Aurora kinase A critically contributes to the resistance to anti-cancer drug cisplatin in JAK2 V617F mutant-induced transformed cells

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A R T I C L E   I N F O

Article history:
Received 25 January 2011
Revised 22 April 2011
Accepted 26 April 2011
Available online 8 May 2011

Keywords:
JAK2
V617F mutation
Myeloproliferative neoplasm
Aurora kinase A
Cisplatin

A B S T R A C T

JAK2 V617F mutant induces transformation through aberrant activation of various transcription factors. We found that the expression of Aurora kinase A (Aurka) was significantly induced by mutant JAK2 through c-Myc expression. Interestingly, mutant JAK2 enhanced resistance to cisplatin (CDDP)-induced DNA damage, and effectively suppressed apoptosis. Ectopic expression of Aurka in Ba/F3 cells exhibited similar resistance to CDDP, and this required kinase activity. Conversely, knockdown and inhibition of Aurka in cells expressing mutant JAK2 abolished the resistance to CDDP. Taken together, Aurka is most likely critical for resistance to DNA damage in cells transformed by JAK2 V617F mutant.

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1. Introduction

Janus kinase 2 (JAK2) is a non-receptor tyrosine kinase and an essential signal transducer of various cytokine signaling, including erythropoietin (Epo) [1]. Dysregulation of the JAK2 signaling pathway promotes cell growth and prevents apoptosis in a variety of hematological malignancies [2]. Recently, a novel somatic mutation of JAK2, V617F, was identified in myeloproliferative neoplasms (MPNs), including 95% polycythemia vera (PV) patients and ~50% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) [3].

JAK2 V617F mutant is constitutively active and induces cytokine-independent survival of JAK2-deficient erythroid progenitor cells [4]. In addition, in the presence of erythropoietin receptor (EpoR), JAK2 V617F mutant exhibits tumorigenesis in nude mice, suggesting that JAK2 V617F mutant functions as a potent oncogene in the presence of EpoR as a signaling scaffold [5]. Furthermore, JAK2 V617F mutant exhibited resistance to a DNA cross-linking drug, mitomycin C, suggesting that JAK2 V617F mutant activates survival signals against apoptosis induced by not only cytokine removal but also DNA damage [6]. JAK2 V617F mutant caused aberrant activation of various transcription factors, including signal transducers and activators of transcription 5 (STAT5) [5], and induced the expression level of c-Myc [7]. It is simple speculation that the expression of target genes regulated by these transcription factors should be constitutively enhanced by JAK2 V617F mutant, and some could contribute to transformation; however, it is still unclear which gene expression harbors an essential role in transforming activity.

Aurora kinase A (Aurka) is a member of the serine/threonine kinase family and is required for assembly of the mitotic spindle [8]. Amplification and overexpression of Aurka are observed in several types of human tumors and are more frequently associated with tumor progression as well as resistance of the cells to chemotherapy [9]. Recently, it has been reported that the expression of Aurka is directly induced by c-Myc and that an Aurora kinase inhibitor, VX-680, exhibited life-extending effectiveness in mice transplanted with lymphoma elicited by overexpression of c-Myc [10]. This indicates that Aurka functions as not only an important mediator in oncogenesis caused by Myc but also as an attractive therapeutic target for cancers [11].

Here, we found that the expression of Aurka was induced through c-Myc downstream of JAK2 V617F mutant. In order to clarify the role of Aurka in DNA damage-induced apoptosis, we examined the effect of Aurka on DNA damage induced by cisplatin (CDDP). Interestingly,
we showed that Aurka significantly contributed to the tolerance to CDDP of cells expressing JAK2 V617F mutant.

2. Materials and methods

2.1. Reagents

Recombinant human erythropoietin (Epo) (ESPO® 3000) and murine IL-3 were purchased from Kirin Brewery Co. and PEPRO-TECH, respectively. CDDP and Aurora kinase inhibitor II were purchased from Nihon-Kayo and Calbiochem, respectively. Anti-Aurka antibody and anti-Flag antibody (M2) were purchased from Sigma. Anti-β-actin antibody and anti-c-Myc antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-HA antibody was purchased from Roche. Peroxidase-conjugated rabbit anti-mouse and goat anti-rabbit secondary antibodies were purchased from Dako.

2.2. Plasmids

Murine Aurka N-Flag was subcloned into MSCV-Puro. Mutagenesis of amino acid residue, K175R in Aurka, was performed using a site-directed mutagenesis kit (Stratagene). The sequences of oligonucleotides used for constructing shRNA retrovector were as follows: sh-A1: 5’-gatccggcagagccaccaaatcaagagatgcttgtcgtttcctttttta-3’ and 5’-agcttaaaaaagagcaggaacactttgtaaatttggg-3’. sh-A2: 5’-gatccggcagagccaccaaatcaagagatgttcaagagagaccccacactttcctttttta-3’ and 5’-agcttaaaaaagaaacaaatgaagccaagattgctgttgcg-3’. sh-A3: 5’-gatccggcagagccaccaaatcaagagatgttcaagagagaccccacactttcctttttta-3’ and 5’-agcttaaaaagagcaggaacactttgtaaatttggg-3’. sh-c-Myc: 5’-gatccggcagagccaccaaatcaagagatgttcaagagagaccccacactttcctttttta-3’ and 5’-agcttaaaaagagcaggaacactttgtaaatttggg-3’.}

(Underlined sequences correspond to the sequence of murine Aurka and c-Myc.)

2.3. Cell cultures and retroviral infection

Ba/F3 cells were infected with empty virus (−), wild-type JAK2 (WT), JAK2 mutant (V617F) and EpoR, which had been established previously [5]. Ba/F3 cells were infected with retrovirus encoding Aurka and its kinase dead mutant (KD). Ba/F3 cells expressing JAK2 (WT) or JAK2 mutant (V617F) and EpoR were infected with retrovirus harboring shRNA against Luciferase (Luc), c-Myc and Aurka. These cells were cultured in RPMI-1640 supplemented with 10% FBS and 2 ng/ml IL-3.

2.4. Ba/F3 cell growth assay

Transduced and exponentially growing Ba/F3 cells were washed twice with PBS and incubated with RPMI-1640 supplemented with 1% FBS in the presence of IL-3 (0.01 ng/ml) or Epo (0.01 U/ml) for the indicated periods. Living cells were counted using a Beckman Coulter VI-Cell.

2.5. DNA fragmentation assay

Genomic DNA was prepared for gel electrophoresis as described previously [5]. Electrophoresis was performed on a 1% (W/V) agarose gel in Tris–boric acid buffer.

2.6. Immunoblotting

Cells were lysed in NP-40 lysis buffer (50 mM Tris–HCl pH 7.4, 10% glycerol, 50 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 20 mM NaF, 0.2 mM Na3VO4) supplemented with protease inhibitors. Denatured samples were run on 10% SDS–PAGE and transferred to PVDF membranes. Immunoblotting was performed as previously described [5].

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT was performed using an oligo (dT)20 primer and 2 μg total RNA for first-strand cDNA synthesis. The PCR primer sequences were as follows: c-Myc 5’-tgccgacagagagaaattt-3’ (upstream) and 5’-aaccctggcaatacagcctt-3’ (downstream); Aurka 5’-aacaggagagctgtt-3’ (upstream) and 5’-gctcttcgttgctcaagg-3’ (downstream); GAPDH, 5’-acttcaaccagcataaatc-3’ (upstream) and 5’-ccctccacaatgcaaatg-3’ (downstream).

3. Results

3.1. JAK2 V617F mutant constitutively induced Aurka expression

In order to observe the alterations of the gene expression induced by JAK2 mutant (V617F), total RNA was prepared from
WT/EpoR cells and V617F/EpoR cells cultured without Epo for 12 h and then DNA micro array analysis was performed. Compared with WT/EpoR cells, the induction of Aurka was observed as well as c-Myc in V617F/EpoR cells (Fig. 1A). In cells expressing EpoR, Epo stimulation significantly enhanced the expression of c-Myc mRNA and Aurka mRNA. In contrast, in V617F/EpoR cells, a high expression of c-Myc and Aurka mRNAs was observed regardless of Epo stimulation (Fig. 1B). Furthermore, protein levels of c-Myc and Aurka were also markedly elevated in V617F/EpoR cells in the presence and absence of Epo stimulation (Fig. 1C).

3.2. JAK2 V617F mutant induced Aurka expression through c-Myc activation

A recent study demonstrated that c-Myc directly induces the expression of Aurka [10]. To investigate whether the JAK2 V617F mutant-induced expression of Aurka is also mediated by c-Myc, we established Ba/F3 cells expressing wild-type c-Myc and c-Myc mutant (In373), which carries an insertion in the DNA-interacting region and fails to bind to DNA (Fig. 2A and B) [12]. In unstimulated cells, endogenous Aurka was slightly observed in empty virus-infected cells. In contrast, while c-Myc significantly induced the expression of Aurka, In373 reduced the expression level of endogenous Aurka. Interestingly, IL-3 stimulation induced the expression of endogenous c-Myc and Aurka in empty virus-infected cells. Moreover, In373 completely inhibited IL-3-induced expression of Aurka (Fig. 2B). In addition, whereas ectopic expression of c-Myc and IL-3 stimulation significantly induced the expression of Aurka mRNA, In373 failed to induce its expression and inhibited IL-3-induced expression of Aurka mRNA, suggesting that Aurka was transcriptionally induced by c-Myc (Fig. 2C). Furthermore, knockdown of c-Myc significantly resulted in a marked decrease in the levels of Aurka mRNA and protein in both Epo-stimulated WT/EpoR cells and unstimulated V617F/EpoR cells (Fig. 2D and E). Fig. 2F shows the location of Myc-responsive CACGTG and CATGTG E-box sequences in Aurka gene locus. The presence of these E-boxes suggests that the expression of Aurka is most likely to be directly regulated by c-Myc downstream of JAK2 V617F mutant.

3.3. JAK2 V617F mutant reduced cisplatin sensitivity

Next, we investigated the effect of JAK2 V617F mutant on DNA damage induced by CDDP. After CDDP treatment, while -/EpoR cells showed high sensitivity to CDDP, WT/EpoR cells slightly reduced its sensitivity. Compared to these cells, in V617F/EpoR cells, sensitivity to CDDP was significantly reduced (Fig. 3A). Interestingly, CDDP induced cell cycle arrest at the G2/M of V617F/EpoR cells in a dose-dependent manner (Fig. 3B). Furthermore, the sensitivities of these cells to CDDP were inversely correlated with the expression levels of Aurka and c-Myc (Fig. 3C). These results suggest that Aurka is transcriptionally induced by c-Myc downstream of JAK2 V617F mutant.

**Fig. 2.** c-Myc is critical for JAK2 V617F mutant-induced expression of Aurka. (A) Scheme of wild-type c-Myc and c-Myc mutant (In373). (B and C) Ba/F3 cells were infected with empty virus (−), retroviruses encoding wild-type c-Myc and c-Myc inhibitory mutant (In373), which carries an insertion in the DNA-interacting region and fails to bind to DNA (Fig. 2A and B) [12]. In unstimulated cells, endogenous Aurka was slightly observed in empty virus-infected cells. In contrast, while c-Myc significantly induced the expression of Aurka, In373 reduced the expression level of endogenous Aurka. Interestingly, IL-3 stimulation induced the expression of endogenous c-Myc and Aurka in empty virus-infected cells. Moreover, In373 completely inhibited IL-3-induced expression of Aurka (Fig. 2B). In addition, whereas ectopic expression of c-Myc and IL-3 stimulation significantly induced the expression of Aurka mRNA, In373 failed to induce its expression and inhibited IL-3-induced expression of Aurka mRNA, suggesting that Aurka was transcriptionally induced by c-Myc (Fig. 2C). Furthermore, knockdown of c-Myc significantly resulted in a marked decrease in the levels of Aurka mRNA and protein in both Epo-stimulated WT/EpoR cells and unstimulated V617F/EpoR cells (Fig. 2D and E). Fig. 2F shows the location of Myc-responsive CACGTG and CATGTG E-box sequences in Aurka gene locus. The presence of these E-boxes suggests that the expression of Aurka is most likely to be directly regulated by c-Myc downstream of JAK2 V617F mutant.

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expression of p53 tumor suppressor protein was effectively decreased in V617F/EpoR cells (Fig. 3C). According to the previous report that p53 is stabilized by DNA damage and regulates apoptosis [13], our data in Fig. 3C well fit our observation that JAK2 V617F mutant exhibits resistance to DNA damage. In addition, while CDDP-induced activation of caspase 3 was observed in -/EpoR cells and WT/EpoR cells, activation of caspase 3 was not detected in V617F/EpoR cells (Fig. 3D). Also, CDDP induced DNA internucleosomal fragmentation in a concentration-dependent manner in -/EpoR cells and WT/EpoR cells but not V617F/EpoR cells (Fig. 3E).

3.4. Kinase activity of Aurora kinase is required for resistance to cisplatin

In order to investigate how Aurora functions in CDDP-induced apoptosis, Ba/F3 cells were infected with retroviruses encoding wild-type Aurora (WT) and its kinase dead mutant (KD), in which an ATP-binding site, lysine at 175, was substituted to arginine (Fig. 4A and B). There was no significant difference of the proliferation rate in these cells, suggesting that Aurora is not involved in proliferation and survival (Fig. 4C). Interestingly, compared with Ba/F3 cells infected with empty virus (−), while cells expressing Aurora reduced sensitivity to CDDP, cells expressing Aurora KD mutant slightly enhanced sensitivity to CDDP (Fig. 4D). As shown in Fig. 4B, wild-type Aurora markedly reduced the expression of p53. Furthermore, CDDP-induced caspase 3 activation and DNA fragmentation were inhibited by the expression of wild-type Aurora. On the other hand, Aurora KD mutant enhanced the expression of p53 more than that detected in empty virus-infected cells and, as a result, induced lower viability and higher induction of apoptosis in the presence of CDDP (Fig. 4B–F). These results suggest that kinase activity is required for downregulation of p53 by Aurora.

![Fig. 3.](image-url) JAK2 V617F mutant exhibits resistance to cisplatin-induced apoptosis. (A and B) Transduced Ba/F3 cells were treated with various concentrations (0, 0.5, 1, 2, and 4 μg/ml) of CDDP in the presence of Epo (0.01 U/ml) for 24 h. (A) Viable cell numbers were counted. Results are the mean ± S.D. of three independent experiments. (B) Cells were fixed, treated with propidium iodide, and subjected to FACS analysis. (C) Whole cell lysates were immunoblotted with antibodies against p53 or β-actin. (D and E) Transduced Ba/F3 cells were treated with CDDP in the presence of Epo (0.01 U/ml) for 24 h. (D) Whole cell lysates were immunoblotted with anti-cleaved caspase 3 antibody or anti-β-actin antibody. (E) DNA was isolated from cells and subjected to agarose gel electrophoresis.
3.5. Aurka is critical for resistance to cisplatin in cells expressing JAK2 V617F mutant

To gain further insight into the role of Aurka, endogenous Aurka was knocked down in V617F/EpoR cells using shRNA. As a control, we used the shRNA expression vector against luciferase (sh-Luc). Two different shRNAs (sh-A2 and sh-A3) effectively reduced the expression of Aurka in V617F/EpoR cells (Fig. 5A). The viable cells infected with sh-Luc as a control and shRNAs for Aurka were counted; however, there was no difference in the cell proliferation rate (data not shown). Interestingly, knock down of Aurka markedly enhanced the sensitivity to CDDP and increased the expression level of p53, compared to when infected with sh-Luc (Fig. 5A and B). In addition, in cells infected with shRNA for Aurka, CDDP markedly induced the activation of caspase 3 and DNA fragmentation at a lower concentration (Fig. 5C and D). Furthermore, we examined the effect of Aurka inhibitor on the resistance of V617F/EpoR cells to CDDP. Interestingly, Aurka inhibitor slightly reduced the viability of V617F/EpoR cells and significantly enhanced the sensitivity of V617F/EpoR cells to CDDP (Fig. 5F). In addition, Aurka inhibitor enhanced the expression of p53 in V617F/EpoR cells (Fig. 5E). This observation well fits the result shown in Fig. 4 and emphasizes that kinase activity of Aurka is critical for the regulation of p53 stability. Furthermore, both the activation of caspase 3 and DNA fragmentation were slightly detected in V617F/EpoR cells treated with Aurka inhibitor, and treatment...
with Aurka inhibitor markedly enhanced CDDP-induced apoptosis in V617F/EpoR cells (Fig. 5G and H). Taken together, it is suggested that Aurka is critical for resistance to DNA damage in cells transformed by JAK2 V617F mutant and that Aurka inhibitor is an effective drug for MPNs.

4. Discussion

In the current study, we identified Aurka as an essential gene induced by JAK2 V617F mutant and clarified that the expression of Aurka is regulated by c-Myc. Our results demonstrated that the expression of c-Myc is also upregulated by JAK2 V617F mutant, although it remains to be clarified how the expression of c-Myc is induced by JAK2 V617F. Interestingly, the expression level of p53 was down-regulated by overexpression of Aurka and up-regulated by knockdown of Aurka (Figs. 4B and 5A). Previously, in vitro studies have demonstrated
that Aurka phosphorylates p53 at Ser315, leading to its ubiquitination by Mdm2 and proteolysis. They also showed that silencing of Aurka results in less phosphorylation of p53 at Ser315 and enhances the stability of p53 [14]. In the current study, we observed that the expression level of p53 was increased when Aurka KD mutant was expressed or endogenous Aurka was inhibited by its specific inhibitor (Fig. 4B and 5E), indicating that kinase activity of Aurka strongly contributes to the instability of p53 downstream of JAK2 V617F mutant. When considering these results, it is thought that Aurka KD mutant functions as a dominant negative mutant in p53 expression, although the mechanism by which Aurka KD mutant inhibits the downregulation of p53 expression has not been elucidated in this study. Moreover, Mao et al. reported that the status of p53 locus influenced the function of Aurka by utilizing p53-deficient mice [15]. These reports strongly support a significant interaction between Aurka and p53; therefore, in considering treatment for MPNs, not only examining the presence of JAK2 V617F mutation in patients but also checking the status of their p53 locus will become important in the future.

However, overexpression of Aurka failed to completely mimic the effect of JAK2 V617F mutant (Fig. 4). We currently do not have any additional data to explain this discrepancy; however, in the course of DNA-array analysis, we observed that the expression of FANCC mRNA was also highly elevated by JAK2 V617F mutant in STAT5 dependenty (data not shown), FANCC is closely related to Fanconi anemia (FA), a recessive genomic instability syndrome. In fact, when endogenous FANCC was knocked down using shRNA in V617F/EpoR cells, sensitivity to CDDP was markedly increased, suggesting that FANCC is also involved in resistance to CDDP downstream of JAK2 V617F mutant (data not shown). Clarification of the requirement of Aurka and FANCC in JAK2 V617F mutant-induced resistance to DNA damage is a future problem to be elucidated.

Previous reports have shown that the enhancement of Aurka expression was associated with tumor progression [9]. In addition, immortalized rodent cell lines transfected with Aurka form colonies in vitro, and tumors when injected into nude mice [16], suggesting that Aurka can promote transformation in certain settings; however, conversely, in another cases, the overexpression of Aurka induces mitotic abnormalities and hyperplasia in mammary glands in transgenic mice [17]. Combining these results, it is difficult to summarize the functions of Aurka in tumorigenesis and tumor progression. In our study, Aurka strongly contributed to the resistance to CDDP; however, overexpression of Aurka or kinase dead mutant of Aurka (KD) in Ba/F3 cells could not induce cytokine-independent cell growth (Fig. 4C). We also made a similar observation that the proliferation rate of V617F/EpoR cells was not changed when Aurka was knocked down (data not shown). In addition, we tested whether overexpression of Aurka in Ba/F3 cells causes accumulation of 4N DNA content in the G2/M phases of the cell cycle, and induces polyploidy with >4N DNA content. However, the increase of aneuploidy was not observed in Ba/F3 cells expressing not only wild-type Aurka but also the kinase dead mutant of Aurka, as shown in Supplementary data Fig. S1. These data suggest that Aurka alone is insufficient to induce cellular transformation to a JAK2 V617F mutant.

In this study, it was strongly suggested that Aurka could be essential for the progression of a tumor induced by JAK2 V617F, and the combination of CDDP and Aurka inhibition would be effective to treat patients with MPDs induced by JAK2 V617F mutant; therefore, Aurka is a candidate target for the development of anti-cancer drugs.

Acknowledgements

We thank Dr. J.N. Ihle for the retroviral vectors of JAK2 and EpoR. This work was supported in part by grants (21790090) from MEXT and Takeda Science Foundation.

Appendix A. Supplementary data


References