Biochemical and Biophysical Research Communications 456 (2015) 367–372

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Adiponectin signals through Adiponectin Receptor 1 to reverse imatinib resistance in K562 human chronic myeloid leukemia cells

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ARTICLE INFO

Article history:
Received 18 November 2014
Available online 2 December 2014

Keywords:
Adiponectin
Adiponectin Receptor
Chronic myeloid leukemia
Imatinib
Resistance
Bcr-Abl

ABSTRACT

Adiponectin, a member of adipokines, is a functional ligand for Adiponectin Receptor-1 (AdipoR1) and Adiponectin Receptor-2 (AdipoR2), and has been found to be linked to the risk of CML. Imatinib has undoubtedly revolutionised the management and outcome of chronic myeloid leukemia (CML), however imatinib resistance has been recognized as a major problem in CML therapy. In this study, we first established imatinib-resistant K562 CML cells, and then evaluated the effect of Adiponectin in reversing imatinib resistance. The data presented here demonstrated that Adiponectin was able to reverse K562 resistance to imatinib *in vitro* and *in vivo*. Additional data with molecular approaches suggested that the reversion of Adiponectin in imatinib resistance signals through AdipoR1 but not AdipoR2 to down-regulate Bcr-Abl expression and effect in imatinib-resistant K562 CML cells. Taken together, our data showed that Adiponectin can reverse imatinib resistance in CML, and to a certain extent elucidate the mechanism of Adiponectin reversing imatinib resistance that may provide a new and promising approach in imatinib resistance management in CML therapy.

1. Introduction

Adiponectin, also called GBP-28, apM1, AdipoQ, and Acrp30, has been discovered to be an adipocyte secreted hormone. It is a protein of 244 amino acids and the product of the apM1 gene, which is specifically expressed in human adipocytes [1]. Adiponectin, a member of adipokines, has been found to be an important negative regulator of hematopoiesis and linked mainly to the risk of Chronic myeloid leukemia (CML) [2]. It is well established that Adiponectin has modulated cell proliferation and apoptosis via two distinct receptors: Adiponectin Receptor-1 (AdipoR1) and Adiponectin Receptor-2 (AdipoR2) [3,4]. Moreover, a recent report has revealed that AdipoR1 expression was significantly increased in the mononuclear cells in CML patients, and this increase was similar in newly diagnosed and in imatinib treated CML patients [5], on the other hand, imatinib could increase Adiponectin secretion through the suppression of PI3 kinase signaling [6]. Furthermore, Adiponectin levels are elevated in CML patient plasma after imatinib therapy [7]. Taken together, it is tempting to hypothesize that Adiponectin and its functional receptor AdipoR1 might be involved in regulation of imatinib responding in CML treatment.

CML is a hematopoietic stem cell disorder with an elevated but immature white blood cell count [8]. CML is generally diagnosed by the presence of an abnormal Philadelphia (Ph) chromosome, which results from a translocation between the long arms of chromosomes 9 and 22. This exchange brings the Bcr gene and the proto-oncogene Abl together [9]. The hybrid gene, Bcr-Abl, encodes for a fusion protein with tyrosine kinase activity leading to uncontrolled growth. One of the major achievements in the treatment of CML has been the development of the first tyrosine-kinase inhibitor imatinib mesylate (STI571, Gleevec), a phenylaminopyrimidine derivative. Imatinib directly occupies the ATP-binding pocket of the Abl-kinase domain, and prevents conformation change of the protein into the active form [10], with the subsequent regulation on transcription of several genes involved in the control of cell cycle, cell adhesion, and cytoskeleton organization, leading to apoptosis of target cells [11]. Despite high rates of hematological and cytogenetic responses to therapy, the emergence of resistance to imatinib has been recognized as a major problem in CML treatment [12,13]. So far, mechanisms identified in development of imatinib-resistance include overexpression of Bcr-Abl associated with amplification or mutation of Bcr-Abl, and overexpression of

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http://dx.doi.org/10.1016/j.bbrc.2014.11.089
0006-291X/

the multidrug-resistant P-glycoprotein (MDR-1) [14–16]. Although considerable progress has been made in the elucidation of the mechanisms of resistance, we are still far from understanding the cause of this resistance and the proper solution to reverse imatinib resistance in human CML cells [13].

Involvement of Adiponectin in the regulation of imatinib resistance in human CML cells has not been hitherto examined. Therefore, in this study, we first established imatinib-resistant K562 CML cells, and then evaluated the effect of Adiponectin in reversing imatinib resistance. The data presented here showed that Adiponectin was able to reverse K562 resistance to imatinib *in vitro* and *in vivo*. Additional data with molecular approaches suggested that the reversion of Adiponectin in imatinib resistance signals through AdipoR1 but not AdipoR2 to downregulate Bcr-Abl expression and effect in imatinib-resistant K562 CML cells.

2. Materials and methods

2.1. Cell lines and culture conditions

Human chronic myelogenous leukemia K562 cells (ATCC) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone). Cells were cultured at 37 °C in 5% CO_2.

Development of imatinib-resistant K562 cells: cells were exposed to stepwise increasing concentrations of imatinib (Novartis Pharmaceuticals, Switzerland) starting with a concentration of 50 nM. Sub-populations of cells that were able to grow in the presence of 500 nM imatinib, were selected. Then, the inhibitory concentration 50 (IC_{50}) values of imatinib were determined and compared with control sensitive parental cells by MTT assay.

2.2. Cell proliferation assay

Cell proliferation was measured by MTT assay. In brief, cells (5×10^4 cells/well) were plated into 96-well plates containing 100 μL of the growth medium in indicated concentrations of imatinib at 37 °C in 5% CO_2 for 96 h. They were then treated with 5 μL of MTT (5 mg/mL) for 4 h. The reaction was stopped by using 100 μL of 0.1 N HCl in anhydrous isopropanol. Cell growth was evaluated by measuring the absorbance at 570 nm, using an automated plate reader.

2.3. Cell cycle analysis

Cells were fixed in 70% ethanol, for at least 2 h at 4 °C, and stained with 20 μg/mL propidium iodide (PI) containing 10 μg/mL RNase A for 30 min at room temperature. Fluorescence cell analysis was performed with a FACSCalibur (Becton–Dickinson, CA, USA) and sub-G_0/G_1 cell populations were considered apoptosis [17].

2.4. SiRNAs transfection

Human AdipoR1, AdipoR2, and non-targeting (scrambled) siRNAs were synthesized by Qiagen. The sequences of AdipoR1 and AdipoR2 siRNAs were used as previously described [18]. Transfection of K562 cells was performed using Transmessenger Transfection Reagent (Qiagen) as described by the manufacturer.

2.5. Real-time PCR

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions, 1 μg of total RNA was converted to cDNA by SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Life Technologies). PCR was performed on ABI Prism 7000 using corresponding primers and SYBR gene PCR Master Mix (Invitrogen). The primer sequences were as follows: AdipoR1-forward (5′-CGGTGGAACTGGCTGAACTG-3′), AdipoR1-reverse (5′-CCGCACCTCCTCCTCTTCTT-3′); AdipoR2-forward (5′-ACGGAGTTGTCAGCACTCAC-3′) AdipoR2-reverse (5′-GCCATCGTCTTGTACCTCAC-3′); Bcr-Abl-forward (5′-GGGAGCAGCAGAAGAAGTGT-3′), Bcr-Abl-reverse (5′-AAAGGTTGGGGTCATTTTCAC-3′) and GAPDH-forward (5′-GAAGGTGAAGGTCGGAGTC-3′), GAPDH-reverse (5′-AGATGGTGATGGGATTTC-3′). Template cDNA was denatured at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The quantification data were analyzed with ABI Prism 7000 SDS software. The cycle time values were normalized to GAPDH of the same sample.

2.6. Western blot

Whole-cell lysates were prepared and subjected to western blot analysis. Equal amounts of the cell lysates were resuspended in 5× Tris–glycine SDS sample buffer, electrophoresed on 8–15% SDS–PAGE, and transferred to nitrocellulose membranes (Amersham Pharmacia). The detection of proteins was performed with anti-CrkL antibody (Cell signaling Technology), anti-Phospho-CrkL (Tyr207) antibody (Cell signaling Technology), anti-c-Abl antibody (Santa Cruz Biotechnology), and anti-β-actin antibody (Cell signaling Technology), followed by corresponding IDRy second antibody. The blots were scanned using an Odyssey Imaging System (LI-COR Bioscience, USA).

2.7. IMR-K562 xenograft model

BALB/c nu/nu female mice (4–6 weeks of age; vital river, China) were used in this study. All animal experiments were done in accordance with protocols approved by the Institutional Authority for Laboratory Animal Care of Wenzhou Medical University.

1.0×10^7 IMR-K562 cells in a total volume of 0.1 mL were injected subcutaneously into the single flanks of the mice. Mice bearing IMR-K562 cell xenografts were divided randomly into nine groups ($n = 8$ in each group) when the bearing tumor reached approximately 20 mm^3, including (a) an equal volume of saline (buffer control); (b) rhAdiponectin (10 mg/kg); (c) rhAdiponectin (50 mg/kg); (d) imatinib (100 mg/kg); (e) imatinib (100 mg/kg) plus rhAdiponectin (10 mg/kg) and (f) imatinib (100 mg/kg) plus rhAdiponectin (50 mg/kg). Recombinant human Adiponectin was from Peprotech.

Imatinib was given orally by gavage and rhAdiponectin were given by intraperitoneal (i.p.) injection.

Treatments began on day 14, when the average tumor volume was 200 mm^3, and were given every day, for a total of ten dosages. Tumor volumes were calculated using the following formula: $\pi ls^2/6$, in which l represents the long diameter of the tumor, and s represents the short diameter.

Tumor inhibition rate of tumor growth was calculated as (1 − average tumor weight of treated group/average tumor weight of saline control group) × 100%.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 software. IC_{50} values were calculated by nonlinear regression using the sigmoidal dose–response equation. Statistical analysis of the differences among different treatment modalities was performed using the unpaired Student's t-test. $P < 0.05$ was considered statistically significant.