Resveratrol induces apoptosis of human chronic myelogenous leukemia cells in vitro through p38 and JNK-regulated H2AX phosphorylation

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Aim: The phosphorylation of histone H2AX, a novel tumor suppressor protein, is involved in regulation of cancer cell apoptosis. The aim of this study was to examine whether H2AX phosphorylation was required for resveratrol-induced apoptosis of human chronic myelogenous leukemia (CML) cells in vitro.

Methods: K562 cells were tested. Cell apoptosis was analyzed using flow cytometry, and the phosphorylation of H2AX and other signaling proteins was examined with Western blotting. To analyze the signaling pathways, the cells were transfected with lentiviral vectors encoding H2AX-wt or specific siRNAs.

Results: Treatment of K562 cells with resveratrol (20–100 µmol/L) induced apoptosis and phosphorylation of H2AX at Ser139 in time- and dose-dependent manners, but reduced phosphorylation of histone H3 at Ser10. Resveratrol treatment activated two MAPK family members p38 and JNK, and blocked the activation of another MAPK family member ERK. Pretreatment with the p38 inhibitor SB202190 or the JNK inhibitor SP600125 dose-dependently reduced resveratrol-induced phosphorylation of H2AX, which were also observed when the cells were transfected with p38- or JNK-specific siRNAs. Overexpression of H2AX in K562 cells markedly increased resveratrol-induced apoptosis, whereas overexpression of H2AX-139m (Ser139 was mutated to block phosphorylation) inhibited resveratrol-induced apoptosis. K562 cells transfected with H2AX-specific siRNAs were resistant to resveratrol-induced apoptosis.

Conclusion: H2AX phosphorylation at Ser139 in human CML cells, which is regulated by p38 and JNK, is essential for resveratrol-induced apoptosis.

Keywords: resveratrol; chronic myelogenous leukemia; K562 cell; H2AX; apoptosis; p38; JNK; siRNA; SB202190; SP600125

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Introduction
Chronic myeloid leukemia (CML) is a clonal disease of hematopoietic stem cells that occurs because of a reciprocal translocation between chromosomes 9 and 22[1, 2]. This chromosomal arrangement results in a BCR–ABL fusion gene that encodes a BCR–ABL fusion oncoprotein, which possesses constitutively active tyrosine kinase activity and promotes leukemogenesis. Multiple signaling pathways are engaged by this abnormal BCR–ABL kinase, including Myc, Ras/mitogen-activated protein kinase (MAPK), c-Raf, MAPK/extracellular signal-regulated kinase (ERK), stress-activated protein kinase (SAPK)/JNK, signal transducer and activator of transcription (STAT), nuclear factor-κB (NF-κB), phosphatidylinositol 3-kinase (PI3-K), c-Jun, c-bcl, CrkL, Janus kinase (Jak)/STAT and Src pathways[3, 4]. Clinically, CML has been successfully treated with imatinib mesylate, which is a selective small-molecule protein kinase inhibitor that specifically targets the oncogenic BCR–ABL fusion protein kinase[5, 6]. However, ~30% of CML patients develop intolerance or resistance to imatinib owing to a high frequency of point mutations or gene amplifications of the BCR–ABL fusion gene[3]. Although some of these mutations can be overcome by treatment with next-generation tyrosine kinase inhibitors such as dasatinib or nilotinib, one mutation, T315I, causes complete insensitivity to all currently available tyrosine kinase inhibitors and is present in ~15% of patients after failure of imatinib therapy[8–10]. More recently, other types of resistance that are independent of BCR–ABL and linked to the overexpression of the Src tyrosine kinases Lyn and Hck have also been demonstrated[11, 12]. Thus, the search for novel and effective drugs against CML is an...
attractive aim.

Resveratrol (3',4',5-trans-trihydroxystilbene), which is a naturally polyphenolic phytoalexin, is found in >70 plant species, primarily in red grapes, red wine, peanuts, certain berries, dark chocolate, and other cocoa products[13, 14]. Early research found that resveratrol elicits several beneficial effects related to human diseases, notably in the prevention of cardiovascular diseases[15]. This compound is currently being used in Phase I studies to treat obesity and diabetes[16, 17]. Recently, an increasing number of studies have revealed that resveratrol exhibits potent anti-cancer properties in certain epithelial tumors and leukemia[18, 19]. Its potential chemopreventive and chemotherapeutic activities have been demonstrated in all three stages of carcinogenesis (initiation, promotion, and progression) in various murine models of human cancer[19]. Several in vitro studies have demonstrated that resveratrol can inhibit proliferation and induce apoptosis in various leukemic cell lines[20]. Resveratrol has also been demonstrated to induce apoptosis in B lineage leukemic cells (acute lymphoblastic leukemia) through the CD95-independent and mitochondria/caspase-9-specific pathway[21]. Another study has demonstrated that resveratrol induces the apoptosis of CML cells, including imatinib-resistant CML cells, in a caspase-independent manner[22]. Recently, Puissant et al found that resveratrol promotes autophagic cell death in CML cells[23]. Despite these promising in vitro and in vivo effects, resveratrol has not yet been analyzed as a potential therapeutic agent for the treatment of CML. To accelerate its clinical application, the detailed molecular mechanism of resveratrol against CML cells requires further clarification.

H2AX is a variant of the histone H2A family. H2AX is known as a novel tumor suppressor protein owing to its role in the regulation of cancer cell apoptosis[23]. Our previous studies have demonstrated that H2AX phosphorylation at Ser139 is related to apoptosis in CML cells (K562 cells)[24, 25]. In the current study, we used resveratrol to induce the apoptosis of K562 cells and to investigate the signaling pathways involved in the regulation of H2AX phosphorylation. Finally, we evaluated the role of H2AX phosphorylation in resveratrol-induced apoptosis of K562 cells, revealing the antileukemic effects of resveratrol. We demonstrated that resveratrol induced dramatic phosphorylation of H2AX during apoptosis in a time- and dose-dependent manner. We also observed that the MAPK family members p38 and JNK were activated by resveratrol, which coincided with H2AX phosphorylation. The inhibition of p38 and JNK activity or the knockdown of p38 and JNK reduced H2AX phosphorylation. Overall, these data demonstrated that resveratrol-induced H2AX phosphorylation in K562 cells is regulated by the p38 and JNK pathways. Furthermore, we provided evidence to confirm that H2AX phosphorylation at Ser139 is essential for the resveratrol-induced apoptosis of K562 cells.

Materials and methods

Reagents

Resveratrol (trans-3',4',5-trihydroxystilbene), the p38 inhibitor SB202190 and the JNK inhibitor SP600125 were purchased from Sigma-Aldrich (St Louis, MO, USA). The antibodies against γH2AX (at Ser139), H2AX, caspase-3, cleaved caspase-3, phosphorylated JNK1/2 (pJNK1/2), total JNK1/2, phosphorylated ERK1/2 (pERK1/2), total ERK1/2, phosphorylated p38 (p38), total p38, H3, phosphorylated H3 at Ser10 [pH3 (Ser10)], Bim, Bcl-2, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). H2AX siRNA was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and p38- and JNK-specific siRNAs were obtained from Cell Signaling Technology.

Lentivirus preparation

HIV-based lentiviral expression plasmids, 293Ta lentiviral packaging cells and a Lenti-Pac HIV Expression Packaging Kit were obtained from GeneCopoeia (Rockville, MD, USA). At 48 h after seeding, the 293Ta packaging cells were transfected with lentiviral vectors encoding H2AX-wt (wild type; EX-B0074-LV201), H2AX-139m (Ser139 was mutated to stop phosphorylation; CCS-B0074-LV201), or empty vector (EX-NEG-LV201) using a Lenti-Pac™ HIV Expression Packaging Kit according to the manufacturer’s instructions. The lentiviruses were harvested by collecting the pseudovirus-containing culture medium at 48 h post-transfection. K562 cells were transduced with the harvested lentiviral particles in the presence of 5 µg/mL polybrene (Santa Cruz Biotechnology) and cultured in selection medium with 2 µg/mL puromycin for ~2 weeks to allow the cells to grow to 90% confluence.

siRNA transfection

To knock down the endogenous expression levels of H2AX, p38, or JNK, siRNAs targeting H2AX, p38, or JNK were transfected into K562 cells according to the manufacturer’s instructions, and the effect of knockdown was assessed by Western blotting. K562 cells transfected with control siRNA served as controls.

Cell culture and drug treatment

K562 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in a 37 °C, 5% CO2 incubator. Before each experiment, K562 cells, K562 cells transfected with siRNAs targeting p38, JNK, or H2AX, and stable K562 cells transduced with the lentiviral particles (overexpression of H2AX-wt and H2AX-139m) were seeded at 1×10⁶ cells/mL in 10-cm dishes and treated with resveratrol, p38 inhibitor SB202190, or JNK inhibitor SP600125 1 h before resveratrol treatment.

Whole cellular protein or histone extraction and Western blot analysis

Whole cellular proteins were extracted after drug treatment by disrupting the cells in lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, 1 mmol/L Na3VO4, 1 mmol/L NaF, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, and 1 mmol/L PMSF). To extract the histones after drug treat-
ment, the cells were lysed in NETN buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L Tris, pH 8, and 0.5% NP-40) and centrifuged for 5 min. The histones were extracted from the pellets with 0.1 mol/L HCl. The protein samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked at room temperature for 1 h with 5% nonfat milk or BSA in Tris-buffered saline containing Tween 20 (TBST). Primary antibodies were incubated with the membranes in TBST with 5% nonfat milk or BSA at 4 °C overnight. The membranes were incubated with the appropriate secondary antibody in TBST with 5% nonfat milk or BSA for 1 h at room temperature. Proteins were detected using SignalFire ECL Reagent (Cell Signaling Technology).

**Flow cytometry**

After drug treatment, K562 cells (1×10^6) were washed with 1×binding buffer at room temperature. The cells were incubated with Annexin V for 15 min. The cells were then incubated with propidium iodide (PI) before detection, followed by analysis with flow cytometry (FACS-Calibur; Becton-Dickinson, San Jose, CA, USA). All reagents were obtained from an Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA).

**Statistical analysis**

The data are expressed as the mean±SD of three independent determinations, and Student’s t-test or ANOVA was performed using SPSS version 17.0 software (SPSS INC, Chicago, IL, USA). The significance levels of all tests were set at P<0.05.

**Results**

**Resveratrol induces H2AX phosphorylation and apoptosis in K562 cells**

Previous data, including our own, have demonstrated that histone H2AX phosphorylation at Ser139 is involved in apoptotic regulation. In particular, H2AX phosphorylation at Ser139 has an important role in the apoptosis of tumor cells. Hence, we investigated whether resveratrol could induce H2AX phosphorylation in CML cells. Histone samples were extracted from K562 cells after resveratrol treatment at the indicated time and dose, and Western blot analysis was conducted. Resveratrol treatment for 48 h induced dramatic phosphorylation of H2AX (γH2AX) in a dose-dependent manner (Figure 1A). Resveratrol (60 µmol/L) treatment also induced strong phosphorylation of H2AX in a time-dependent manner (Figure 1A). γH2AX was observed as early as 24 h after resveratrol treatment and was maintained for up to 72 h. We also observed that resveratrol strongly inhibited histone H3 phosphorylation at Ser10 (Figure 1A). The detailed mechanism involved in resveratrol-reduced H3 phosphorylation and its function requires further study.

Resveratrol was previously reported to induce apoptosis in CML cells. To test this, we treated K562 cells with resveratrol at the indicated time and detected apoptosis with flow cytometry. Resveratrol stimulated K562 cell apoptosis in a time-dependent manner (Figure 1B), following a parallel response with H2AX phosphorylation (Figure 1A). The percentage of total apoptotic cells (including early and late apoptotic cells) after treatment with resveratrol for 72 h reached >90% (Figure 1B). These data demonstrated that resveratrol induced H2AX phosphorylation during the apoptosis of K562 cells, suggesting that H2AX phosphorylation may be involved in regulating resveratrol-induced apoptosis in CML cells.

**Resveratrol stimulates p38 and JNK activation in K562 cells during apoptosis**

The MAPK family (ERK, p38, and JNK) has been implicated in many human tumors. Therefore, the MAPK pathways have become a focus of intense investigation for developing small-molecule inhibitors as targeted therapeutics. Our previous data demonstrated that p38 MAPK is involved in imatinib-induced apoptosis in CML cells. In the current study, we investigated the role of MAPK pathways (ERK, p38, and JNK) in apoptosis and in H2AX phosphorylation in CML cells induced by resveratrol. K562 cells were incubated with resveratrol for the indicated time, and whole cellular proteins were extracted for Western blot analysis. Resveratrol induced p38 and JNK phosphorylation in a time-dependent manner (Figure 2), following a response similar to that for H2AX phosphorylation (Figure 1A). Unexpectedly, resveratrol strongly inhibited ERK phosphorylation (Figure 2), which is activated by BCR–ABL with constitutively active tyrosine kinase activity in CML cells. This finding is not consistent with the results reported by Mustafi et al, indicating that resveratrol stimulates ERK activity in K562 cells. Additionally, we used the whole cellular proteins extracted from resveratrol-treated K562 cells to analyze apoptosis with Western blotting. Resveratrol increased caspase-3 cleavage to form cleaved caspase-3 (active caspase-3), which is a hallmark of apoptosis. We also observed that the expression of another potent proapoptotic protein, Bim (B-cell lymphoma 2 interacting mediator of cell death), was increased by resveratrol treatment. However, the level of Bcl-2 (B-cell lymphoma2), which exerts an antiapoptotic effect, was not affected by resveratrol treatment (Figure 2). These results, together with those discussed above (Figure 1), indicated that resveratrol treatment triggered p38 and JNK activation, followed by H2AX phosphorylation in K562 cells during apoptosis, suggesting that p38 and JNK are involved in regulating H2AX phosphorylation.

**p38 and JNK regulate resveratrol-induced H2AX phosphorylation in K562 cells**

We investigated whether p38 and JNK are actually involved in regulating H2AX phosphorylation. K562 cells were pre-incubated with the p38 inhibitor SB202190 or with the JNK inhibitor SP600125 before resveratrol treatment, and the γH2AX level was analyzed by Western blotting. p38 and JNK phosphorylation was also detected to confirm the effects of SB202190 and SP600125 on their targeted kinases. Treatment
with the p38 inhibitor SB202190 or with the JNK inhibitor SP600125 inhibited p38 or JNK activity, respectively, and at the same time dramatically suppressed resveratrol-induced H2AX phosphorylation (level of γH2AX) (Figure 3A and 3B). To definitively establish the roles of the p38 and JNK signaling pathways in H2AX phosphorylation, we used p38- and JNK-specific siRNAs to inhibit p38 and JNK expression, respectively, and analyzed the effects of p38 and JNK knockdown on resveratrol-induced H2AX phosphorylation. Consistent with the findings using SB202190 and SP600125 in K562 cells, transfection with p38- and JNK-specific siRNAs, but not with control siRNA, inhibited resveratrol-induced H2AX phosphorylation (Figure 3C). p38- and JNK-specific siRNAs were each confirmed to suppress the expression of their respective targeted kinases (Figure 3C). These data demonstrated that p38 and JNK regulate resveratrol-induced H2AX phosphorylation in K562 cells.

**H2AX phosphorylation is required for the resveratrol-induced apoptosis of K562 cells**

We have previously reported that H2AX phosphorylation has a critical role in apoptotic regulation[26]. Apoptosis is almost completely blocked in H2AX–/– mouse embryonic fibroblasts (MEFs). The transfection of H2AX-wt, but not H2AX-139m (Ser139 was mutated to block H2AX phosphorylation), into H2AX–/– MEFs restored the ability to

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**Figure 1.** Resveratrol induces H2AX phosphorylation and apoptosis. (A) K562 cells were treated with different concentrations of resveratrol (RSV) for 48 h (left panel) or treated with 60 µmol/L resveratrol for different time periods (right panel). Cells not treated with resveratrol served as negative controls. The histone samples were extracted for Western blot analysis using antibodies against γH2AX (phosphorylated H2AX at Ser139), total H2AX, pH3 (Ser10) (phosphorylated H3 at Ser10), and total H3. (B) K562 cells were treated with 60 µmol/L resveratrol. Cells were harvested at the indicated time and then subjected to flow cytometry after Annexin V/PI staining to assess apoptosis (upper panel). The mean±SD of three independent determinations is shown (lower panel). *P<0.05, †P<0.01.
induce apoptosis. Increasing evidence has also demonstrated that H2AX phosphorylation is involved in the apoptosis of tumor cells\cite{23, 25, 27}. To investigate the important role of γH2AX in the resveratrol-induced apoptosis of CML cells, we assessed the effects of H2AX overexpression and knockdown on the apoptosis of CML cells. The stable K562 cell lines transduced with either the lentiviral vectors encoding H2AX-wt or H2AX-139m, or the empty vector control were used to detect resveratrol sensitivity. After 24 h of continuous treatment with resveratrol, the above cells were analyzed by flow cytometry for apoptosis. The stable K562 cells without resveratrol treatment served as controls (Figure 4A). The data demonstrated that H2AX-wt overexpression strongly increased K562 cell apoptosis (Figure 4A). The percentage of apoptotic K562 cells harboring empty vectors reached only 40% (including early and late apoptosis) after resveratrol treatment; however, the percentage of apoptotic K562 cells overexpressing H2AX-wt reached almost 90% after the same treatment (Figure 4A). By contrast, the K562 cells with H2AX-139m overexpression were resistant to resveratrol-induced apoptosis, and the percentage of apoptotic cells reached 30%, which is a lower percentage than that of K562 cells harboring empty vectors (Figure 4A). These results were also confirmed by Western blot analysis (Figure 4B). H2AX-wt overexpression increased the resveratrol-induced expression of Bim compared with that of the empty vector control (Figure 4B). H2AX-139m overexpression inhibited the resveratrol-induced expression of Bim compared with that of the empty vector control or H2AX-wt overexpression (Figure 4B). Consistent with the above data (Figure 2), Bcl-2 expression levels did not differ between these stable K562 cells (Figure 4B). Seemingly, H2AX-139m overexpression reduced endogenous H2AX (Ser139) phosphorylation and inhibited resveratrol-induced apoptosis in K562 cells. Furthermore, H2AX knockdown in K562 cells transfected with H2AX-specific siRNA strongly inhibited resveratrol-induced apoptosis. The apoptotic percentage of K562 cells with H2AX knockdown decreased from 38%, the apoptotic percentage of K562 cells transfected with control siRNA, to 10% (Figure 5A). H2AX knockdown also blocked resveratrol-induced Bim expression (Figure 5B). These findings demonstrate that H2AX phosphorylation is required for the apoptosis of CML cells.

**Discussion**

Resveratrol has been demonstrated to induce apoptosis in CML cells, including imatinib-resistant CML cells, suggesting that this compound could act against CML by bypassing the BCR–ABL fusion protein, which possesses constitutively active tyrosine kinase activity and promotes leukemogenesis. In this study, we found a novel mechanism to be involved in the antileukemic effect of resveratrol. The resveratrol-induced apoptosis of K562 cells is related to the phosphorylation of the tumor suppressor protein H2AX at Ser139. Resveratrol treatment stimulated H2AX phosphorylation and apoptosis in K562 cells (Figure 1). H2AX overexpression in K562 cells increased resveratrol-induced apoptosis; however, overexpression of Ser139-mutated H2AX (which blocks phosphorylation) resulted in K562 cells that were resistant to apoptotic induction with resveratrol (Figure 4). These data demonstrated that H2AX phosphorylation is essential for resveratrol-induced apoptosis. This conclusion was confirmed in K562 cells with H2AX knockdown. Compared with control siRNA, H2AX-specific siRNA strongly reduced resveratrol-induced apoptosis (Figure 5), which is consistent with the finding in K562 cells overexpressing H2AX-139m (Figure 4). The above results indicated that histone H2AX can serve as a novel and important molecule that can be targeted by resveratrol. In searching for the upstream signaling pathways that regu-
late H2AX phosphorylation, we found that resveratrol could activate p38 and JNK. When these two kinases were inhibited by their inhibitors or by siRNA for knockdown, H2AX phosphorylation was also inhibited (Figure 3). Overall, these data demonstrated that the p38 and JNK pathways regulate H2AX phosphorylation that is induced by resveratrol in K562 cells. Previously, we reported that UVA radiation induces H2AX phosphorylation by the JNK pathway in MEFs and that imatinib induces H2AX phosphorylation by the p38 pathway in K562 cells[25, 26]. The current study revealed that resveratrol triggered both the p38 and JNK pathways, which are involved in H2AX phosphorylation. These findings demonstrated that the signaling network regulating H2AX phosphorylation is complex. We also found that another MAPK family member, ERK, was strongly activated in K562 cells, characterized by high levels of phosphorylated ERK1/2 (Figure 2). Unexpectedly, resveratrol treatment could dramatically block ERK1/2 phosphorylation in K562 cells (Figure 2). The role of ERK inhibition by resveratrol in the regulation of K562 cell function is interesting and requires further investigation.

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Author contribution
Xin-pin WU and Min XIONG performed most of the experiments. Cheng-rong LU and Lian-ning DUAN designed the study and analyzed the results. Cheng-rong LU wrote the manuscript, which was revised by Lian-ning DUAN. Cheng-
Figure 4. H2AX phosphorylation increases resveratrol-induced apoptosis. (A) K562 cells were transfected with lentiviral vectors encoding H2AX-wt, H2AX-139m (blocking Ser139 phosphorylation) or the empty vector alone control. After selection, the stable cells were treated with 60 μmol/L resveratrol for 0 and 24 h and subjected to flow cytometry for the analysis of apoptotic cells (early and late apoptosis). (B) The stable K562 cells harboring vector encoding H2AX-wt or H2AX-139m were treated with 60 μmol/L resveratrol for 24 h and then harvested for Western blot analysis with antibodies against Bim and Bcl-2. β-Actin was used to confirm equal whole cellular protein loading. The mean±SD of three independent determinations are shown. *P<0.01.
Figure 5. H2AX knockdown inhibits resveratrol-induced apoptosis. (A) To detect the effect of H2AX phosphorylation on resveratrol-induced apoptosis, K562 cells were transfected with H2AX-specific siRNA or control siRNA as a negative control. The transfected cells were treated with 60 µmol/L resveratrol for 24 h and subjected to flow cytometry for analysis of apoptotic cells (early and late apoptosis). (B) K562 cells transfected with H2AX-specific siRNA or control siRNA were treated with 60 µmol/L resveratrol for 24 h and then harvested for Western blot analysis with antibodies against Bim and Bcl-2. β-Actin was used to confirm equal whole cellular protein loading. The mean±SD of three independent determinations are shown. *P<0.01.

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