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ORIGINAL ARTICLE

c-Jun-NH_2-terminal kinase potentiates apoptotic cell death in response to carboplatin in B lymphoma cells

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Abstract
Purpose Exposure to carboplatin (CBDCA) has been demonstrated to result in apoptotic and/or necrotic cell death, but molecular mechanisms underlying CBDCA-induced apoptosis or necrosis remain largely unclear. Here, we examined whether activation of c-Jun NH_2-terminal kinase (JNK) modulates the mode of cell death induced by CBDCA in CD31 B lymphoma cells.
Methods The mode of cell death (apoptosis versus necrosis) was investigated by flow cytometry using 7-amino-actinomycin D (7-AAD) and annexin-FITC probes. To evaluate the role of JNK1 in CBDCA-induced cell death, CH31 B lymphoma cells overexpressing dominant-negative form of JNK1 (dnJNK1) or constitutively active form of JNK1 (MKK7-JNK1) were established. Intracellular accumulation of superoxide anion (O_2^-) was determined by flow cytometry using the fluorescent probe dihydroethidium (DHE).
Results The CBDCA-induced primary apoptosis and secondary necrosis were abrogated in the dnJNK1-overexpressing CH31 cells, while it was somewhat enhanced in the MKK7-JNK1-overexpressing cells. In contrast, the CBDCA-induced primary necrosis was reduced by MKK7-JNK1, with a concurrent decrease in production of O_2^-. The superoxide anion scavenger for butylated hydroxyanisol (BHA) partially reduced the CBDCA-induced O_2^- production and necrotic, but not apoptotic, death in both wild type and dnJNK1-overexpressing CH31 cells.
Conclusions Prolonged activation of JNK1 appears to be involved in CBDCA-induced apoptosis with prevention of necrosis induction, and the induction of necrosis appears to correlate with CBDCA-induced O_2^- production, which is partially blocked by co-culture with BHA. These observations provide valuable information for understanding molecular mechanisms underlying CBDCA-induced cell death, and hopefully for the design of novel treatment modalities for patients with tumors.

Keywords Apoptosis · Necrosis · B lymphoma cells · c-Jun NH_2-terminal kinase

Abbreviations

CBDCA	Carboplatin
JNK	c-Jun NH_2-terminal kinase
MAPK	Mitogen-activated protein kinase
ROS	Reactive oxygen species
dnJNK	Dominant-negative JNK
BHA	Butylated hydroxyanisol
MKK	MAPK kinase
7-AAD	7-Amino-actinomycin D
DHE	Dihydroethidium

Introduction

Carboplatin (CBDCA), a less nephrotoxic analog of *cis*-diamminedichloroplatinum (cisplatin), has been used for the treatment of multiple malignancies, including lymphoma, ovarian, head and neck, and non-small cell lung cancer [1–3]. Although CBDCA is highly effective against some tumors, it has serious problems such as side effects

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and/or inherent and/or acquired resistance during treatment [4, 5]. Chemotherapeutic agents including carboplatin are believed to induce cell death through induction of mitochondrial membrane permeabilization (MMP) [6, 7]. We and others have recently demonstrated that CBDCA initiates necrotic as well as apoptotic cell death depending on dose and cell types [8, 9]. Necrotic cell death can be distinguished from apoptotic death by morphological characteristics, DNA strand breaks, and flow cytometry [10, 11]. However, the molecular mechanisms by which apoptosis or necrosis is initiated remain largely unresolved. Sustained activation of c-Jun NH_2-terminal kinases (JNK), a member of mitogen activated protein kinases (MAPKs), is involved in the induction of apoptosis in a variety of cell types including B lymphoma cells [12–17]. The activated JNK migrates from the cytosol to mitochondria, where JNK may phosphorylate one or more substrates including Bim [18, 19]. The BH3-only protein Bim in combination with Bax-α causes mitochondrial depolarization [20], favoring generation of reactive oxygen species (ROS). The three major ROS include superoxide anion (O_2^-), hydroxylradical (OH^-), and hydrogen peroxide (H_2O_2) [21, 22]. Reactive oxygen species has been demonstrated to cause activation of apoptosis-inducing signal kinase 1 (ASK1) through dissociation from thioredoxin [23, 24], and/or inhibition of MAPK phosphatase [25], creating a positive feedback loop. Whether JNK and ROS contribute to apoptotic or necrotic cell death in response to multiple stimuli including CBDCA remains controversial [9, 22, 26–29].

In the present study, we examined whether CBDCA-induced JNK activation is involved in the induction of apoptosis and/or necrosis. The dominant-negative form of JNK (dnJNK1) reduced the CBDCA-induced apoptosis and concurrent enhancement of necrotic cell death. CBDCA produced superoxide anion, which was blocked by JNK activation and the anti-oxidant butylated hydroxyanisol (BHA). Pretreatment with BHA substantially reduced CBDCA-induced necrosis, but not apoptosis. Thus, there is a differential requirement for JNK and ROS in the CBDCA-induced apoptosis versus necrosis. Moreover, cells dying by necrosis cause inflammatory reaction, whereas those dying from apoptosis could be rapidly removed by phagocytic cells prior to membrane lysis [30]. These findings have some implications for the design of CBDCA regimens for patients with tumors.

Materials and methods

Cell culture

Murine B lymphoma cell line CH31 was maintained at 37°C in 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 μM 2-mercaptoethanol (2-ME), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 100 μg/ml kanamycin. CH31 cells overexpressing dnJNK1, MAPK kinase (MKK)7-JNK1, or the control vector alone were established as previously described [14, 15].

Flow cytometric analysis of apoptosis, secondary necrosis, and primary necrosis

Flow cytometric analysis using annexin-FITC and 7-amino-actinomycin D (7-AAD) was performed as previously described [8]. In some experiments, CH31 cells pretreated with 100–200 μM BHA (dissolved in ethanol) (Sigma-Aldrich, St. Louis, MO) or vehicle alone for 1 h were exposed to 100 μg/ml CBDCA for 24 h.

In vitro kinase assay

CH31 cells cultured in the presence of 100 μg/ml CBDCA for the indicated times were lysed in a lysis buffer. Samples were used for in vitro kinase assay using c-Jun as a substrate, as previously described [15].

Western blot analysis

Western blot analysis was performed as previously described [14]. Briefly, cells cultured with 100 μg/ml CBDCA for the indicated times were lysed in a lysis buffer. Samples were resolved on SDS-PAGE and transferred to PVDF membranes. Blots were incubated with anti-JNK1 antibodies (Abs) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-actin serum (Sigma-Aldrich). After several washes, the blots were developed with horseradish peroxidase-labeled anti-rabbit IgG and enhanced chemiluminescence according to the manufacturer's recommendation (Amersham Biosciences, Piscataway, NJ).

Identification of cells producing superoxide anion and hydrogen peroxide by flow cytometry

Intracellular accumulations of superoxide anion and hydrogen peroxide were measured using fluorescent probes dihydroethidium (DHE) and 5- (and -6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester (H_2DCFDA) (Molecular Probes, Eugene, OR, USA). Cells were stimulated with or without 100 μg/ml CBDCA for the indicated periods and washed once with RPMI-1640 without phenol red. Cells resuspended in phenol red-free RPMI-1640 were loaded with 2 μM DHE for 15 min or 1 μM H_2DCFDA for 30 min at 37°C, and samples were analyzed on a flow cytometer.

Statistical analysis

Data were expressed as the mean ± SD for each group. Statistical significance was determined by Student's *t*-test, and a difference of $P < 0.05$ was considered significant.

Results

CBDCA induces necrotic as well as apoptotic cell death in CH31 B lymphoma cells

CH31 B lymphoma cells were exposed to increasing concentrations of CBDCA for 24 h, followed by an assay for cell death. Inhibitory concentration 50% (IC_{50}) value of CBDCA in CH31 cells was 100 μg/ml, which is equal to the highest dose used in this study, as assessed by trypan blue dye exclusion method (Takada et al. unpublished observation). Flow cytometric analysis distinguished apoptotic from necrotic cell death using the probes 7AAD and annexin V, respectively [8, 31]. Cells that are annexin-positive and 7AAD-negative were considered apoptotic, while annexin$^-$ and 7AAD$^+$ cells were considered necrotic (Figure S1A in Electronic supplementary material). Annexin$^+$7AAD$^+$ cells were necrotic subsequent to apoptosis (secondary necrosis). As a control for primary necrotic and apoptotic cell death, cells treated with 0.3% formaldehyde for 30 min or treated with CD95L were employed (Figure S1A in ESM). Low concentrations of CBDCA (20–50 μg/ml) rapidly induced primary apoptosis and secondary necrosis, but not primary necrosis, whereas primary necrosis occurred in addition to primary apoptosis and secondary necrosis in response to higher concentration of CBDCA (100 μg/ml) (Fig. 1). Primary apoptotic and primary necrotic cell death induced by CD95L and formaldehyde were confirmed by forward light scatter (FSC, Figure S1B in ESM) and May-Giemsa staining (Figure S1C in ESM), respectively. These results indicate that CBDCA induces both apoptotic and necrotic cell death, depending on the dose employed.

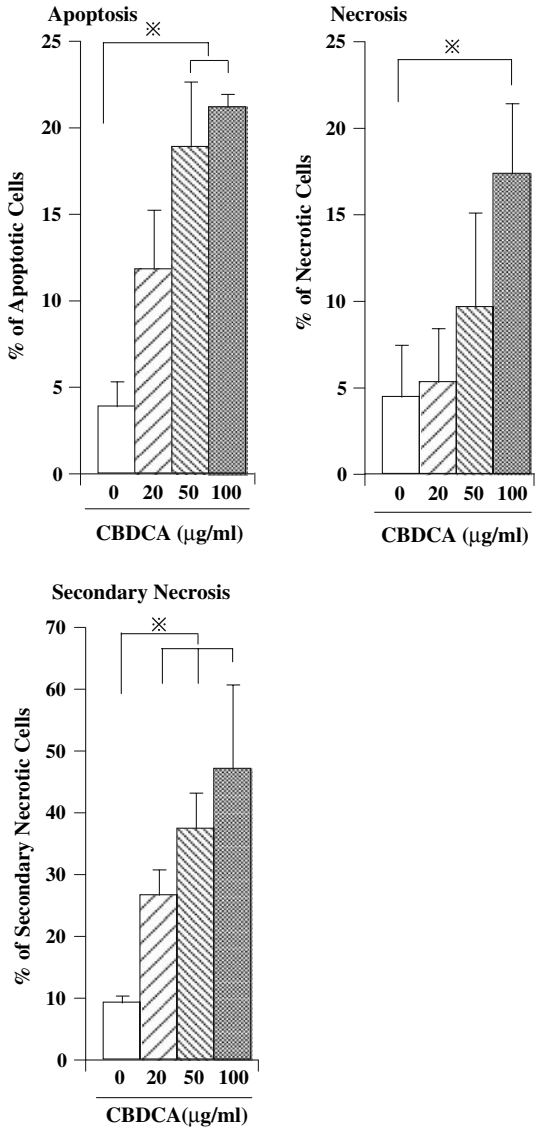

Fig. 1 CBDCA induces both apoptotic and necrotic cell death in CH31 B lymphoma cells depending on the dose. CH31 B lymphoma cells were exposed to the indicated concentrations of CBDCA (0–100 μg/ml) for 24 h and assayed by flow cytometry using 7AAD and annexin V staining. The percentages of cell deaths induced by primary apoptosis (annexin$^+$7AAD$^-$), primary necrosis (annexin$^-$7AAD$^+$), and secondary necrosis (annexin$^+$7AAD$^+$) in response to CBDCA exposure are shown. Results are the mean ± SD from three independent experiments. *Significantly different from the control medium alone

JNK activation is involved in primary apoptotic, but not primary necrotic cell death following CBDCA exposure

A variety of apoptotic stimuli have been shown to induce JNK activation leading to cell death in multiple cell types [16, 32]. Treatment of CH31 cells with CBDCA resulted in substantial activation of JNK at 0.5 h, with elevated levels up to 24 h (Figure S2 in ESM), as assessed by in vitro kinase assay using c-Jun as a substrate. Anti-JNK1 Ab detected mainly 46 kDa form of JNK1 in CH31 cells, although JNK1 comprises two forms, 46 and 54 kDa. To address the requirement of JNK activation for CBDCA-induced cell death, CH31 cells overexpressing dnJNK1 were employed. Both primary apoptosis and secondary necrosis induced by 100 μg/ml CBDCA were substantially abrogated in a dnJNK1 cell line (dnJNK1-#5, Fig. 2a). Two additional clones (dnJNK1-#8 and -#12) behaved like dnJNK1-#5 cells (Figure S3 in ESM). In contrast, CBDCA-induced primary necrosis was moderately enhanced in the dnJNK1 cells, in comparison with the control Neo alone. To further evaluate the role of JNK activation in the

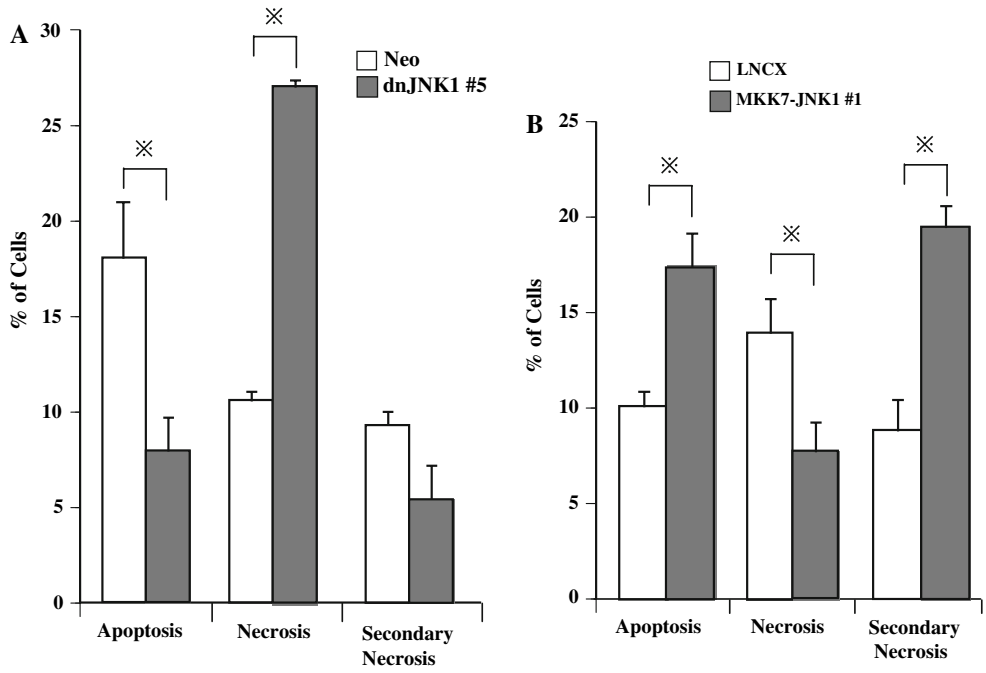

Fig. 2 CBDCA-induced apoptotic cell death is partially inhibited by a concurrent increase in primary necrosis in dnJNK1-overexpressing cells, whereas it is somewhat increased by a concurrent decrease in MKK7-JNK1-overexpressing cells. CH31 cells over-expressing dnJNK1 (**a**) or MKK7-JNK1 (**b**) were exposed to 100 μg/ml CBDCA for 24 h, followed by assays for cell death using flow cytometry. Results are the mean ± SD from three independent experiments. *Significantly different from the control vector alone

CBDCA-induced cell death, CH31 cells expressing a constitutively active form of JNK1, MKK7-JNK1, were employed [14]. MKK7-JNK1-expressing CH31 cells (MKK7-JNK1-#1) displayed greater levels of apoptosis and secondary necrosis than controls, indicating concurrent inhibition of primary necrosis in comparison with control LNCX (Fig. 2b; Figure S4 in ESM). These results suggest that CBDCA-induced activation of JNK is involved in apoptosis, with prevention of primary necrosis.

CBDCA-induced superoxide anion production is diminished in MKK7-JNK1-expressing cells

Production of ROS is closely related to chemotherapy-induced cell death [29, 33]. CH31 cells generating superoxide anion and hydrogen peroxide were detected by flow cytometry using probes DHE and H_2DCFDA, respectively. Considerable numbers of O_2^- producing cells were detected in 20–24 h after stimulation with CBDCA, whereas H_2O_2-producing cells were undetected up to 24 h (Fig. 3). The CBDCA-induced primary necrotic as well as primary apoptotic cell death was detected 24 h after stimulation (Figure S5 in ESM). The proportion of cells generating superoxide anion was decreased in MKK7-JNK1-expressing cells compared with the control (Fig. 4). Although the fraction of O_2^- producing cells was slightly higher in the dnJNK1-overexpressing cells in comparison with the control, the difference was not statistically significant. These results suggest that activation of JNK partially abrogates CBDCA-induced O_2^- production in CH31 B lymphoma cells.

Superoxide anion scavenger BHA partially reduces CBDCA-induced primary necrotic, but not apoptotic, death in dnJNK1-overexpressing CH31 cells

The ROS scavenger BHA has been demonstrated to prevent necrosis in some cell types after various stimulations [34, 35]. As expected, BHA reduced the proportion of O_2^- producing cells induced by the high (100 μg/ml) concentration of CBDCA (Fig. 5a). Although CBDCA-induced primary apoptosis was unaffected by pretreatment with BHA (Fig. 5b), the CBDCA-induced primary necrotic cell death was substantially abrogated (Fig. 5c). The proportion of primary necrotic cell death was increased in the dnJNK1 cells in comparison with control Neo-expressing cells, which was also prevented by pretreatment with 200 μM BHA (Fig. 6; Figure S6 in ESM). Together, these results suggest that activation of JNK1 inhibits CBDCA-induced primary necrosis, but not apoptosis, at least in part through prevention of O_2^- production.

Discussion

The platinum complex CBDCA has been widely used for the treatment of multiple cancers including ovarian cancer,

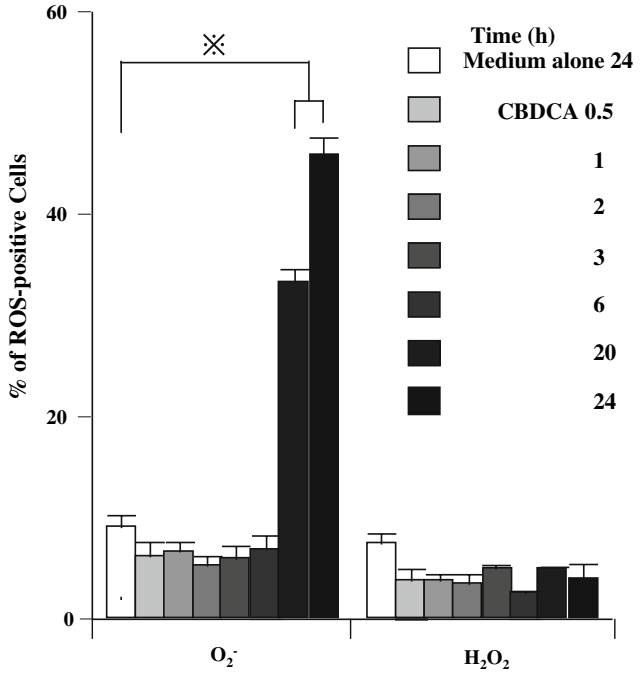

Fig. 3 CBDCA induces production of superoxide anion, but not hydrogen peroxide in CH31 B lymphoma cells. CH31 cells were exposed to 100 μg/ml CBDCA for the indicated time periods, and assayed for production of superoxide and hydrogen peroxide using flow cytometry. Percentages of cells producing superoxide and hydrogen peroxide were shown. Results are the mean ± SD from three independent experiments. *Significantly different from the control medium alone

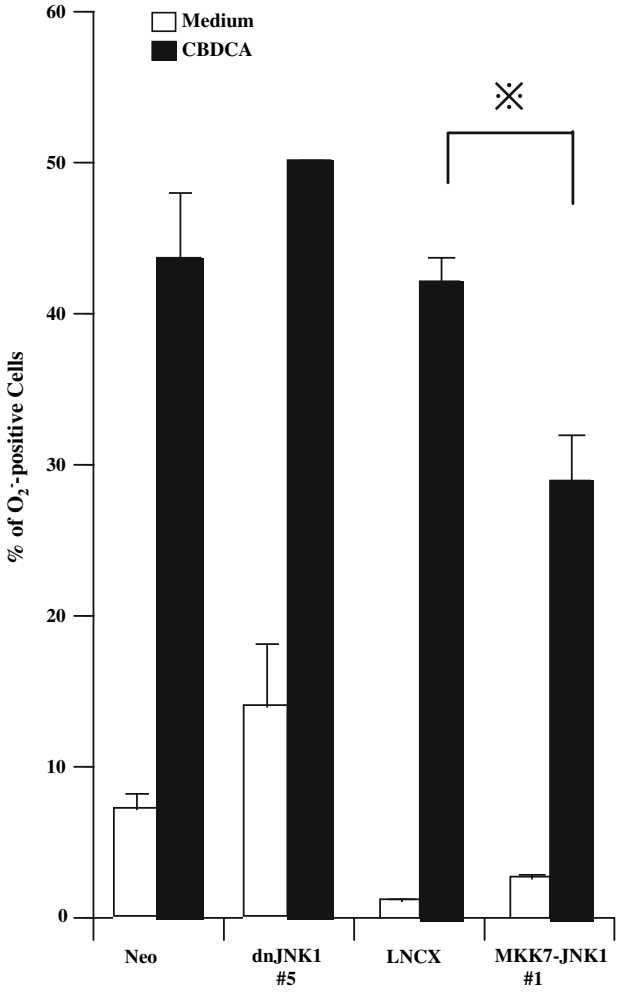

Fig. 4 CBDCA-induced O_2^- production is slightly enhanced in dnJNK1-expressing cells, whereas it is substantially reduced in MKK7-JNK1-expressing cells. The dnJNK1-overexpressing cells and MKK7-JNK1-overexpressing cells were exposed to 100 μg/ml CBDCA for 24 h, followed by assay for superoxide-producing cells. Results are the mean ± SD from three independent experiments. *Significantly different from the control vector alone

head and neck squamous cell carcinoma, non-small cell lung cancer, and lymphoma [1–3]. Although CBDCA is highly effective against some cancers, it has major drawbacks during treatment, such as inherent and/or acquired resistance to this drug and severe adverse effects [4, 5], which limit its clinical use. To develop other pharmaceutical drugs with improved properties, understanding of the molecular mechanisms underlying CBDCA-induced cell death is urgently required.

We and others have recently demonstrated that CBDCA induces necrotic as well as apoptotic death in some cell types, including melanoma cells [8, 36]. Apoptotic cell death can be distinguished from necrotic cell death by morphological features, DNA degradation, caspase activation, and flow cytometry [10, 11]. Flow cytometry using staining with annexin V and 7-AAD revealed that low concentrations of CBDCA (20–50 μg/ml) induced primary apoptosis and secondary necrosis, while higher concentration of CBDCA (100 μg/ml) induced both primary apoptotic and necrotic cell death in CH31 B lymphoma cells (Fig. 1). Although some form of necrotic cell death occurs when intracellular ATP levels drop below a critical threshold [37], the CBDCA-induced primary necrotic cell death occurred without a substantial decrease of ATP levels (Takada et al. unpublished observation). Necrotic cells induce inflammatory responses by secreting inflammatory cytokines as well as by leaking their intracellular contents [38]. In contrast, apoptotic cells are immediately ingested by phagocytic cells, resulting in production of anti-inflammatory cytokines such as TGF-β and IL-10 [38]. If primary apoptotic cells are not removed by phagocytic cells, they are considered to lose plasma membrane integrity and to leak intracellular contents, behaving like necrotic cells (secondary necrosis). Thus, the mode of cell death affects the types of immune responses.

The molecular mechanisms underlying CBDCA-induced apoptosis versus necrosis remain incompletely understood. Sustained activation of JNK is reported to be involved in the induction of apoptosis [12, 14, 15], while activation

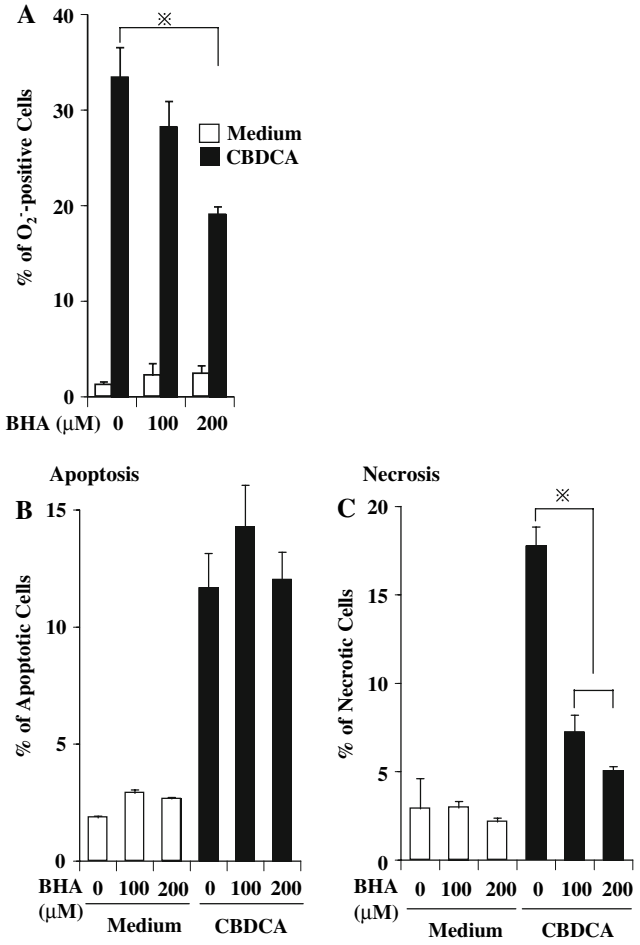

Fig. 5 Superoxide anion scavenger BHA reduces CBDCA-induced necrotic cell death without effect on apoptosis in CH31 cells. CH31 cells pretreated with BHA for 1 h were exposed with 100 μg/ml CBDCA for 24 h, and then assayed for O_2^- production (**a**) and induction of primary apoptotic (**b**) and primary necrotic cell death (**c**). Results are the mean ± SD from three independent experiments. * Significantly different from the control vehicle alone

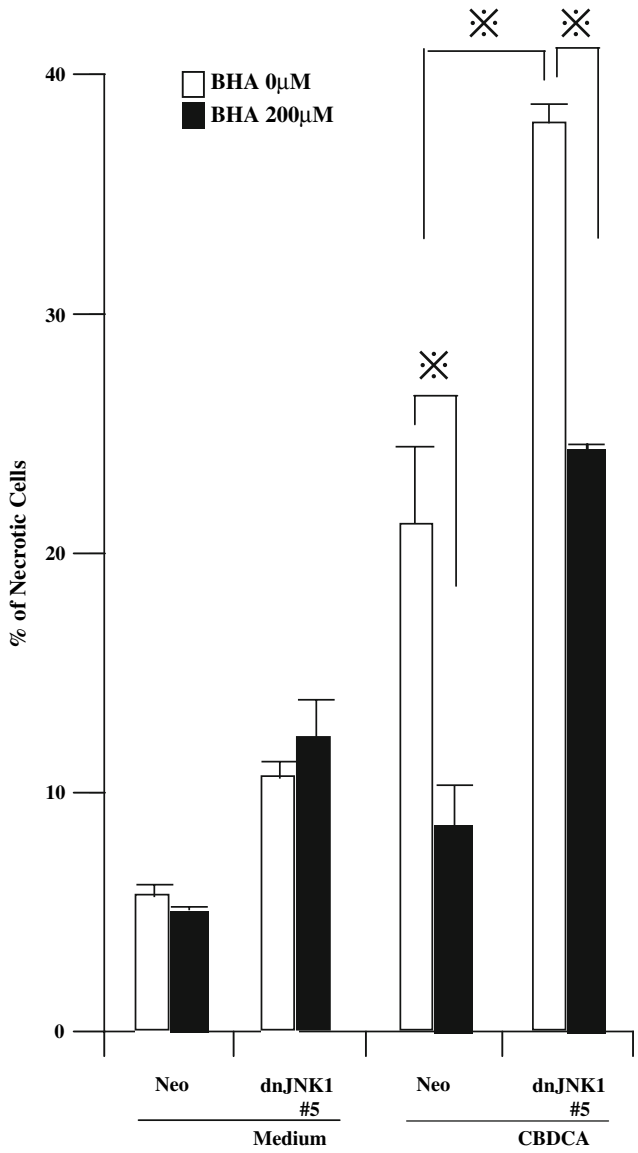

Fig. 6 The enhanced CBDCA-induced primary necrosis in the dnJNK1-overexpressing cells is also reduced by pretreatment with BHA. CH31 cells overexpressing dnJNK1 or control vector alone were exposed to 100 μg/ml CBDCA for 24 h, followed by assays for induction of primary necrosis. Results are the means ± SD from three independent experiments. * Significantly different from the control vehicle alone

of ERK promotes proliferation and cell survival [13]. CBDCA induced a prolonged activation of JNK in CH31 B lymphoma cells (Figure S2 in ESM). CBDCA-induced apoptotic cell death was partially abrogated in the dnJNK1-overexpressing cells, with a concomitant increase in primary necrotic death (Fig. 2a). A constitutively active form of JNK, MKK7-JNK1, enhanced the CBDCA-induced apoptosis and secondary necrosis, accompanied by a decrease in the proportion of primary necrosis (Fig. 2b), suggesting that JNK activation is involved in induction of apoptosis with prevention of necrosis. In agreement with our findings, Lopez-Sanchez [39] recently reported that JNK abrogated accumulation of ROS and prevented necrotic damage in neural tumor cells following serum deprivation. In contrast, JNK was reported to potentiate necrotic cell death in response to tumor necrosis factor (TNF) and H_2O_2 in other cell types [27, 40]. Together, depending on the cellular context, CBDCA-induced JNK activation may result in promotion of apoptosis or necrosis.

Unlike apoptosis, the molecular mechanisms underlying necrotic cell death are poorly understood. DNA-damaging agents such as cisplatin/carboplatin cause mitochondrial membrane permeabilization in certain cell types, leading to release of small molecules including cytochrome c and ROS. The major forms of ROS comprise superoxide, hydrogen peroxide, and hydroxyl radical [21]. ROS is implicated in cell death in some cells upon certain stimuli. The number of CBDCA-induced O_2^- producing cells was

substantially reduced in the MKK7-JNK1-expressing cells, whereas they were slightly enhanced in the dnJNK1-expressing cells (Fig. 4). These results suggest that the CBDCA-induced superoxide anion is negatively regulated by JNK1 activation, although the mode of JNK action remains unresolved in the present study.

The CBDCA induction of O_2^- producing cells was blocked by the ROS scavenger BHA (Fig. 5a). Pretreatment with BHA have been demonstrated to abrogate necrosis induced by chemotherapeutic agents and TNF [34, 35]. In agreement with this finding, BHA reduced CBDCA-induced primary necrosis, but not apoptosis (Fig. 5b, c), suggesting that superoxide anion plays some role in the necrosis induction. Osteosarcoma cells exposed to superoxide donor menadione are also reported to switch the mode of cell death from apoptosis to necrosis in a time-dependent manner [41]. Together, CBDCA-induced JNK activation appears to promote apoptosis induction and concomitant inhibition of necrosis, probably through regulation of superoxide anion production. This notion is supported by reports that melanoma cell line(s) resistant to apoptosis by TRAIL were more susceptible to necrosis induced by anti-tumor drugs [42]. Pretreatment with the pancaspase inhibitor Z-VAD-fmk abrogates CBDCA-induced (Takada et al. unpublished observation) and CD95-mediated [34] apoptotic cell death, with a concurrent increase in necrosis induction.

Collectively, the present studies clearly indicate that CBDCA exposure has a dose-dependent effect on cell death, either by apoptosis or necrosis, and that the apoptotic signaling cascade involves JNK activation. JNK activation negatively regulates CBDCA-induced necrosis. These observations will help to understand the molecular mechanisms underlying CBDCA-induced cell death, and hopefully for the design of better strategies for treatment of patients with tumors by chemotherapeutic agents including CBDCA.

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