In addition, Zyxin phosphorylation has been proposed to modulate its head–tail interaction to alter protein binding and thus cell–cell adhesion [16,22].

Altered expression of VASP and its partner Zyxin has been previously described in a variety of epithelial tumors [23–26]; however little is known regarding their involvement in hematopoietic tumors, particularly CML. VASP homologues in Drosophila were identified as substrates of the tyrosine kinase ABL [27]. In mammals, the physical interaction of VASP with ABL and its tyrosine phosphorylation were both demonstrated [28]. However, the connection of VASP and Zyxin to BCR–ABL signaling pathway in the context of CML remains unclear. In addition, the phosphorylation of VASP on Ser157 in CML leukemic cells and how this phosphorylation is affected by Imatinib treatment are not yet known. Herein we described VASP and Zyxin interaction in K562 cells before and after Imatinib treatment and phosphorylation of VASP on Ser157 status in K562 cells and in CML patient bone marrow cells. We characterized VASP and Zyxin participation and action in downstream effectors of BCR–ABL signaling.

2. Materials and methods

2.1. Bone marrow samples

Bone marrow samples were collected from 5 individuals: 1 healthy donor, 1 patient with a diagnosis of chronic myelogenous leukemia (CML), 1 responsive patient in major molecular remission treated with tyrosine kinase inhibitors and 2 patients resistant to treatment. Samples were collected between 2011 and 2012 after informed written consent and the study was approved by the Ethics Committee of the University of Campinas. Total cells from bone marrow samples were obtained after removing erythrocytes by hemolysis.

2.2. Cell culture and reagent chemicals

K562 cells, which are known as a representative human CML cell line, were obtained from ATCC, Philadelphia, PA, USA. Cells were cultured in RPMI containing 10% fetal bovine serum (FBS) and glutamine with penicillin/streptomycin and amphotericin B, and maintained at 37 °C, 5% CO_2. Imatinib mesylate was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland) and prepared as a 50 mM stock solution in dimethyl sulfoxide (Me_2SO_4; DMSO) and used at final concentrations of 0.1, 0.5 or 1 μM, as indicated.

2.3. Lentiviral vectors

K562 cells were transduced with lentivirus-mediated shRNA nonspecific control (sc-108080) or lentivirus-mediated shRNA targeting VASP (shVASP; sc-29516-V) and Zyxin (shZyxin; sc-36370-V) from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, 2×10^5 cells were transduced with lentiviral by spinoculation at multiplicity of infection equal to 3 and selected by puromycin (1.75 μg/mL).

2.4. RNA extraction and Reverse Transcription

Total RNA was extracted using the Trizol® reagent (Invitrogen) according to the manufacturer's instructions. Single stranded cDNA was synthesized from 2 μg of the total RNA preparation using SuperScript® III Reverse Transcriptase kit (Invitrogen Life Technologies).

2.5. Quantitative polymerase chain reaction

Quantitative PCR (qPCR) was performed using ABI 7500 Sequence Detector System (Applied Biosystems, Foster City, CA, USA) with specific primers for *VASP*, *Zyxin* and *HPRT*. Primer sequences and concentrations are described in Supplementary Table 1. The relative gene expression was calculated using the equation, $2^{-\Delta\Delta CT}$ [29].

2.6. Immunoblotting

Pelleted cells were resuspended in RIPA buffer, incubated for 45 min at 4 °C and centrifuged for 30 min at 12,000 rpm at 4 °C. The same amount of protein was loaded on SDS-PAGE and blots were probed with the indicated antibodies and ECL Western Blot Analysis System (Amersham Pharmacia Biotech, UK). Antibodies against ABL (sc-23), p-ERK (sc-7383), P70S6K (sc-8418), CRKL (sc-319), BAX (sc-20067), Actin (sc-1616), p-P70S6K (sc-7984), BCL-XL (sc-8392), BCL2 (sc-492), p-BAD (sc-7999), BAD (sc-943), p-STAT 5 (C11C5), STAT 5 (sc-835), FAK (sc-558), BAK (sc-G23) and VASP (sc-1853) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-CRKL (3181), p-p130 CAS (4011), p130 CAS (12015), p-VASP (3111), and BIM (2819) were from Cell Signaling Technology (Cell Signaling, Danvers, MA, USA) and Anti-ERK1/2 (13-6200) was from Zymed (Invitrogen, Carlsbad, CA, USA). Antibodies against p-JNK (44690G) and JNK (446826) were from Invitrogen and antibody against p-FAK Y397 (ab4803), p-FAK Y576/577 (ab76244), p-FAK Y861 (ab38458), p-FAK Y925 (ab38512), p-Zyxin (ab78910) and Zyxin (ab58210) was from Abcam (Cambridge, MA, USA). The membranes were stripped and reprobed with antibodies for detection of the respective total proteins and of Actin as control of loading.

2.7. Immunoprecipitation

K562 cell lysates were prepared in RIPA buffer containing protease inhibitors, as previously described [30]. Briefly, 500 μg of total K562 cell extracts was incubated overnight with 20 μL anti-VASP antibody (Santa Cruz Biotechnology) or with normal goat immunoglobulin (IgG) as a negative control. The immune complexes were precipitated with protein-G-sepharose 50% slurry (GE), washed in RIPA buffer to remove unspecific proteins, and then analyzed by western-blotting with the antibodies of interest.

2.8. Confocal immunofluorescence microscopy

Confocal imaging was carried out using primary antibodies against VASP or Zyxin (diluted 1:200), as previously described [31]. Actin was stained by Phalloidin (1:1000; Invitrogen). Imaging was performed using a Zeiss LSM 780-NLO confocal on an Axio Observer Z.1 microscope (Carl Zeiss AG, Germany) with a 63× optical zoom.

2.9. Methylthiazoletetrazolium (MTT) assay

Cell proliferation/viability was measured by MTT assay as previously described [32]. Cells were serum-starved in 0.5% FBS for 12 h. A total of 5×10^4 cells per well were then plated in a 96-well plate in RPMI 10% FBS. In brief, 10 μL of a 5 mg/mL solution of MTT was added to the wells and incubated at 37 °C for 4 h. The reaction was stopped by using 100 μL of 0.1 N HCl in anhydrous isopropanol. Cell proliferation/viability was evaluated by measuring the absorbance at 570 nm, using an automated plate reader. All conditions were tested in six replicates.

2.10. Colony formation assay

Colony formation was carried out in semisolid methyl cellulose medium. 1×10^3 cell/mL was seeded in MethoCult 4230 (StemCell