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Original Articles

Anti-cancer fatty-acid derivative induces autophagic cell death through modulation of PKM isoform expression profile mediated by bcr-abl in chronic myeloid leukemia

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ABSTRACT

The fusion gene *bcr-abl* develops chronic myeloid leukemia (CML), and stimulates PI3K/Akt/mTOR signaling, leading to impaired autophagy. PI3K/Akt/mTOR signaling also plays an important role in cell metabolism. The Warburg effect is a well-recognized hallmark of cancer energy metabolism, and is regulated by the mTOR/c-Myc/hnRNP/PKM signaling cascade. To develop a new strategy for the treatment of CML, we investigated the associations among bcr-abl, the cascade related to cancer energy metabolism, and autophagy induced by a fatty-acid derivative that we had previously reported as being an autophagy inducer. Here we report that a fatty-acid derivative, AIC-47, induced transcriptional repression of the *bcr-abl* gene and modulated the expression profile of PKM isoforms, resulting in autophagic cell death. We show that c-Myc functioned as a transcriptional activator of bcr-abl, and regulated the hnRNP/PKM cascade. AIC-47, acting through the PPARγ/β-catenin pathway, induced down-regulation of c-Myc, leading to the disruption of the bcr-abl/mTOR/hnRNP signaling pathway, and switching of the expression of PKM2 to PKM1. This switching caused autophagic cell death through an increase in the ROS level. Our findings suggest that AIC-47 induced autophagic cell death through the PPARγ/β-catenin/bcr-abl/mTOR/hnRNP/PKM cascade.

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Introduction

The fusion gene *bcr-abl* is derived from a translocation between chromosomes 9 and 22, and the chimeric bcr-abl protein has constitutive tyrosine kinase activity that drives the development and maintenance of chronic myeloid leukemia (CML). For example, bcr-abl stimulates PI3K/Akt/mammalian target of rapamycin (mTOR) signaling, resulting in enhanced cell proliferation [1] and impaired macroautophagy [2,3] (which we herein refer to as “autophagy”). Because the downstream molecular pathways of bcr-abl have been revealed, treatment with tyrosine kinase inhibitors (TKIs) has been a successful strategy [4]. However, mutations in the kinase domain of bcr-abl are the most common cause of resistance to therapy with such inhibitors [5]. Therefore, the development of a new strategy for the treatment of CML is needed. Because molecules acting upstream of bcr-abl, such as transcriptional factors of the bcr-abl gene, are still unknown, the identification of such factors could contribute to the development of new molecular targeting therapy.

The tyrosine kinase/PI3K/Akt/mTOR signaling pathway plays a crucial role in regulating not only cell growth and survival, but also metabolism [6]. Unlike normal cells, cancer cells prefer to metabolize glucose by glycolysis rather than by the tricarboxylic acid (TCA) cycle, even in an aerobic environment [7,8]. This phenomenon is the well-known “Warburg effect,” which confers an advantage for the survival and proliferation of cancer cells [9]. The Warburg effect is achieved through regulated expression of pyruvate kinase isoforms,

Abbreviations: CML, chronic myeloid leukemia; mTOR, mammalian target of rapamycin; TKI, tyrosine kinase inhibitor; hnRNP, heterogenous nuclear ribonucleoprotein; PKM, pyruvate kinase M; STAT3, signaling transducer and activator of transcription 3; PPAR, peroxisome proliferator-activated receptor; TCA, tricarboxylic acid; PTB, polypyrimidine tract-binding protein; DMSO, dimethyl sulfoxide; 3-MA, 3-methyladenine; ChIP, chromatin immunoprecipitation; ROS, reactive oxygen species; ESR, electron spin resonance spectroscopy; NAC, *N*-acetylcysteine.

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PKM1 and PKM2 [10]. These 2 isoforms are generated by alternative splicing of the PKM pre-mRNA so as to contain either exon 9 (PKM1) or exon 10 (PKM2) [10]. Three heterogeneous nuclear ribonucleoproteins (hnRNPs), i.e., polypyrimidine tract-binding protein (PTB), hnRNPA1, and hnRNPA2, were identified as alternative splicing repressors of PKM1 [11,12]. David et al. showed that the transcription of hnRNPs is regulated by c-Myc [11]. The overexpression of the c-Myc/hnRNP signaling cascade leads to a low PKM1/PKM2 ratio in cancer cells. On the other hand, c-Myc is known to be transactivated by β-catenin [13], which is regulated by PPARγ [14].

We previously reported that several fatty-acid derivatives induce autophagic cell death, but not apoptosis, in human leukemia K562 cells [15]. We found that 3-decenoic acid derivatives, which are classified as medium-chain fatty-acids, can be novel autophagy inducers. Recently, certain molecules, such as signaling transducer and activator of transcription 3 (STAT3) and peroxisome proliferator-activated receptors (PPARs), were shown to be associated with fatty-acid-induced autophagy [16–19]. However, the mechanism by which fatty-acids induce autophagy flux in bcr-abl-positive cells remains less clear. In this study, we investigated the associations among bcr-abl, cancer energy metabolism, and autophagic cell death induced by a 3-decenoic acid derivative in bcr-abl-positive K562 and KCL-22 cells.

Materials and methods

Synthesis of AIC-47

3-Decenoic acid (0.1 mol; D1186; Tokyo Chemical Industry Co., Tokyo, Japan) was added to 25 mL of thionyl chloride (207–01116; Wako Pure Chemical Industries, Osaka, Japan), and was reacted at room temperature for 2 h. Then, the mixture was kept at 90 °C for 1 h with refluxing. 3-Decenoic acid chloride was obtained by removal of the solvent. 3-Decenoic acid chloride (5 mmol) in 15 mL of tetrahydrofuran (THF) was added to heptamethyleneimine (5 mmol; H0546; Tokyo Chemical Industry Co.) and pyridine (400 mg) in THF. Then, the mixture was heated with refluxing at 90 °C for 3 h. After removal of the solvent, the residue was subjected to liquid–liquid extraction with water and ethyl acetate. (*E*)-1-(azocan-1-yl) dec-3-en-1-one (AIC-47; Fig. 1A), obtained from organic phase after the evaporation, was purified by silica gel column chromatography, using elution with chloroform. Chemical shifts of ^{1}H-NMR spectra were reported in parts-per-million downfield from tetramethylsilane. The abbreviations of the splitting pattern were as follows: d, doublet; t, triplet; br, broad; m, unresolved multiplet. Positive-ion HR-ESI-MS m/z: 294.2756 $[M+H]^+$ (Calcd for $C_{19}H_{36}NO$: 294.2791); ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.88 (3H, t, J = 6.9 Hz), 1.05 (1H, m), 1.11 (1H, m), 1.17 (3H, d, J = 6.9 Hz), 1.22~1.38 (10H, m), 1.47~1.84 (8H, m), 2.03 (2H, br d, J = 5.8 Hz), 3.04 (2H, m), 3.46 (1H, m), 3.96 (1H, m), 5.51 (1H, m), 5.56 (1H, m).

Cell culture and treatment

Human leukemia cell lines K562 and KCL-22 were purchased from Human Science Research Resources (Osaka, Japan). Both cell lines were cultured under an atmosphere of 95% air and 5% CO_2 at 37 °C in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (K562) or 20% (KCL-22) heat-inactivated FBS (Sigma-Aldrich Co., St. Louis, MO, USA). Human peripheral blood lymphocytes from healthy individuals were maintained in RPMI-1640 medium supplemented with 10% FBS, and stimulated with Concanavalin A (75 µg/mL) for 48 h. AIC-47 was dissolved in dimethyl sulfoxide (DMSO). For inhibition of autophagy, K562 or KCL-22 cells were pretreated with 3-methyladenine (Calbiochem, San Diego, CA, USA) or Bafilomycin A1 (Sigma-Aldrich Co.) for 8 h before treatment with AIC-47 (5 µM). Other K562 or KCL-22 cells were pretreated with the free-radical scavenger *N*-acetylcysteine (NAC) for 4 h before treatment with AIC-47 (5 µM). The final concentration of DMSO (<0.3%), 3-MA (150 µM), Bafilomycin A1 (5 nM), or NAC (1 mM) had no significant effect on the growth and differentiation of the cells (data not shown). Viable cell numbers were measured by performing the trypan-blue dye-exclusion test.

Western blotting analysis

Total protein was extracted from whole cells by the procedure described previously [15]. Nuclear and cytoplasmic proteins were extracted by using a CelLytic NuCLEAR Extraction Kit (Sigma-Aldrich Co.), according to the manufacturer's protocol. Protein contents were measured with a DC Protein assay kit (Bio-Rad, Hercules, CA, USA). Five micrograms of protein of each cell lysate was separated by SDS-PAGE by using an adequate percent of polyacrylamide gel and electroblotted onto a PVDF membrane (PerkinElmer Life Sciences, Inc., Boston, MA, USA). The detailed method after blotting was described previously [15]. The antibodies against the following proteins were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA): LC3B (#3868), Akt (#9272), phospho-Akt (Ser473; #4060), mTOR (#2983), Beclin-1 (#3495), PTB1 (#8776), β-catenin (#2698), and Histone H3 (#9715). The following antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA): Bcl-2 (sc-509), c-Abl (sc-131), CrkL (sc-319), c-Myc (sc-40). The antibodies against PKM1 (NBP2-14833SS) and PKM2 (NBP1-48308SS) were purchased from Novus Biologicals (Littleton, CO, USA). The quantity loaded was verified by re-incubating the same membrane with anti-β-actin antibody (A5316; Sigma-Aldrich Co.).

Electron microscopic observation

K562 cells treated or not with AIC-47 (10 µM) were harvested and rinsed with PBS. The cells were fixed for 2 h with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4, PB), rinsed in PB, and postfixed in 2% osmium tetraoxide for 2 h. After dehydration, thin sections (70 nm thickness) were prepared as described previously [15]. Thereafter, they were stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy with a Hitachi-7650 (Hitachi, Tokyo, Japan), operating at 80 kV.

Cell transfection with short interfering RNA

K562 or KCL-22 cells were seeded into six-well plates at the concentration of 0.5×10^5 cells per well. Short interfering RNAs (siRNAs) for *bcr-abl*, *c-Myc*, *PKM2*, *PTB1*, and *β-catenin* (5 or 10 nM) were used for transfection of the cells, which was achieved by using cationic liposomes, Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's Lipofection protocol. The sequences of the siRNAs, all of which were from Invitrogen, were 5′-UUUGUGUUUCAACUGUUCUCGUCGU-3′ (siR-*c-Myc*), 5′-CAGACUUGGUGAGGACGAUUAUGGC-3′ (siR-*PKM2*), 5′-GCAGAGUUCAAAAGCCCUU-3′ (siR-*bcr-abl*), 5′-AUCUCUGGUCUGCUAAGGUCACUUC-3′ (siR-*PTB1*), and 5′-UAAAGAUGGCCAGUAAGCCCUCACG-3′ (siR-*β-catenin*). The sequence of the non-specific control microRNA (Hokkaido System Sciences, Sapporo, Japan) was 5′-GGCCUUUCACUACUCCUCA-3′.

Quantitative reverse transcriptase PCR using real-time PCR

Total RNA was isolated from cells by using a NucleoSpin miRNA kit (TaKaRa, Otsu, Japan) according to the manufacturer's protocol. For determination of the expression levels of mRNAs, total RNA was reverse-transcribed with a PrimeScript RT reagent kit (TaKaRa). Real-time PCR was then performed with primers by using a THUNDERBIRD SYBR qPCR Mix (TOYOBO Co., Osaka, Japan). The sequences of the primers were the following: *bcr-abl*-sense, 5′-TTCAGAAGCTTCTCCCTGACAT-3′; *bcr-abl*-antisense, 5′-TGTTGACTGGCGTGATGTAGTTGCTTGG-3′; *c-Myc*-sense, 5′-ACATCATCATCCAGGACTG-3′; *c-Myc*-antisense, 5′-TTTAGCTCGTTCCTCCTCTG-3′; *PKM2*-sense, 5′-ATTATTTGAGGAACTCCGCCGCCT-3′; *PKM2*-antisense, 5′-ATTCCGGGTCACAGCAATGATGG-3′; *PKM1*-sense, 5′-CGAGCCTCAAGTCACTCCAC-3′; *PKM1*-antisense, 5′-GTGAGCAGACCTGCCAGACT-3′; *GAPDH*-sense, 5′-CAACCCATGGCAAATTCCATGGCA-3′; *GAPDH*-antisense, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. *GAPDH* was used as an internal control. The relative expression level of mRNA was calculated by the $\Delta\Delta Ct$ method.

ChIP assays

K562 and KCL-22 cells were incubated in the presence or absence of 5 µM AIC-47 for 72 h, and ChIP experiments were performed as per the instruction manual for ChIP-IT Express Enzymatic (53009; Active Motif, Carlsbad, CA, USA), and c-Myc antibody (#9402; Cell Signaling Technology). Antibody against Suz12 was used as a positive control; and rabbit IgG (39357; Active Motif), as a negative control. Primers for detection of specific regions of *bcr-abl* promoters were used for real-time PCR.

Docking experiment

The X-ray structure of PPARγ (PDB code 2ZK0) [20] was used for running the docking experiment. Docking was performed by using AutoDock4 [21]. Top-ranked solution was visually inspected and filtered to select the best complex.

Electron spin resonance spectroscopy (ESR)

K562 cells were incubated in the presence or absence of 5 µM AIC-47 for 8 h. Productions of free-radicals were determined using the ESR trapping technique in combination with 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO; D2362; Tokyo Chemical Industry Co.). ESR spectra were observed at room temperature in a quartz capillary tube (i.d. 0.75 mm, JES-LC01) with a JEOL JES-FR30EX free-radical monitor (JEOL Ltd., Akishima Japan). The measurement conditions were as follows: magnetic field, 336.000 ± 5.000 mT; power, 8.98 mW; sweep time, 4 min; modulation, 100 kHz, 0.08 mT; amplitude, 4000; time constant, 0.3 s. The intensity of the spin adduct was evaluated from the peak height of the signal.

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