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Imatinib mesylate induction of ROS-dependent apoptosis in melanoma B16F0 cells

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ABSTRACT

Background: Imatinib mesylate (STI571), a protein tyrosine kinase inhibitor, was shown to reduce the viability of several cancer cell lines via apoptosis induction; however, the role of reactive oxygen species (ROS) in STI571-induced melanoma cell apoptosis is still undefined.
Objective: In this study, we investigated the contribution of ROS to STI571-induced apoptosis in melanoma B16F0 cells, and the apoptotic mechanism elicited by STI571 was illustrated.
Methods: Using an in vitro cell culture system, the effects of STI571 on ROS production, cell cycle progression, caspase activation, and mitochondrial functions were examined via Western blotting, a flow cytometric analysis, an enzyme activity assay, and a DNA integrity assay. In pharmacological studies, the ROS scavenger, N-acetyl cysteine (NAC), the NADPH oxidase inhibitor, dipheylene iodide (DPI), and mitogen-activated protein kinase (MAPK) inhibitors (PD98059, SP600125, and SB203580) were applied to investigate the mechanism.
Results: STI571 reduced the viability of melanoma cells B16F0, but not human skin fibroblasts WS1, via apoptosis induction. Besides, apoptosis induced by STI571 was inhibited by the addition of NAC and DPI, and an increase in the intracellular peroxide level by STI571 was identified in melanoma B16F0 cells. Activation of caspases 3 and 9 enzyme activities accompanied by disrupting the mitochondria membrane potential in according with stimulating JNK and p38 protein phosphorylation was identified in STI571-treated B16F0 cells. STI571-mediated a ROS-dependent apoptosis potentiated by JNK inhibitor SP600125 was first identified in melanoma B16F0 cells.
Conclusion: Our results support the idea that ROS-dependent apoptosis in STI571-treated melanoma cells B16F0. The combination of a JNK inhibitor with STI571 for treating melanomas is suggested for further in vivo studies.

1. Introduction

Melanomas are usually resistant to chemotherapy and radiation therapy; therefore, surgery remains the major treatment in clinical practice. Previous studies showed that the resistance of melanoma cells to apoptosis induction is conferred by activation of survival signaling pathways. The mitogen-activated protein kinase (MAPK) pathway is constitutively activated in melanomas, and has received the most attention in developing therapies for melanomas [1]. Therefore, agents in combination with MAPK inhibitors are critical in treating melanomas, and deserve further investigation [2,3].

Induction of apoptosis through the mitochondrial pathway is a strategy for treating melanomas [4]. Alternative activations of

Abbreviations: Bcl-2, B-cell lymphoma 2; Cyt c, cytochrome c; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMSO, dimethyl sulfoxide; DPI, dipheylene iodide; ERK, extracellular regulated kinase; JNK, c-Jun-N-terminal protein kinase; MAPK, mitogen-activated protein kinase; MTT, tetrazolium dye 3-(4,5,-dimethylthiazol)-2-yl-2,5-diphenyltetrazolium bromide; NAC, N-acetyl cysteine; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; Cyc B1, cyclin B1; Cyc D2, cyclin D2; STI571, imatinib mesylate.

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Bcl-2 family proteins, including anti- and pro-apoptotic proteins, participate in the apoptotic mitochondrial pathway. Decreases in anti-apoptotic proteins such as Bcl-2 or increases in pro-apoptotic proteins promote the release of cytosolic cytochrome (Cyt) c from mitochondria to activate caspases 9 and 3, and induce apoptosis [5,6]. Reactive oxygen species (ROS) are important in tumor cell apoptosis, mitochondrial stability, and chemotherapeutic effects [7–9]. Elevated ROS production was identified in apoptosis induced by various anticancer drugs such as taxol, cisplatin, and doxorubin [10–12]. However, ROS may act as mitogens to stimulate the proliferation and migration of tumor cells [13,14]. Previous studies showed that ROS in melanoma cells are abundant due to melanin metabolism, and they contribute to DNA damage and activation of proto-oncogenes [15]. Therefore, more evidence is needed to verify the role of ROS generators in treating melanomas.

Imatinib mesylate (STI, Gleevec, STI571) is the first small-molecular drug used as targeted therapy which initially focused on the tyrosine kinase receptors such as c-KIT, platelet-derived growth factor (PDGF) α and β and Abl-Bcr [16]. STI571 inhibits activation of these tyrosine kinase receptors through blocking ATP-binding sites and is accepted as the first-line treatment for chronic myelogenic leukemia and gastrointestinal stromal tumors [17–19]. Although many biological actions of STI571 were reported such as improvement of chemotherapeutic drug penetration, down-regulation of telomerase activity, the roles of ROS and the apoptotic mitochondrial pathway in STI571-treated melanoma cells apoptosis are still undefined [20,21]. The aim of this study was to investigate the apoptotic mechanism of STI571 in melanoma cells B16F0, and to elucidate the roles of ROS and MAPKs in STI571-induced apoptosis.

2. Materials and methods

2.1. Chemicals and reagents

STI571 and nilotinib were kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). N-Acetyl cysteine and diphenyleneiodonium (DPI) were from Sigma (St. Louis, MO). Antibodies against Bcl-2, total-JNK, ERK, and p38 (at a 1:1000 dilution) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against pro-PARP, phospho-specific AKT (Ser473/Thr308), ERK (Thr202/Tyr204), JNK (Thr183/Tyr185), p38, and total-AKT (at 1:1000 dilutions) were purchased from Cell Signaling Technology (Danvers, MA). The anti-α-tubulin and Cyt c antibodies were from Neomarker (Fremont, CA). SB203580 and SP600125 were purchased from Calbiochem (La Jolla, CA). D4-GDI, and caspases 3 and 9 were purchased from Imgenex (San Diego, CA) Ten-millimolar DPI stock solutions were prepared in dimethyl sulfoxide (DMSO, Sigma). DPI was diluted in culture medium to treat cells. DMSO (0.01%) diluted in the medium was used as the vehicle control.

2.2. Cell culture

Murine melanoma B16F0 cells and normal human embryonic skin fibroblasts WS1 were grown in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 5 U/ml penicillin, 5 μg/ml streptomycin, and 200 mM L-glutamine at 37 °C in a 5% CO_2 atmosphere.

2.3. MTT assay

Proliferation of cultured cells seeded into 24-well uncoated plastic plates (Costar) at 50,000 cells/well (except where indicated) was quantitated as previously described using a colorimetric method based on the metabolic reduction of the soluble yellow tetrazolium dye, 3-(4,5-dimethylthiazol)-2-yl-2,5-diphenyltetrazolium bromide (MTT), to the insoluble purple formazan by the action of mitochondrial succinyl dehydrogenase [22]. B16F0 cells were then incubated at 37 °C for 4 h under 5% CO_2/95% air, followed by dissolution in 500 μl of a lysis solution (10% sodium dodecylsulfate (SDS) in 0.01 N HCl). The absorbance of the solution was read at 595 nm using a multiplate reader, and cell viability was expressed as the OD_{595} of treated cells.

2.4. LDH release assay

The percentage of LDH release from B16F0 cells under indicated treatments was detected using cytotoxicity detection kit (Roche, Indianapolis, IN, USA), The activity was monitored as the oxidation of the reduced form of nicotinamide-adenine dinucleotide (NADH) at 530 nm, and cytotoxicity was determined by the equation: [(OD530 of the treated group − OD530 of the control group)/(OD530 of the Triton X-100-treated group − OD530 of the control group)] × 100%.

2.5. Detection of chromatin-condensed cells

B16F0 cells after different treatments were fixed by cold methanol followed by adding 10% Giemsa solution for 30 min. Then, the extracellular dye was removed by PBS addition for several times. The chromatin condensed cells were observed microscopically.

2.6. Measurement of ROS generation by intact cells

Intracellular production of ROS by B16F0 melanoma cells was measured by oxidation of DCFH-DA to DCF. DCFH-DA is a non-polar compound that readily diffuses into cells, where it is hydrolyzed to the non-fluorescent polar derivative, DCFH, and thereby trapped within cells. If DCFH-DA is oxidized, it turns into the highly fluorescent DCF. B16F0 cells were incubated in the dark for 10 min at 37 °C with 50 μM DCFH-DA, then harvested, and resuspended in plain medium. The fluorescence was analyzed using a FACScan (Becton Dickinson, Sunnyvale, CA) flow cytometer with excitation at 488 nm and emission at 530 nm.

2.7. Western blot analysis

Cells lysates were prepared by suspending cells in lysis buffer (50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 0.025% sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride). Protein samples (30 μg) were electrophoresed on a 8%, 10%, or 13% SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membrane was blocked with 1% bovine serum albumin (BSA) at room temperature for 1 h and then incubated with specific antibodies (pro-PARP, D4-GDI, caspases 3 and 9, BCL-2, Cyt c, Cyc B1 and D2, Cdc 2, phosphorylated AKT, JNK and p38, and total AKT, JNK, and p38) as indicated for a further 3 h. Protein expression was quantified by incubation with the colorimetric substrates, nitro blue tetrazolium (NBT; Roth, Karlsruhe, Germany) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

2.8. DNA fragmentation assay

B16F0 cells under different treatments were collected, and then lysed in 100 μl of lysis buffer (50 mM Tris, pH 8.0; 10 mM ethylenediaminetetraacetic acid (EDTA); 0.5% sodium sarkosinate,